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# Screening and characterization of high lipid accumulating microalga *Ankistrodesmus* sp. from freshwater environment

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Microalgae based biofuel is an attractive alternative energy source due to its rapid growth rate and high lipid accumulation efficiency. In this study, we screened high lipid content microalga with the favourable fatty acid composition suitable for biodiesel production. Totally twelve different microalgal species were isolated from freshwater habitats. The isolates were identified as *Micractinium* sp., *Chlorella sorokiniana*, *Scenedesmus bajacalifornicus*, *Desmodesmus* sp., *Scenedesmus obliquus*, *Coelastrum proboscideum, Chlamydomonas moewusii*, *Chlamydomonas debaryana*, *Chlamydomonas dorsoventralis*, *Coelastrum* sp., and *Ankistrodesmus* sp. based on morphological features and ITS region similarity. Among the isolates, highest lipid content (33±0.07%) and lipid productivity (0.27±0.06 g $L^{-1}$) were obtained from *Ankistrodesmus* sp. Intracellular lipid droplets of *Ankistrodesmus* sp. were observed through Nile red staining. The lipid content was enhanced up to 45% under the nitrogen deficient (5 mg $L^{-1}$) BG-11 medium. *Ankistrodesmus* sp. fatty acid profile shows the presence of palmitic (16.39%), stearic (15.67%), oleic (25.66%), linolenic (21.62%), and alpha-linoleic acids (14.34%). The oleic acid was the dominant fatty acid 25.66% in the nitrogen deficient condition.

**Keywords:** Biofuel, Microalgae, Fatty acid profile, Nitrogen deficiency

Algae are a diverse group of organisms with vast application potential in numerous industries[1]. Microalgae, in particular, are an excellent feedstock for biodiesel production[2]. The rapid depletion of fossil fuel reserves, escalating demand for diesel, and increasing cost of petroleum fuels necessitate an alternative cost-effective energy source for human consumption. The renewable source should be carbon neutral and able to supplement or replace fossil fuels. Nowadays, biodiesel has received much attention worldwide due to its environmental benefits. Microalgae are potential source of renewable fuel and its biomass may play a key role in future energy supply[3]. It has higher photosynthetic efficiency, more biomass production, and it has ability to fix more atmospheric $CO_2$ from the variety of sources[4,5].

Selection of high lipid content and a favourable fatty acid composition consisting of microalgae is essential for biodiesel production. The selected microalgal strain should have the following properties: (i) rapid biomass productivity; (ii) high lipid productivity; (iii) able to survive in stressful conditions; (iv) high $CO_2$ sinking capacity; (v) limited nutritional requirements; and (vi) provides valuable co-products[6]. In the present study, a freshwater microalgal isolate *Ankistrodesmus* sp. was isolated and further characterized by lipid accumulation and fatty acid profile analysis.

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## Materials and Methods

### Screening of freshwater microalgae

Water samples were collected from different freshwater habitats like the pond, ditch, stream, river, and lakes of Tamil Nadu, India. The samples were filtered to remove any debris and solid particles using double-layered what man (No. 1) filter paper and inoculated into BG-11 (pH 7±0.05) media[7]. The samples were incubated at 25±1°C at a light intensity of 100 µ mol photons $m^{-2}s^{-1}$ and 16:8 light/dark cycle until visible microalgal growth was observed. After visible growth, the broth culture was serially diluted and plated on BG-11 medium to obtain individual colonies. The purity of the culture was checked by repeated plating and by regular microscopic examination (Hund Wizard, Germany). The microscopic identification was done based on the morphological characteristics like shape, colonial, filamentous and motility of the isolates. All the isolates were maintained in BG-11 agar slants.

