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A comprehensive study on enhancement of lipid yield from *Tetradesmus obliquus* MT188616.1

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Abstract−Microalgae are known to produce neutral-lipids such as triacylglycerols (TAGs), a raw material required for biofuel production. The present study aimed to screen the high lipid producing native microalgae strains from freshwater habitats, select appropriate methods to extract lipid from wet algal biomass, and study fatty acid compositions. At first, isolated twenty native strains among them two isolates that exhibited higher lipid content was further subjected to molecular characterization. Results based on the cell morphology, molecular characterization, and phylogenetic analysis indicated that these two strains were *Tetradesmus obliquus* and *Ettlia oleoabundans*. Based on the growth study of screened algal strains, the biomass ranged from 0.65 g/L to 6.03 g/L with *Tetradesmus obliquus* providing the highest biomass and total lipid content of 51% when cultured in a nitrogen-deprived medium. The highest lipid yield was obtained with hexane:isopropanol (2 : 1) solvent mixtures, accompanied by an optimized cell wall disruption method. Additionally, it was found that *Tetradesmus obliquus* had higher contents of saturated and monounsaturated fatty acids, i.e., 36.19% and 31.49%, respectively, in nitrogen-deprived medium (N^-), whereas in nitrogen-containing medium (N^+) was 27.34% and 28.85%, respectively. Hence, this suggests their suitability for biofuel production.

Keywords: *Tetradesmus obliquus*, Molecular Characterization, Nitrogen Starvation, Lipid Extraction, Fatty Acid Profiling

INTRODUCTION

Microalgae have shown their advantages over other feedstock, such as oil from plant and animal sources for biofuel production. They do so because of photosynthetic efficiency, high lipid production under stressful conditions, capable of fixing carbon dioxide, and growth on unfavorable land [1-4]. They can cultivate fast and store high lipid content (20% to 50% of dry weight) of which neutral lipids mainly in the form of TAGs are of interest because they can be transformed directly into biofuel [5]. Selection of high lipid producing microalga strain and suitable culture growth medium is a prime step for the application of the potent strain in biofuel production. Research studies have reported that in nitrogen-deprived medium microalgae are able to accumulate more lipid content than in nitrogen containing medium. Because microalgae cultures can easily acclimatize to the nitrogen starvation condition that is by directing carbon compounds towards the accretion of carbon reserves such as lipids [6,7].

Concurrently, the success of commercializing microalgae as a suitable feedstock stems from both upstream and downstream processes, such as high microalga growth rate, high neutral lipid producing strains, optimal cell disruption technique for releasing intracellular lipids, optimal neutral lipid extraction methods, the suitability of extraction solvent, and the economics of the process, etc [8]. Since the concept of biofuel production from microalgae has been introduced, many significant developments have already been made in the upstream process to produce more algal biomass for high lipid yield. In the case of the downstream process, accurate methods for lipid extraction continue to be challenging despite the availability of many standardized processes with higher lipid yield due to variability in the cell wall structures [9]. Hence, for each newly isolated microalga strain, suitable lipid extraction techniques need to be optimized to commercialize a higher lipid yield process.

In the present study, native freshwater microalgae cultures were isolated and characterized up to a molecular level from various habitats in Bengaluru, India, and further examined to screen the high lipid producing microalga strain. Among twenty isolated microalgae cultures, *Tetradesmus obliquus* was selected because of its ability to accumulate high amounts of lipids in a nitrogen-deficient medium. Further, the extraction competence of various solvent combinations was examined, accompanied by a comparison of multiple cell disruption methods to enhance this strain's lipid yield. Subsequently, lipid extract was transesterified to fatty acid methyl esters (FAMEs) and studied their fatty acid compositions by Gas chromatography-Mass spectrometry (GC-MS) analysis to confirm it as the best biofuel feedstock.

MATERIALS AND METHODS

1. Chemicals and Reagents

All chemicals, reagents, and solvents used in this study were ob-

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Table 1. Composition and preparation of Hutner's reagent

Component	Concentration (gm L^{-1})	Dissolved in known amount of water (ml)
EDTA	50	250
$ZnSO_4 \cdot 7H_2O$	22	100
H_3BO_3	11.4	200
$MnCl_2 \cdot 4H_2O$	5.06	50
$FeSO_4 \cdot 7H_2O$	4.99	50
$CoCl_2 \cdot 6H_2O$	1.61	50
$CuSO_4 \cdot 5H_2O$	1.57	50
$(NH_4)6Mo_7O_{24} \cdot 4H_2O$	1.1	50

Above all solutions were prepared separately and mixed together except EDTA. Solution mixture was brought to boil condition and added EDTA solution. It turned green colour and allowed to cool to 70 °C. Next, 85 mL of hot 20% KOH solution was added, finally solution was made up to 1 L by adding 115 mL of distilled water. Allowed to settle for few days until solution turned purple colour. Later it was filtered using two layers of Whatman No. 1 filter paper and stored at refrigerator condition for further use.

tained from Sigma Aldrich (India) and SD Fine (India), which were analytical grades.

2. Preparation of ATCC 897 Broth and Agar Media

According to lgcstandards-atcc.org, ATCC 897 broth and agar media were prepared using 50 ml/L of Beijerinck's solution (10 g NH_4Cl, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 1 L distilled water), 50 ml/L of Phosphate buffer (28.8 g K_2HPO_4, 14.4 g KH_2PO_4, 1 L distilled water), 1 ml/L of Hutner's trace elements (composition and preparation, as shown in Table 1), 2 g/L of sodium acetate in distilled water for broth medium and added 1.5% agar for agar medium (pH 7.2). ATCC 897 nitrogen-deprived medium was devoid of NH_4Cl.

3. Isolation of Microalgae

Freshwater samples were collected from various freshwater bodies in Bengaluru, Karnataka state, India, and cultivated in a salt medium, namely ATCC 897, for 10 to 20 days at 25 °C, 50-100 μEinsteins/m^2/s irradiance (cool fluorescent white light), which was maintained under a 14 h light - 10 h dark photoperiod. After growth, a known volume of sample was serially diluted using 0.9% saline medium (0.9 g NaCl in 100 ml of distilled water). The required aliquots of appropriate serial dilutions of the sample were spread plated on sterile pre-poured plates of ATCC 897 agar medium. These inoculated agar plates were incubated at the standard conditions for 7 to 15 days. Pure colonies isolated by successive plating processes were evaluated by microscopic observation (Labomed, OPTICX model, India). To maintain the culture viability and purity, screened cultures were sub-cultured in the respective medium at regular intervals of 30 days, followed by storage at 4 °C. Microalgae were identified by the conventional method, i.e., by observing microscopical structural characteristics. All isolates were subjected for estimation of total lipids to screen the higher lipid producing strain [10].

4. Molecular Characterization - PCR Detection for Genus Specificity

Further, the molecular characterization of screened microalgae cultures was carried out by the target gene sequence homology study. For this, microalgae cultures were subjected to PCR analysis to amplify the 28S rRNA gene fragments for which universal eukaryotic primers were used to estimate and analyze the *Chlorophyta* variety. In the first step, microalgae cultures were cultivated in ATCC 897 broth medium at the standard condition of 25 °C, 50-100 μEinsteins/m^2/s irradiance, and maintained under a 14 h light - 10 h dark photoperiod for five days. Aliquots of the culture broth were plated on ATCC 897 agar medium to obtain discrete colonies. Single and well-isolated colony grown on the agar medium were regrown in ATCC 897 broth at the same standard condition. This was followed by harvesting of alga cells in the course of the exponential growth period, and the extraction of genomic DNA using the conventional method [11]. Targeted region of 28S rRNA (size - 1,000 bp) gene fragments was amplified by uniplex PCR with genus-specific primers, i.e., Forward - GGACAGAAAGACCCTATGAA with Reverse - TCAGCCTGTTATCCCTAGAG [12]. PCR analysis was performed by preparing 50 μl of reaction mixture containing $MgCl_2$ (2 mM), DNA (50 ng), dNTPs (200 μM), primers (0.6 μM each) and Taq DNA polymerase (1.25 U) [13]. PCR analysis was carried out by maintaining the following conditions, i.e., 1st cycle at 94 °C for 5 min followed by 32 cycles at 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1.30 min, and final cycle at 72 °C for 7 min. Amplified PCR products were purified by using the purification kit (Sigma Aldrich, India), later submitted to the Eurofins Genomics India Pvt. Ltd for gene sequencing. It was carried out by using the BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Subsequently, the acquired nucleotide sequences were examined by using the basic local alignment search tool (BLAST) with the databases of National Centre for Biotechnology Information (NCBI) to evaluate the percentage of homology with closely related strains, which were documented in the GenBank. A phylogenetic tree was constructed using Phylogeny.fr.

5. Growth Study

All cultures were grown independently in 250 ml Erlenmeyer flasks containing 120 ml of ATCC 897 broth medium. They were grown at 25 °C, 120 rpm, 50-100 μEinsteins/m^2/s irradiance, and 14 h light -10 h dark photoperiod for about ten days. Growth was monitored every 24 hours by measuring cell counts and optical density (OD) at 680 nm. The cell count was measured using Neubauer hemocytometer with 0.05% Evans blue dye under a microscope (Labomed, OPTICX model, India) [14]. The culture growth was monitored until there was a drop in OD at 680 nm using a spectrophotometer (Eppendorf, India).

6. Cultivation of Microalgae in Nitrogen Deprived Medium

The algae cultures, i.e., initial concentration of 1×10^6 cells/ml were inoculated into 500 ml Erlenmeyer flasks containing 250 ml of normal ATCC 897 broth medium and allowed to cultivate at standard conditions till the end of the exponential period. Grown cultures were centrifuged at 6,000 rpm for 10 min at 4 °C to harvest the alga cells. The resultant cell pellet was washed thrice with sterile distilled water, followed by transferring into 250 ml of ATCC 897 broth medium with nitrogen-free for lipid accumulation, and incubated at standard condition. Later, the cells were harvested between 1st to 10th days at 24 h intervals. The growth was monitored using OD at 680 nm at 24 h intervals.

7. Optimization of Solvent Mixture for Lipid Extraction

Four organic solvents like hexane, isopropanol, chloroform, and

methanol--were combined in ratios of 1:1, 1:2, and 2:1 for lipid extraction. It was done by modifying Chen and Vaidyanathan method [15] as follows: 0.5 g of wet biomass was suspended in 1.5 ml of 1 M Tris-HCl (pH 7.8) and 8.5 ml of saponification reagent (5% of 1 M NaOH in a mixture of isopropanol and water ratio of 40 : 60), later vortexed for 5 min, this was followed by incubation of sample at 90 °C for 30 min, this protocol was considered as a control for further experimental study. After this, 8.5 ml of each combination of the solvent mixture in different ratios was mixed with the saponified sample. The mixture was vortexed for 3 min and centrifuged at 6,000 rpm for 10 min. The supernatant comprising of the lipids in the organic phase was transferred to a pre-weighed glass vial and vaporized at room temperature. The lipid content (% dry weight) was determined gravimetrically [15].

8. Optimization of Cell Wall Disruption

The following five cell wall disruption techniques were employed to investigate the optimal lipid release from the cells of Strain1; (i) grinding with sand, (ii) bead vortexing, (iii) thermolysis, (iv) mixing on a magnetic stirrer, and (v) combination of grinding with sand and saponification method [16,17]. Step by step experimental studies were carried out as shown in Fig. 1 and 2. For all the methods, 0.5 g of wet biomass was taken and suspended in 1.5 ml of 1 M Tris-HCl (pH 7.8), followed by disruption of the microalga cell wall. Thorough cell lysis was confirmed by observing under a light microscope. After the disruption of the cell wall, the sample was blended with 8.5 ml of optimized hexane and isopropanol (2 : 1) solvent mixture to extract lipids. The mixture was vortexed for 3 min, then centrifuged for 10 min at 6,000 rpm. The supernatant, which was the organic phase with the lipids, was transferred to a pre-weighed glass vial and vaporized at room temperature. After vaporization, the lipid content (% dry weight) was determined gravimetrically [15]. For control, the protocol that was mentioned in step 7 was followed using the optimized solvent mixture.

8-1. Grinding with Sand

To the wet biomass, 0.2 g of sand (Neutralized one) was added, later ground with mortar and pestle for 5 min followed by solvent extraction of total lipids using the optimized solvent mixture.

8-2. Bead Vortexing

Approximately 50 mg of 0.1 mm glass beads was added to the wet biomass. Cell disruption was promoted by vigorous vortexing for 10 min, in between sample was kept on ice, followed by solvent extraction of total lipids using the optimized solvent mixture.

8-3. Thermolysis

The wet biomass was placed in a preheated water bath at 90 °C for 30 min to induce the thermolysis, followed by solvent extraction of total lipids using the optimized solvent mixture.