**Molecular characterization**

Colony PCR protocol was followed as reported by Radha *et al*.[8]. The internal transcribed spacer (ITS-2) region was amplified using universal primer[9]. The primer sequences for the ITS-2 region includes forward 5'-ATGCGATACTTGGTGTGAAT-3' and reverse 5'-GACGCTTCTCCAGACTACAAT-3'. The PCR reaction components for 50 µL reaction mix includes 1 µL of DNA template, 1U of *Taq* DNA polymerase (Bangalore Genei, India), 5 µL of 10X PCR buffer, 1 µL dNTP mix (10mM each nucleotide) (Bangalore Genei, India), 1 µL of primer (5 picomoles of each, forward and reverse) (Bioserve, India). The conditions for PCR used were initial denaturation at 95ºC for 5min, annealing at 55ºC for 30 s, extension at 72ºC for 1 min for 35 cycles of ITS-2. The PCR reaction was carried out using Thermal Cycler (Applied Biosystems, USA). The PCR products were examined on 1% (w/v) agarose gel. The size of the amplified products was confirmed with a 100 bp molecular marker. The amplified products were purified using EZ-10 column purification kit (Biobasic Inc., USA) and subjected for sequencing using Automated DNA Sequencer AB1 3130 XL (Genetic Analyser, Applied Biosystems, USA). The obtained sequences were analysed using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Microalgae cultivation and total lipid extraction**

The isolated individual microalgal strains (18.95 cells/mL × $10^{-3}$) were inoculated into 1000 mL of BG-11 broth and incubated at 23±1°C for 16:8 light: dark conditions for growth for 21 days (until the culture reach the stationary phase). After incubation, the cells were harvested by centrifugation at 4000 rpm for 5 min and washed twice with de-ionized water. The pellet was collected and the algal biomass was dried using a freeze dryer and the dry weight was estimated[10]. The total lipid was extracted from microalgal biomass using Bligh and Dyer method[11]. Lipid productivity and biomass productivity was calculated for all the isolated species[12]. Experiments were performed in triplicate, and data were expressed as mean standard deviation (±SD).

**Nile Red staining of high lipid accumulating strain**

High lipid accumulating strain was subjected to Nile Red staining as per Matsunaga *et al*.[13]. Algal cells were collected by centrifugation at 1500 ×g for 10 min and washed with physiological saline solution (0.5 mL) several times. The collected cells were resuspended in the same solution (0.5 mL), the Nile Red solution (0.1 mg/mL in acetone) was added to cell suspensions (1:100 v/v) and incubated for 10 min. After washing once, stained microalgal cells were observed by a confocal microscope (Olympus Fluoview FV1000).

**Effect of nitrogen on biomass and lipid accumulation of high lipid accumulating strain**

The effect of nitrogen on BG-11 medium was studied with high lipid accumulating strain[14]. The isolate (18.95 cells/mL ×$10^{-3}$) was inoculated into modified BG-11 medium with sodium nitrate (mg$L^{-1}$) 375, 90, 23, 11, 5, 2.5, 1.25 and control without sodium nitrate. The culture was incubated for 18 days and cells were harvested by centrifugation and the biomass was collected and dried. Dry biomass weight was measured gravimetrically and lipid content was analyzed[11]. Triplicate experiments were carried out for all the experiments. Statistical significant differences were determined using analysis of variance (ANOVA) with probability limit of $P$ <0.05.

**Fatty acid profile analysis**

Fatty acid profile analysis was carried out for high lipid content strain grown on BG-11 and BG-11 with nitrogen (5 mg$L^{-1}$) medium. The extracted lipid was trans esterified with 2 mL 1% $H_2SO_4$ dissolved in methanol at 80°C for 60 min. The sample was cooled; 2 mL n-hexane and 5 mL water were added. The upper phase (n-hexane) was separated by centrifugation and used for fatty acid composition analysis. The fatty acid profile was analysed using a Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a flame ionization detector (280°C) and a fused silica column (30 m, 0.25 mm), helium was used as a carrier gas. Temperature conditions were 170-225°C for 55 min, the injection port temperature was 250°C and the sample volume was 1 µL. The components were identified by comparing their retention times and fragmentation patterns of standards. The following fatty acid components were used as the internal standards such as palmitic (C16:0), margaric (C17:0), stearic (C18:0), oleic (C18:1), linolenic (C18:2), alpha linoleic (C18:3) and moroctic acid (C18:4)[15].

## Results and Discussion

**Screening and characterization of freshwater microalgae**

Microalgae are simple autotrophic and photosynthetic organisms that are able to use energy from sunlight through the photosynthesis to combine with water and carbon dioxide to produce biomass.