8-4. Mixing on Magnetic Stirrer

8.5 ml of ethanol was directly added to the wet biomass, which

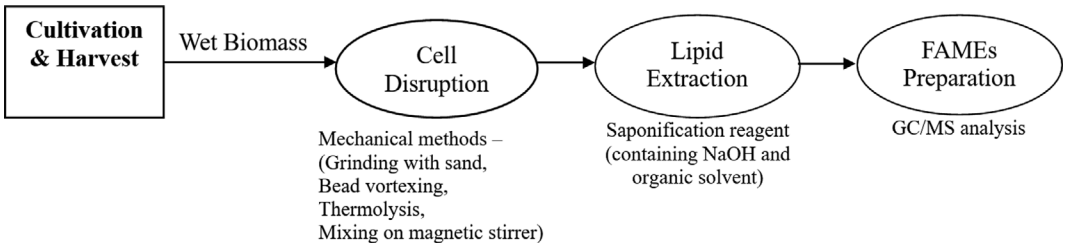

Fig. 1. Brief overview of experimental methods used to study the lipid content in microalgae.

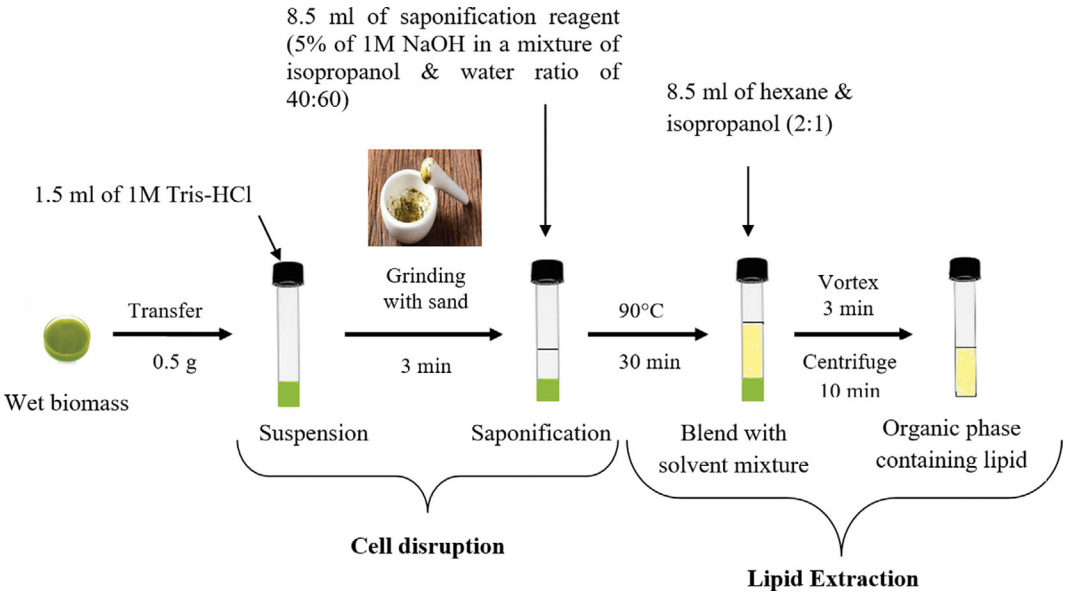

Fig. 2. Microalgae cell disruption and lipid extraction optimized method.

was then vortexed for 5 min and incubated at room temperature overnight with continuous mixing on magnetic stirrer. This was followed by the solvent extraction of total lipids using the optimized solvent mixture.

8-5. Combined Method

The wet biomass (0.5 g) was suspended in 1.5 ml of 1 M Tris-HCl solution (pH 7.8) and ground with neutralized sand for 3 min followed by saponification step as described in step 7. This was followed by the solvent extraction of total lipids using the optimized solvent mixture.

9. Total and Neutral Lipid Estimation

9-1. Saponification and Extraction of Cellular Lipids

To extract the lipid content from alga cells, 2 ml of seven days old culture broth was taken in an Eppendorf tube and centrifuged for 10 minutes at 6,000 rpm at 4 °C. The obtained cell pellet was suspended in 40 µl of Tris-HCl solution (1 M, pH 7.8) and 1,960 µl of saponification reagent (5 g of NaOH in 100 ml of isopropanol and water ratio of 40 : 60). Subsequently, the sample was macerated by adding a trace amount of sand and vortexed for 5 min to rupture the cell wall, so complete lysis of alga cells was confirmed by observing under light microscope. This was followed by incubation of sample for 30 min at 90 °C during this process every 5 min once the sample was vortexed to hydrolyze the ester bonds of membrane lipids and triglycerides. Further, 5 ml of hexane and isopropanol (2 : 1) solvent mixture was added to the saponified mixture, which was mixed thoroughly by vortexing for 3 min and centrifuged at 6,000 rpm for 10 min. Resultant supernatant containing lipids in the organic phase, was used for the estimation of total and neutral lipids [15].

9-2. Estimation of Total Lipids

The amount of fatty acids was estimated as follows: 500 µl of the organic phase containing lipids from step 9.1 was taken in an Eppendorf tube. To this, an equal volume of copper reagent (9 vol. aq. 1 M triethanolamine (TEA), 1 vol. 1 M acetic acid, 10 vol. 6.45% w/v $Cu(NO_3)_2 \cdot 3H_2O$) was added followed by vortexing and centrifugation at 6,000 rpm for 5 min at 4 °C. The supernatant containing the organic phase was collected and transferred to a fresh tube. While transferring the organic phase, caution was taken to avoid the transfer of aqueous phase containing copper ions since it produce false signal during spectrophotometric detection. In the next step, an equal volume of color-developing reagent (1% sodium diethyldithiocarbamate in 2-butanol w/v) was added, and absorbance was measured at 440 nm by spectrophotometer (Eppendorf, India). For the standard curve, 5 mM palmitic acid stock solution was used to obtain 60, 240, 480, 720, 960, 1,200 µg of the sample, and the volume was made up to 2 ml with a solvent mixture of hexane and isopropanol (2 : 1). 500 µl of these dilutions was then used to obtain the standard curve following the above-mentioned procedure. Simultaneously, blank was prepared as mentioned for sample except milli-Q water was used instead of the sample [10].

9-3. Estimation of Triacylglycerides

The amount of neutral lipids, i.e., triacylglycerides, was estimated using 1.67 mM tripalmitin as standard and without the use of Zeolite [18].

500 µl of organic phase containing lipids was taken in an Eppendorf tube. To this 1 ml of freshly prepared sodium metaperiodate - SMI (Main stock: 0.025 M SMI in 1 N acetic acid. Working stock: 2.4 ml of 0.025 M SMI in 4 ml of isopropanol and 13.6 ml of 1 N acetic acid) and 0.5 ml of acetyl acetone reagents were added. The sample was mixed well, followed by incubation at 60 °C for 30 min. The sample was allowed to cool before the absorbance at 405 nm was measured by a spectrophotometer (Eppendorf, India). For the standard curve, 1.67 mM tripalmitin stock solution was used to obtain 60, 240, 480, 720, 960, 1,200 µg of the sample, and the volume was made up to 2 ml with a solvent mixture of hexane and isopropanol (2 : 1). 500 µl of these dilutions was then used to obtain the standard curve following the above-mentioned procedure. Blank was prepared by following the above-mentioned steps except milli-Q water was used instead of the sample.