They are present in all existing ecosystems such as aquatic, terrestrial, freshwater, brackish water, sea water, and wastewater[16]. So far, only a few freshwater species have been characterized for biodiesel production. In this study, seven different genera were isolated and identified based on microscopic observation (Hund wizard, Germany) (Fig. 1). Based on the cell shape and size, the isolated strains were identified as *Desmodesmus* (oval in shape), *Scenedesmus* (colonial forming of cells around 2-4 joined together and lying parallel to each other), *Ankistrodesmus* (cells are colonial, curved or sigmoid, with gradually tapered ends and it has autospore formation), *Coelastrum* (spherical or ovoid, pyramidal or cuboid, free-floating), *Chlorella* (spherical in shape), *Micractinium* (spherical in shape), *Chlamydomonas* ( motile and oval in shape)[17]

**Molecular characterization**

Internal transcribed spacer (ITS) region has been used in barcoding studies of fungi[18], diatom and green algae[9]. ITS regions are found between the 18S and 5.8S gene subunits and these regions are divergent and unique[19]. ITS region of the nuclear rDNA cistrons is one of the more frequently utilized regions for phylogenetic analysis at the genus and species levels[20]. ITS-2 region was amplified from the microalgal species and products were column purified and sequenced. The obtained sequences were subjected to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences were submitted to Genbank (Table 1). Of the 11 isolates, 9 species were identified at the genus and species level with more than 97% sequence similarity. However, three species were identified only at the genus level as *Ankistrodesmus* sp. (97%), *Chlamydomonas* sp. (99%) and *Coelastrum* sp. (99%)

**Biomass, lipid content and lipid productivity of microalgal species**

The 11 microalgal isolates were tested for their total lipid content by evaluating biomass and lipid productivity. Table 2 shows the biomass, lipid

Table 1 — List of isolated microalgal species with accession number and its percentage similarity

| Microalgal strain | Accession number | % Similarity |
|---|---|---|
| *Coelastrum proboscideum* SRM06 | JX485653 | 100 |
| *Desmodesmus* sp. SRM04 | JQ782747 | 99 |
| *Micractinium* sp. SRM08 | JX290027 | 98 |
| *Chlamydomonas dorsovantralis* SRM14 | JX041603 | 98 |
| *Scenedesmus bajacalifornicus* SRM09 | JX456466 | 99 |
| *Chlorella sorokiniana* SRM02 | JQ898145 | 98 |
| *Scenedesmus obliqus* SRM01 | JX290026 | 97 |
| *Chlamydomonas moewusii* SRM12 | JX290025 | 99 |
| *Ankistrodesmus* sp. SRM11 | JX456463 | 97 |
| *Chlamydomonas* sp. SRM03 | JX456467 | 99 |
| *Coelastrum* sp. SRM13 | JX456464 | 99 |