10. Analysis of Fatty Acids Methyl Esters (FAMEs)

The condensed lipid extract (100 µl) was transmethylate with 500 µl of 2% H_2SO_4/methanol solution (v/v) in a 2 ml Eppendorf tube by incubating at 80 °C for 2 h. Once the sample cooled, 500 µl of n-hexane and 500 µl of 0.9% (w/v) NaCl solution were added into the tube to create separate layers. Resultant upper n-hexane layer was collected to analyze the FAMEs by using GC-MS system [19]. The analysis was performed by the Indian Institute of Chemical Technology (IICT), Hyderabad, India. The specific fatty acid compositions on the chromatogram were identified by comparing with authentic standards and quantified from the peak areas using known fatty acid as the internal standard. Finally, total fatty acid content was calculated by adding all the individual fatty acid contents.

11. Statistical Analysis

All data are calculated and expressed as mean ± the standard deviation. Microsoft Excel 2010 (USA) was used for regression analysis.

RESULTS

1. Isolation and Identification of Microalgae

In this study, isolated 20 native freshwater microalgae from different habitats in Bengaluru and subjected to microscopic observation, which exhibited their colony existence and purity. Most of the screened microalgae were belong to the genus *Tetradesmus*, *Ettlia*, *Coelastrella*, *Scenedesmus*, *Chlorella*, *Acutodesmus*, *Chlorococcum*, *Botrydiopsis*, *Desmodesmus,* and *Dictyochloris*, which were identified based on their cell morphology. Generally, characterization up to species level based on morphological data is not reliable. Therefore, molecular characterization work was carried out for two potent isolates, which exhibited high lipid productivity among 20 screened strains (Table 2). These two strains were labeled as Strain1 and Strain2. Both strains grew very well in ATCC 897 broth medium and were maintained in the same medium.

2. Molecular Characterization - PCR Detection for Genus Specificity

As confirmatory evidence, PCR analysis was carried out with universal eukaryotic forward and reverse primers to identify the specific genus of microalgae. For this, microalga DNA was isolated and evaluated its quality on 1% Agarose Gel. A single band of high molecular weight DNA was observed, as shown in Fig. 3. Subsequently, fragments of 28S rRNA regions were amplified by PCR. A single distinct PCR amplicon band of ~1,000 bp was observed when resolved on 1% Agarose gel, as shown in Fig. 3. In the next step, the

Table 2. Total lipid content of 20 microalgae strains in normal ATCC 897 broth medium

Serial No.	Strain name	Source	Lipid content %
1	*Tetradesmus obliquus* Strain1	Pond, Bengaluru	42.01
2	*Ettlia oleoabundans* Strain2	Pond, Bengaluru	37.89
3	*Coelastrella* sp. Strain3	Pond, Bengaluru	35.34
4	*Scenedesmus obliquus* Strain4	Pond, Bengaluru	25.02
5	*Scenedesmus obliquus* Strain5	Pond, Bengaluru	23.90
6	*Scenedesmus quadricauda* Strain6	Pond, Bengaluru	22.39
7	*Scenedesmus* sp. Strain7	Pond, Bengaluru	32.75
8	*Chlorococcum* sp. Strain8	Lake, Bengaluru	31.02
9	*Chlorella vulgaris* Strain9	Lake, Bengaluru	32.63
10	*Chlorella vulgaris* Strain10	Lake, Bengaluru	19.05
11	*Chlorella vulgaris* Strain11	Lake, Bengaluru	22.90
12	*Botrydiopsis* sp. Strain12	Lake, Bengaluru	20.30
13	*Scenedesmus dimorphus* Strain13	Lake, Bengaluru	21.65
14	*Chlorococcum* sp. Strain14	Pond, Bengaluru	17.56
15	*Chlorella ellipsoidea* Strain15	Pond, Bengaluru	29.21
16	*Chlorococcum* sp. Strain16	Lake, Bengaluru	28.43
17	*Chlorella* sp. Strain17	Lake, Bengaluru	34.56
18	*Scenedesmus* sp. Strain18	Lake, Bengaluru	15.28
19	*Desmodesmus* sp. Strain19	Pond, Bengaluru	32.01
20	*Dictyochloris* sp. Strain20	Pond, Bengaluru	21.78

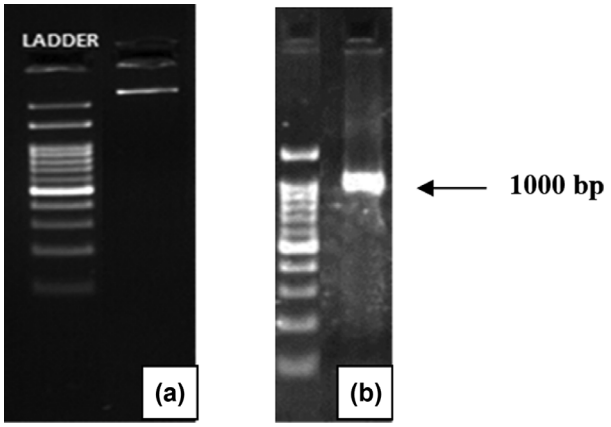

Fig. 3. Agarose gel electrophoretic pattern of (a) Genomic DNA, (b) PCR amplicon with 28S rRNA gene fragment in a native isolates of *Tetradesmus obliquus* and *Ettlia oleoabundance*.

amplified PCR product was purified to remove the impurities. This was followed by the gene sequencing of purified PCR amplicon with F and R primers using the BDT v3.1 Cycle sequencing kit on the ABI 3730xl Genetic Analyzer. The consensus sequence of the amplified product was generated from forward and reverse sequence data using aligner software. The 28S rRNA region sequences were analyzed using the nucleotide BLAST tool with the database of NCBI GenBank. Based on maximum identity score, the starting ten sequences were selected and aligned using nucleotide sequence alignment software program FASTA with default settings. Accession number was obtained for both strains by submitting the resultant aligned sequences in NCBI Bankit, for Strain1 accession number was MT188616.1 and Strain2 MT110991.1. Finally, the distance matrix was generated, and the phylogenetic tree was constructed using Phylogeny.fr. The resultant phylogenetic tree revealed the evolutionary relationships of Strain1 and Strain2 with closely related microalgae isolates deposited in NCBI GenBank. Strain1 showed 96-94% homology with the following isolates: *Tetradesmus obliquus* (MF661972.1) and *Scenedesmus obliquus* (HM103383.1). Similarly, Strain2 showed 93-95% homology with the following genera: *Ettlia oleoabundance* (JX415205.1), *Scenedesmus obliquus* (HE861890.1), *Acutodesmus obliquus* (HE965013.1) and *Tetradesmus obliquus* (KY741858.1). After molecular characterization and phylogenetic analysis, screened isolates of Strain 1 and 2 were identified to be *Tetradesmus obliquus* and *Ettlia oleoabundans*, respectively, as appearing in the generated tree as shown in Fig. 4. Both strains were belonging to *Chlorophyta*.

3. Growth Study

After molecular characterization of the microalgae, Strains 1 and 2 were subjected to growth study. Fig. 5 shows the growth behavior and linear relationship between OD at 680 nm and the average cell concentration (cells/mL) of Strain1 and Strain2. From which one can observe that Strain1 was fast-growing and reached a maximum growth by day 8^{th}. Both, OD at 680 nm and the cell concentration measured using hemocytometer, were monitored for the growth study. A similar growth curve and the linear relationship between OD at 680 nm and the average cell concentration were obtained for Strain2. It grew to the maximum by day seven for a photoperiod of 14 h light - 10 h dark and 50-100 μEinsteins/m^2/s irradiance. The specific growth rate (μ day^{-1}), calculated for Strain1 and Strain2 was 6.13 μday^{-1} and 1.29 μday^{-1}, respectively. To calculate the specific growth rate of microalgae the following formula was used,

$$\text{Maximum growth rates } (\mu\text{max}) = (\text{In } (X_m - X_0))/(t_m - t_0)$$

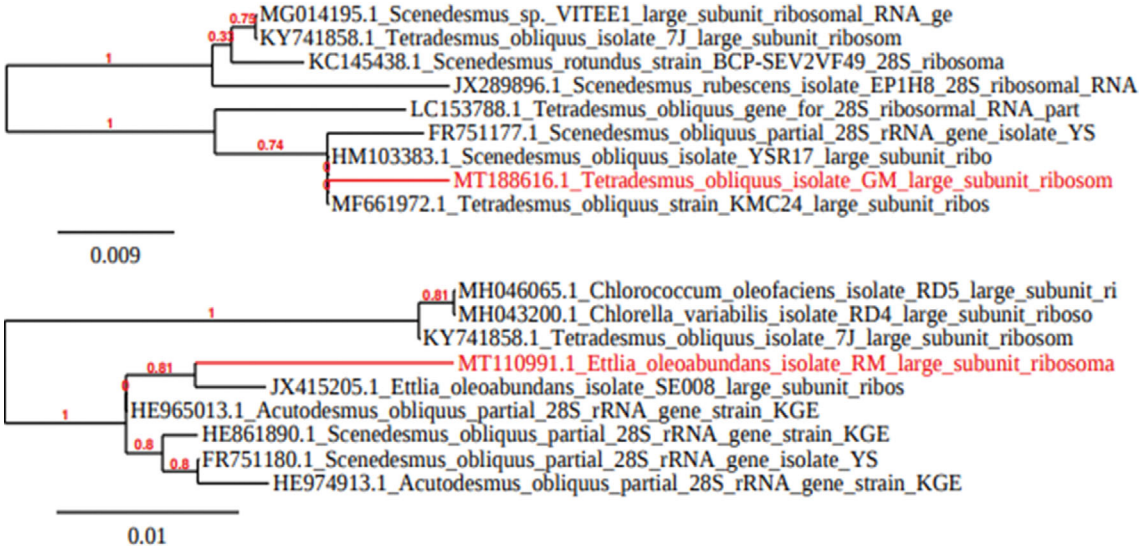

Fig. 4. Phylogenetic relationships of isolated *Tetradesmus obliquus* (MT188616.1) and *Ettlia oleoabundans* (MT110991.1) based on large subunit ribosomal RNA gene.

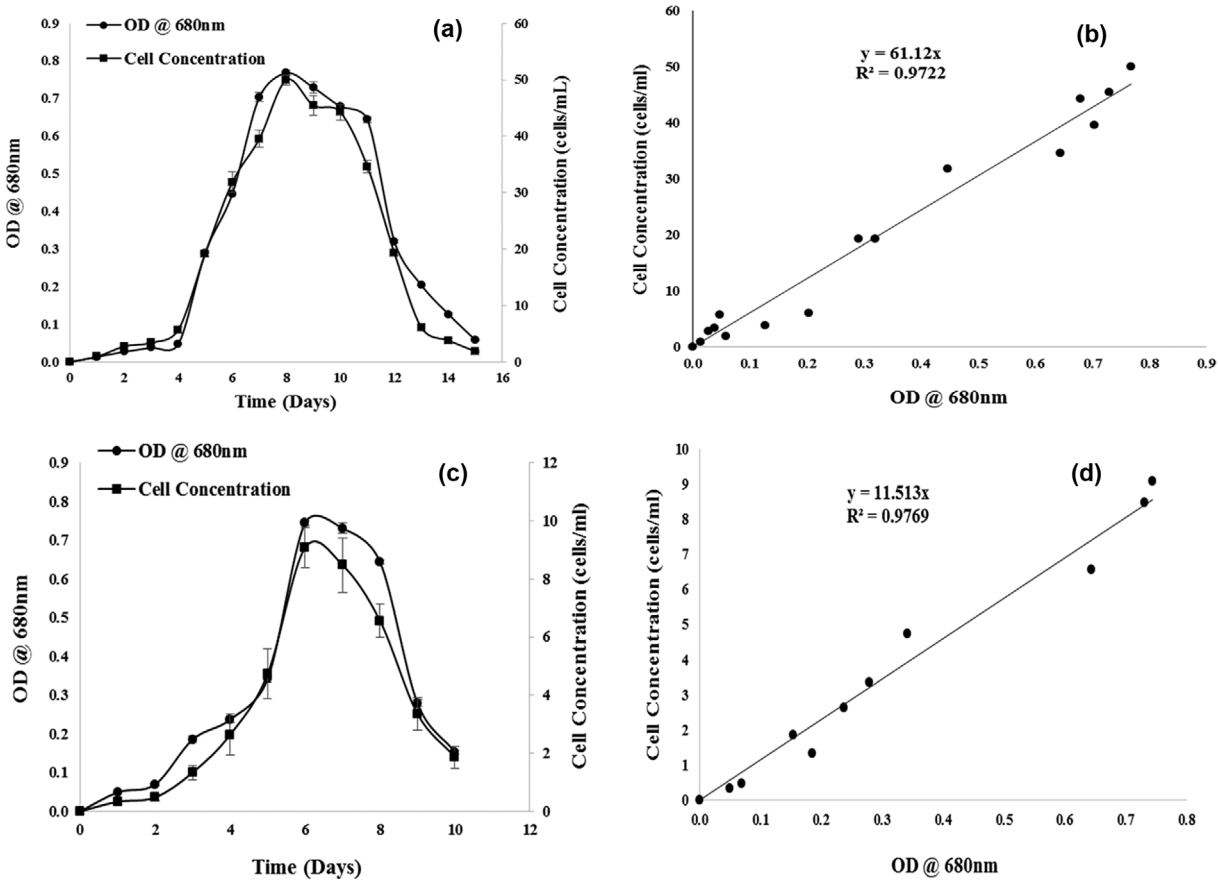

Fig. 5. Growth curve of (a) *Tetradesmus obliquus* and (c) *Ettlia oleoabundans* Linear relationship between OD at 680 nm and the average cell concentration (cells/mL) of (b) *Tetradesmus obliquus* and (d) *Ettlia oleoabundans*.

where, X_m is a maximum cell number, X_0 is an initial cell number, t_m is the day of maximum cell number and t_0 is day of initial cell number.