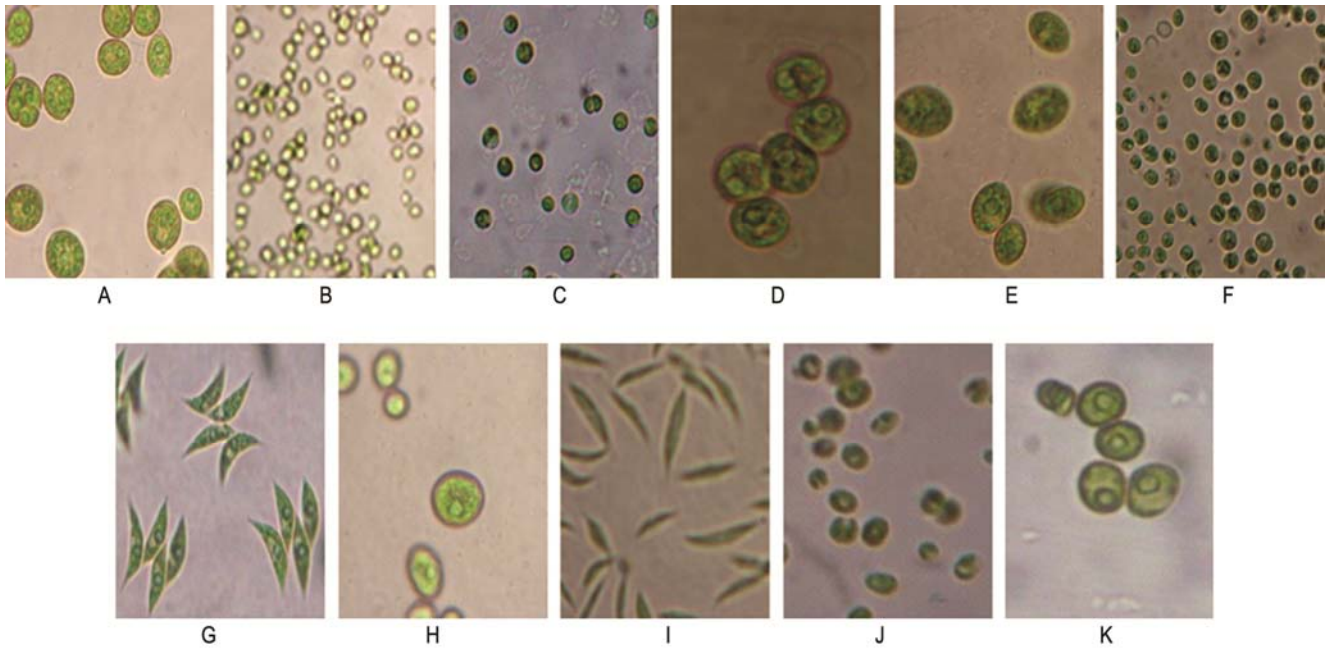


Fig. 1 — Light microscopic (40X) observation of isolated microalgal species. (A) *Coelastrum proboscideum* (B) *Desmodesmus* sp. (C) *Micractinium* sp. (D) *Chlamydomonas dorsovantralis* (E) *Scenedesmus bajacalifornicus* (F) *Chlorella sorokiniana* (G) *Scenedesmus obliqus* (H) *Chlamydomonas moewusii* (I) *Ankistrodesmus* sp. (J) *Chlamydomonas* sp.; and (K) *Coelastrum* sp.

content, and lipid productivity of isolated microalgal species. Biomass productivity of all the isolates was found to be ranging from 0.46±0.02 to 0.84±0.07 g dwt $L^{-1}$ and the total lipid content ranged from 10±0.25 to 33±0.07% based on dry wt. The highest lipid yield of 33±0.07% was obtained for *Ankistrodesmus* sp. and the lowest lipid yield of 11±0.18% was obtained for *S. bajacalifornicus.* The highest lipid productivity was 0.27±0.06 $gL^{-1}$ for *Ankistrodesmus* sp. and the lowest was 0.061± 0.08 $gL^{-1}$ for *Micractinium* sp. In the present work, the percentage of lipid content and lipid productivity for *Ankistrodesmus* sp. was high as 33±0.07% and 0.27±0.06 $gL^{-1}$, respectively.

A review by Mata *et al.*[21] stated that *Ankistrodesmus* sp. has the ability to accumulate 24-31% of lipid. In a similar study carried out by Talebi *et al.*[22] the lipid content was found to be only 17.5% by the freshwater *Ankistrodesmus* sp. UTEX 202/27 in Moh202 medium after 35 days of growth. However, in the present study the isolate, *Ankistrodesmus* sp. lipid content was reached 33±0.07% at the end of 21 days in BG-11 medium. The variation in the lipid accumulation could be due to medium components, growth period and conditions. In another study by Sukkrom *et al.*[23], *Ankistrodesmus* sp. was cultivated for the production of microalgal lipid where they found that around 30% lipid production could be enhanced when 5% by vol. of $CO_2$ was mixed with the air supply. The ratio of N and P could influence the biomass productivity and lipid yield[24].

**Nile Red staining**

Nile Red (9-diethylamino-5H-benzo (α) pheno-xazine-5-one) staining is a quick method to quantify intracellular lipids in microalgal cells. It is a red lipophilic dye that, permeates the microalgal cell membrane, interacts with intracellular lipid droplets, and shows yellow gold or green fluorescence[25]. In the present study, based on the high lipid content (33±0.07%), *Ankistrodesmus* sp. was chosen for intracellular lipid analysis. Fig. 2 shows the Nile Red staining of *Ankistrodesmus* sp. under the confocal microscope (100X). A lipid body emits green fluorescence within the cell upon interaction with Nile Red dye. The green spots indicate the presence of neutral lipids within *Ankistrodesmus* sp. Zhang *et al.*[26] demonstrated that the stronger fluorescence intensity is related to the oil productivity in *Botryococcus braunii.* Due to adjusting excitation and emission filters of confocal microscope; neutral lipid fraction detects green fluorescence. Therefore, the algal lipid bodies are visible green in colour. However, longer exposure to fluorescence could lead to experimental errors due to the quenching of fluorescence[27].