The maximum average cell concentration for Strain2 was around 9.1×10^6 cells/mL, and Strain1 was 50×10^6 cells/mL, indicating that Strain1 had more cell density than Strain2. Both strains were further subjected to lipid accumulation under nitrogen-deficient medium and measured for lipid content.

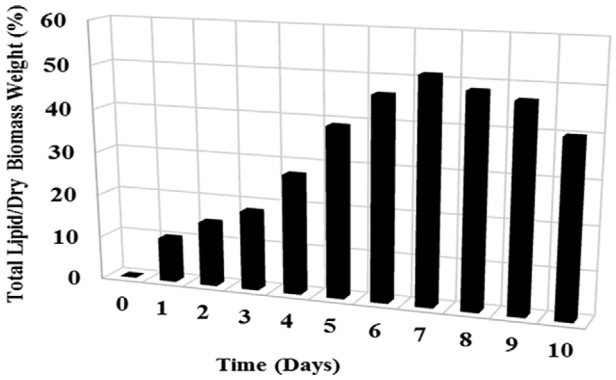

Fig. 6. Lipid accumulation study for *Tetradesmus obliquus* in nitrogen-deprived medium.

4. Lipid Accumulation in Nitrogen Deprived Medium

Strain1 and Strain2 were subjected to lipid accumulation study by growing in nitrogen deprived medium for ten days. The lipid content in algae cultures was obtained by the solvent extraction method and measured gravimetrically. The total lipid content of Strain1 extracted from the organic solvent mix was increased steadily in percentage till the seventh day and then dropped down gradually, as shown in Fig. 6. Day seven gave the highest yield of total lipid of 51% based on dry cell weight. Hence, the optimal lipid content was obtained on day seventh after the growth period of Strain1. Though Strain2 also showed an increase in lipid accumulation in nitrogen deprived medium that was comparatively less than Strain1. Hence, Strain1 *Tetradesmus obliquus* was selected for optimizing the suitable solvent mixture for lipid extraction as well as cell wall disruption methods.

5. Lipid Extraction with Different Solvent Mixtures

To test the best solvent mixture for lipid extraction from *Tetradesmus obliquus* was performed by selecting different solvent mixtures outlined in Step 7. The percentage of total lipid extraction was obtained using the different solvent mixtures, as shown in Fig. 7(a). The combinations of hexane/isopropanol and chloroform/methanol in the ratios of 1 : 1, 1 : 2, and 2 : 1 were used in the study. The ratio of 2 : 1 for both the combinations gave the highest lipid content, i.e., the mixture of hexane/isopropanol and chloroform/methanol gave 52% and 47% of total lipids, respectively, while all other combinations gave less total lipid percentage. Since the mixture of hexane/isopropanol (2 : 1) gave the highest yield of total lipids, the same combination was used for lipid extraction in the further experimental study.

6. Different Cell Wall Disruption Techniques

Enhancement of lipid yield from microalga cells with a cost-effective procedure can be achieved by applying an appropriate cell disruption technique. If the employed technique releases the highest amount of intracellular lipids from microalga cells, then with a suitable solvent mixture, lipid recovery can improve. Hence, different cell wall disruption techniques were evaluated for releasing the optimal lipid content from *Tetradesmus obliquus*. Fig. 7(b) shows the results of total lipid content in percentage for the different techniques. Among them, the combined method gave the highest total lipid yield of 62%. Other methods such as grinding with sand, mixing on a magnetic stirrer, thermolysis, and bead vortexing ex- tracted 42.5%, 38.5%, 33.5%, and 19% of total lipids. By this result, it confirmed that the method which was mentioned in the step 8.5 as an optimized condition for releasing the lipid contents from *Tetradesmus obliquus*.

7. Lipid Analysis and Characterization using FAME

The lipid from the cell pellet of *Tetradesmus obliquus* was subjected to FAME analysis at IICT, Hyderabad, India. The results of lipid characterization using FAME analysis are listed in Table 3. From the retention time achieved by GC-MS, peak values were examined and observed as, lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), gadoleic acid (C22:0), lignoceric acid (C24:0), palmiloteic acid (C16:1), oleic acid (C18:1), eichosenoic acid (C20:1), and linoleic acid (C18:2). It was found that *Tetradesmus obliquus*, which was grown in nitrogen deprived medium (N^-), had shown higher contents of saturated and monounsaturated fatty acids, i.e., 36.19% and 31.49%, respectively, than in nitrogen containing medium (N^+) were 27.34% and 28.85%, respectively. The major fraction of the FAMEs consisted of oleic acid (24.74±0.04), linoleic acid (21.44±0.04) and palmitic acid (19.7±0.02) in nitrogen containing medium (N^+).

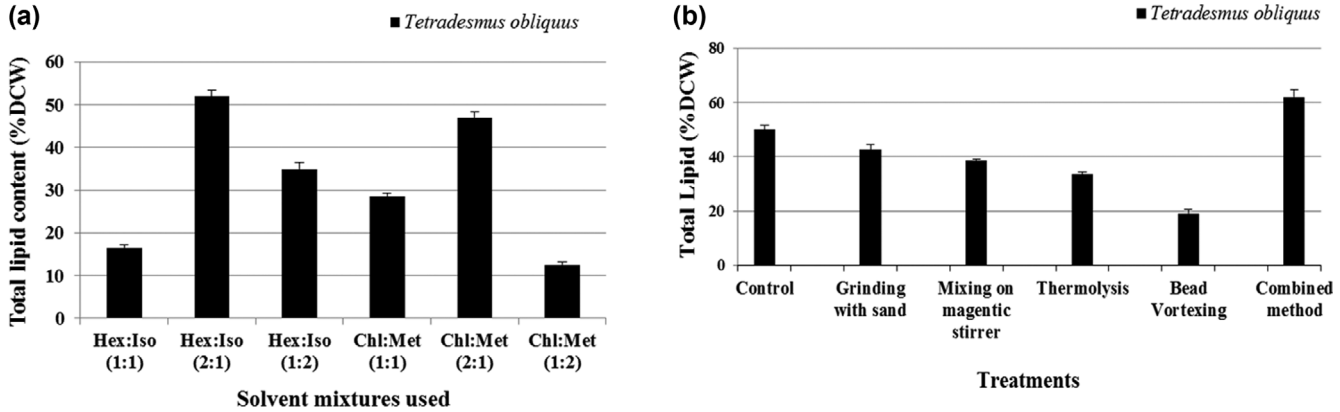

Fig. 7. Effect of different (a) solvent mixtures and (b) cell disruption methods for total lipid extraction from *Tetradesmus obliquus*. The symbol represent Hex (hexane), Iso (isopropanol), Chl (chloroform), Met (methanol), DCW (dry cell weight). Error bars represent ±SD.

Table 3. Fatty acid methyl esters (FAMEs) profile of *Tetradesmus obliquus* cultured under N containing and N deficient media

Identified fatty acids	N containing medium	N deficient medium
C12:0 (Lauric Acid)	0.14±0.02	0.68±0.09
C14:0 (Myristic Acid)	0.56±0.02	0.48±0.08
C16:0 (Palmitic Acid)	19.7±0.02	25.69±0.74
C16:1 (Palmiloteic Acid)	3.81±0.02	3.14±0.09
C16:3 (Hexadecatrieonic acid)	3.19±0.02	2.03±0.08
C17:0 (Margaric acid)	3.76±0.02	2.28±0.24
C18:0 (Stearic Acid)	1.8±0.02	5.11±0.38
C18:1 (Oleic acid)	24.74±0.04	27.68±0.28
C18:2 (Linoleic acid)	21.44±0.04	17.31±0.20
C18:3 (Gamma Linolenic acid)	1.56±0.02	0.93±0.18
C18:3 (Alpha Linolenic Acid)	12.14±0.02	8.28±0.30
C20:0 (Arachidic acid)	0.39±0.02	0.53±0.05
C20:1 (Eichosenoic acid)	0.30±0.02	0.67±0.28
C22:0 (Gadoleic acid)	0.67±0.01	0.74±0.12
C24:0 (Lignoceric acid)	0.32±0.02	0.68±0.02
ΣSFAs	27.34	36.19
ΣMUFAs	28.85	31.49
ΣPUFAs	38.33	28.55

SFA saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids. Values are presented as means±standard deviations

While, in nitrogen deprived medium (N^-) the content of oleic acid (27.68±0.28) and palmitic acid (25.69±0.74) were increased, but there was decrease in linoleic acid (17.31±0.20). As well, polyunsaturated contents were high in nitrogen containing medium (N^+) compared with nitrogen deprived medium (N^-).

DISCUSSION

The present situation in the field of bioenergy is mainly focussed on renewable energy, which can be obtained from materials derived from biological sources. Since microalgae have a potentiality in oil production, many industrial and research institutes are working on processes to enhance the oil content in microalgae as well as oil extraction procedures. Hence, the present study was focussed on screening the potent microalgae strains that have high lipid producing property, in order to convert the lipid into biofuel from the carbon-neutral source. Initially, 20 microalgae strains were isolated from different habitats in Bengaluru, India and characterized based on their cell morphological study. Most of the screened isolates belong to the family *Scenedesmaceae*, *Chlorellaceae*, *Chlorococcaceae*, and *Botrydiopsidaceae*. Further, the high lipid producing strains were subjected to molecular characterization by using large subunit ribosomal RNA gene universal primers. Strains 1 and 2 were identified as *Tetradesmus obliquus* and *Ettlia oleoabundans*, respectively, as these strains are closely related to respective genera based on 93 to 95% sequence similarities. The growth rate of *Tetradesmus obliquus* and *Ettlia oleoabundans* was determined by measuring the cell count and optical density at 680 nm at an interval of 24 h. The exponential period was seen until ten days and entered a stationary phase, which was followed by a declining phase. In the case of other studies, isolates belong to the *Scenedesmaceae* family showed a longer exponential period till 20 days. This type of variation observed in the growth pattern may be due to adaptation to their respective environmental conditions. Whereas higher growth rate of *Ettlia oleoabundans* was recorded at 360 μEinsteins/m^2/s irradiance, but not faster than other algal species [20]. While in the present study, *Tetradesmus obliquus* and *Ettlia oleoabundans* showed a faster growth rate at lower irradiance (50 μEinsteins/m^2/s irradiance) in selected ATCC 897 culture medium. Therefore, the fast-growing ability can be beneficial for the open cultivation of microalgae on a large scale, and also helpful in the avoidance of contamination problems. However, after days of the growth pH 7.2 of the culture medium increased to pH 10, a similar variation in pH was documented in other studies [21,22].

After standardizing the growth conditions, selected strains were grown in nitrogen-deprived medium to enhance the lipid production. This study was conducted to find the actual duration at which the maximum amount of lipid could be accumulated in the nitrogen-deprived medium, i.e., from 1st to 10th day of growth. For this, *Tetradesmus obliquus* and *Ettlia oleoabundans* were cultivated in a two-step process to uphold the enhancement of lipid production. In the first step, each microalga was grown in a nitrogen-containing medium and allowed to grow till the end of the exponential phase to ensure the higher biomass production. In the second step, cell biomass was harvested and washed, followed by transferring biomass into a nitrogen-free medium to enhance the lipid content. Similar type of cultivation technique was employed and had achieved higher lipid production in *Chlorella vulgaris* SDEC-3M, *Scenedesmus* sp. CCNM 1077, and *Nannochloropsis oceanica* [23-26]. The lipid content of these cultures was promptly augmented when the biomass from a nitrogen-rich medium was transferred to a nitrogen-deficient one. Even the present study also observed that during the

growth period, each strain exhibited considerable differences in the accumulation of lipid content. Among the tested strains, *Tetradesmus obliquus* accumulated the highest amount of total lipid content of 51% on day seven based on dry biomass. The *Ettlia oleoabundans* accumulated the maximum amount of lipid contents i.e., 43% on the eighth day of the growth period. Both strains exhibited a higher accumulation of total lipid content in the exponential growth phase. But in normal ATCC 897 broth medium lipid content was moderately less, so increased lipid yield is an expected response to stressful conditions as recorded in many studies. In one of the reports *Chlorella pyrenoidosa* produced a higher amount of total lipid content of 51% of dry biomass after 15 days of the incubation period [5]. But in the present study, *Tetradesmus obliquus* produced a higher amount of lipid within a shorter incubation period in a nitrogen-deprived medium. Besides, it exhibited neutral lipid content about 67% of total lipid, i.e., 51% of dry weight. Some microalgae such as *Nannochloropsis* sp., *Isochrysis* sp., *Dunaliella salina*, *Haematococcus pluialis*, and *Neochloris oleoabundans* exhibited synthesis of neutral lipids ranging from 23-80% of total lipid ranging from 2-60% of dry weight [1,27-32].