Table 2 — Biomass, lipid content, and lipid productivity of isolated microalgal species

| Microalgal strain | Biomass productivity (g dry wt $L^{-1}$) | Lipid productivity (g $L^{-1}$) | Lipid content (%) |
|---|---|---|---|
| *C. proboscideum* | 0.79±0.17 | 0.22±0.31 | 28±0.27 |
| *Desmodesmus* sp. | 0.64±0.09 | 0.115±0.12 | 18±0.21 |
| *Micractinium* sp. | 0.47±0.51 | 0.061±0.08 | 13±0.21 |
| *C. dorsovantralis* | 0.55±0.09 | 0.066±0.13 | 12±0.08 |
| *S.bajacalifornicus* | 0.57±0.03 | 0.063±0.01 | 11±0.18 |
| *C. sorokiniana* | 0.52±0.03 | 0.073±0.17 | 14±0.24 |
| *S. obliquus* | 0.49±0.13 | 0.078±0.02 | 16±0.10 |
| *C. moewusii* | 0.68±0.08 | 0.136±0.15 | 20±0.17 |
| *Ankistrodesmus* sp. | 0.81±0.11 | 0.27±0.06 | 33±0.07 |
| *Chlamydomonas* sp. | 0.48±0.11 | 0.056±0.02 | 12±0.21 |
| *Coelastrum* sp. | 0.84±0.07 | 0.21±0.04 | 25±0.06 |

**Effect of nitrogen on lipid accumulation**

The impact on biomass yield and the lipid content percentage with different concentrations of nitrogen was depicted in Fig. 3. The original nitrogen concentration in the BG-11 control medium was 1.5 $gL^{-1}$. The total lipid content was found to be 29–45% under different nitrogen concentrations ranging from 1.25

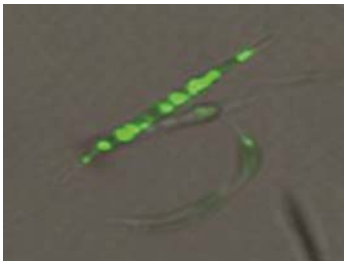

Fig. 2 — Nile Red staining of *Ankistrodesmus* sp. using confocal microscope (100X).

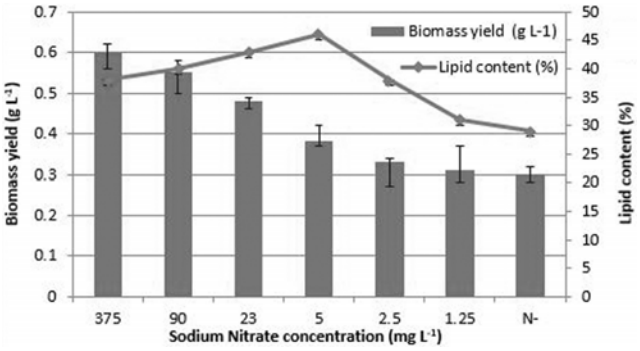


Fig. 3 — Effect of nitrogen on *Ankistrodesmus* sp. biomass and lipid content with different concentration of sodium nitrate ($mgL^{-1}$) and N- indicates absence of sodium nitrate in the modified BG-11 medium.

$mgL^{-1}$ to 375 $mgL^{-1}$ and without nitrogen. Among the six different concentrations of nitrogen and absence of nitrogen, 5 $mgL^{-1}$ showed the maximum lipid content as 45% and the minimum at 29% in the absence of nitrogen (nitrogen starvation). When nitrogen concentration was reduced from 375 to 5 $mgL^{-1}$, biomass productivity also gradually reduced from 0.6 to 0.3 $gL^{-1}$ owing to loss of algal pigments. Based on our results, the highest lipid content of 45% was achieved for 5 $mgL^{-1}$ of nitrogen concentration with low biomass productivity for 0.3 g $L^{-1}$.

Sheehan *et al*.[28] reported that the microalgal species biomass content increases in the nitrogen sufficient medium; however, when inoculated into the nitrogen deficient medium the biomass content got reduced and instead, oil content increases. This is because, in the nitrogen deficient condition, nitrogen containing macromolecules are converted into fat (TAGs)[29]. Therefore, the lipid content of *Ankistrodesmus* sp. could have increased under nitrogen deficient condition.

Another study stressed on the significance of combined stress of moderate nitrogen (750 $mgL^{-1}$) and high iron supplementation (9 $mgL^{-1}$) which resulted in increased lipid productivity by 25.56% when compared to the normal BG11 medium[30]. Using response surface methodology, they showed that nitrogen was the most influential factor followed by iron for increased lipid productivity.

**Fatty acid composition of *Ankistrodesmus* sp.**

The fatty acid composition of microalgae makes them a prospective source for biofuel production. The fatty acid composition of C16 and C18 series has properties similar to diesel[30]. In the current study, fatty acid profile analysis was carried out for *Ankistrodesmus* sp. using the lipid extracted from BG-11 (1.5 $gL^{-1}$) and nitrogen deficient (5 $mgL^{-1}$) medium. The extracted lipid was trans esterified and the fatty acid composition was analysed by gas chromatography. Table 3 indicates the fatty acid composition of *Ankistrodesmus* sp. lipids cultivated in BG-11 as compared with nitrogen deficient medium. The fatty acid composition of *Ankistrodesmus* sp. in BG-11 medium was found to be palmitic (15.67%), stearic (20.34%), oleic (20.44%), linolenic (22.65%), and alpha-linolenic acid content (17.89%), respectively. Lipid extracted from the nitrogen deficient medium showed less stearic (15.67%) and alpha-linoleic acid (14.34%) content as compared with BG-11 medium. Fatty acid like palmitic (15.67; 16.39%) and linolenic acid (22.65; 21.62%) content were similar in BG-11 and nitrogen deficient medium. The oleic acid content was high as 25.66% in the nitrogen deficient condition compared with BG-11 medium.

Table 3 — Fatty acid profile of *Ankistrodesmus* sp. cultivated in BG-11 (1.5 g $L^{-1}$) as compared with nitrogen deficient medium (5 mg $L^{-1}$)

| Fatty acid composition | BG-11 medium (%) | Nitrogen deficient medium (%) |
|---|---|---|
| Palmitic acid (C16:0) | 15.67 | 16.39 |
| Stearic acid (C18:0) | 20.34 | 15.67 |
| Oleic acid (C18:1) | 20.44 | 25.66 |
| Linolenic Acid (C18:2) | 22.65 | 21.62 |
| Alpha linolenic acid (C18:3) | 17.89 | 14.34 |
| Moroctic acid (C14:0) | ND | 1.45 |

[ND, Not detected]

Talebi *et al*.[21] analyzed the fatty acid profile of freshwater *Ankistrodesmus* sp. UTEX 202/27 grown on Moh202 medium (35 days) with nitrogen (0.8 $gL^{-1}$) concentration and found that the major fatty acid composition were oleic (17.66%) and linolenic acid (8.48%). In this study too, the most dominant fatty acids of *Ankistrodesmus* sp. were oleic (20.44) and linoleic acid (22.65%) in the BG-11 medium (21 days) with nitrogen (1.5 $gL^{-1}$). In nitrogen deficient condition oleic acid was the dominant fatty acid in the *Ankistrodesmus* sp. constituting up to 25.66% of total fatty acids. This is a commonly observed phenomenon for green algae[31]. Even though the species were same, the variation in the fatty acid composition could be due to growth medium and the concentration of nitrogen used for algal growth.

## Conclusion

High lipid accumulating strain was isolated from freshwater and it was characterized as *Ankistrodesmus* sp. (JX456463) based on the ITS-2 sequence similarity. Medium with nitrogen deficient condition (5 $mgL^{-1}$) enhanced the lipid content from 33 to 45%. The fatty acid profile analysis of nitrogen deficient condition (5 $mgL^{-1}$) showed higher oleic acid content (25.66%) which could improve the property of the algal based biodiesel fuel. However, further investigation is required to improve the total lipid and fatty acid content through outdoor cultivation condition.

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## Conflict of interest

The authors declare no conflict of interest.

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