Further, to enhance the total lipid yield from microalgae, the suitable solvent should be selected for extraction, since the free fatty acids present in the lipid mainly manage the polarity. Generally, each microalga has a different fatty acid composition, so a universal solvent cannot be used for all types of microalgae. By this, it can be stated that the extraction of total lipid yields differs with solvent polarity [33]. For this reason in the present study, four solvents and their combinations were selected to comprehend the effectiveness of organic solvents in lipid extraction from *Tetradesmus obliquus*. Among the selected solvent combinations, the mixture of hexane : isopropanol (2 : 1) exhibited higher lipid yield (52%) than with other combinations of solvents. In other study, species of *Chlorella* and *Schizochytrium* exhibited 22% of total lipid yield with the mixture of chloroform : methanol (2 : 1) than with the hexane combine with other non-polar solvents, i.e., chloroform [34,35]. The individual solvent also attained less lipid yield than with a mixture of polar and non-polar solvents [36,37]. Hence, in this study polar and non-polar solvent combinations were chosen since a range of polar and non-polar lipids is usually present in the microalgae. Similarly, many research studies have shown that a combination of polar and non-polar solvent mixtures extracted more lipids from microalga cells. Presently, many methods have already been reported, but few methods are extensively using for the extraction of lipids from endogenous cells, i.e., the Folch method is implemented by the use of chloroform : methanol in the ratios of 2 : 1 by volume, as well as the Bligh and Dyer method is performed by using 1 : 2 (v/v) chloroform : methanol solvent mixtures [38,39]. Both methods deliver better recovery of nearly all major classes of lipids. One more method is performed by using methyl-tert-butyl ether : methanol : water (10 : 3 : 2.5, v/v/v) and dissolved lipid extract in chloroform : methanol : water (60 : 30 : 4.5, v/v/v) to attain complete lipid recovery [40]. In other cases, usage of chloroform : ethanol solvent mixtures in a ratio of 1 : 1 gave maximum lipid yield from *Chlorella* sp. [35]. These methods, generally use chloroform as the extracting solvent. However, the lipid extraction is very effective, but the application of these methods at large-scale is highly dangerous to the environment. Hence, research studies have suggested using less-toxic alternative solvents, such as ethanol, hexane, isopropanol, butanol, and acetic acid esters for lipid extraction from microalgae cells [41].

Various cell disruption techniques were compared to improve the recovery of total lipid yields, including grinding with sand, bead vortexing, thermolysis, mixing on magnetic stirrer, and a combination of grinding with sand and saponification. All the cell disruption methods attempted in this study were able to disrupt alga cells, even though the lipid yield varied. The highest lipid extraction yield was obtained using a combination of grinding with sand and saponification, i.e., 62% from *Tetradesmus obliquus*. The saponification step was proved to be very effective in removal of chlorophyll. Similar method was applied to optimize the condition for removal of chlorophyll from *Scenedesmus* biomass using saponification reagent, mainly consisting of ethanol-NaOH and resulted in the chlorophyll removal rates of 92% with the lowest rate of loss in total lipid contents [42]. One of the studies had shown that the osmotic shock technique was suitable for the disruption of cells of *Schizochytrium* sp. and *Thraustochytrium* sp. [34]. Other techniques, such as microwave, grinding with liquid nitrogen, and sonication, enabled to extract high lipid contents from *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp. [43-45]. In another research study had tested various cell disruption techniques, including autoclaving, microwaving, osmotic shock, and pasteurization in the seven day old microalgae cultures like *Chlorococcum* sp. MCC30, *Botryococcus* sp. MCC31, *Botryococcus* sp. MCC32, and *Chlorella sorokiniana* MICG5; the obtained lipid content (on dry weight) ranged from 11.15 to 48.33% [46]. These results suggest that, to improve the lipid extraction yield from microalgae, selection of suitable cell wall disruption technique is highly applicable. Since each microalga has a different cell wall structure, all of them not respond the same to different types of cell wall disruption techniques. Hence, research studies have suggested that results achieved from one species could not be compared with other species [47].

The microalga oil belonging to the *Tetradesmus obliquus* examined for fatty acid compositions using GC-MS analysis exhibited the following fatty acid profiles: C12:0, C14:0, C16:0, C16:1, C16:2, C16:3, C16:4, C17:0, C18:0, C18:1, C18:2, C18:3, C18:4, C20:0, C20:1, C22:0, and C24:0. Main fatty acids like palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) were found in both nitrogen-containing and nitrogen-deficient media wherein microalga was cultivated. The C16-C18 fatty acid components are essential for the production of high-quality biofuel to exhibit good performance in combustion and low-temperature flow [48]. The proportion of C16-C18 fatty acids exceeded 95% of the total fatty acids in both tested media. One of the studies reported that the proportion of C16-C18 fatty acids increased to 80% by following a two-step cultivation method [23]. Whereas in the present investigation, by employing the two-step cultivation method, the proportion of main components like palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) were increased considerably in the nitrogen-deficient medium. But the contents of hexadecatrieonic acid (C16:3), linoleic acid (C18:2), α-linolenic acid, and γ-linolenic acid (C18:3) were decreased in the same medium. According to European biodiesel standard (EN14214)

for biodiesel production, it should contain high levels of saturated and monounsaturated fatty acids with low levels of polyunsaturated fatty acids, particularly C18:3 or C16:3 [44]. Thus, the lesser in a proportion of these components in a nitrogen-deficient medium proved to be acceptable for the production of good quality biofuel. Shao et al. [49] reported that *Tetradesmus obliquus* grown in medium containing 10^{-1} mmol L^{-1} ferric iron had significantly higher amount of saturated and monounsaturated fatty acids than in medium containing a lower amount of iron concentration, indicating that changes in fatty acid components in each type of microalgae strains mainly depend upon the presence of nutrient components in a growth medium [49]. The present study demonstrated that the cultivation of microalga in the nitrogen-deficient medium could improve the production of desirable fatty acid components, which could be applied to biofuel production.

CONCLUSIONS

An attempt was made to grow microalgae in nitrogen deficient medium to improve the lipid production in cells. Suitable cell disruption and lipid extraction techniques were implemented to achieve higher lipid and fatty acid yield. The outcomes of this study conclusively established that microalgae cultures with high lipid producing characteristics are present in diversified habitats. They need to be well characterized and exploited commercially toward the production of renewable biofuel for the wellness of the society. Finally, we can conclude that among screened isolates, *Tetradesmus obliquus* Strain1 produced the highest total lipid content, suggesting that it has significant potential for biofuel production and can be used for the same.

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