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Research Article
Lipid Droplet Synthesis in *Chlorococcopsis minuta* Mediated by Nitrogen Deprivation for Higher Lipid Productivity

Shoaib Hamid and G. Sibi

Department of Biotechnology, Indian Academy Degree College-Autonomous, Bengaluru, India

Abstract

Background and Objectives: Physiological response in micro-algae triggered by nutrient stress to induce triacylglycerol accumulation is a promising solution to produce micro-algae based biofuel. The main objective of the study was to determine the response of micro-algae to nutrient stress by means of lipid accumulation. **Materials and Methods:** This study investigated the effect of nitrogen sufficient and nitrogen deficient conditions (N+ and N-) in micro-algal biomass and lipid productivity to determine the response to nutrient stress by micro-algae. A micro-alga was isolated from fresh water lake and amplification of 18S rDNA genes with the primers NS1 and NS4 resulted in 1300 bp fragments. The 18s rDNA sequences obtained were compared against sequences in the GenBank nucleotide collection through the Basic Local Alignment Search Tool (BLAST). **Results:** The micro-algae were identified as *Chlorococcopsis minuta* based on sequence similarities. In the case of micro-algae grown in nutrient limited conditions (N- media), the biomass content was lower with the yield of 8.4 g L^{-1}, however, there was a 62.5% increase in the total lipid content (11.7%) compared to nutrient sufficient conditions. Confocal imaging of lipid droplets stained by Nile red showed that lipid droplets was increased in N deficient (N-) conditions when compared to N sufficient (N+) conditions. **Conclusion:** Increase in lipid content of *C. minuta* was confirmed by accumulation of more lipid droplets under nutrient stress conditions.

Key words: Micro-algae, neutral lipid, biofuel, nitrogen starvation, *Chlorococcopsis*, nile red

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Corresponding Author: G. Sibi, Department of Biotechnology, Indian Academy Degree College-Autonomous, Bengaluru, India Tel: +91 99864 52875

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Biofuel production using micro-algae focuses on screening of potential micro-algae species, using wastewater to cultivate micro-algae, optimizing culture conditions and recombinant micro-algae. In order to make the industrial scale application of micro-algae based biofuel as cost effective, optimizing the culture conditions to trigger triacylglycerol accumulation is a widely recognized and promising solution. Various culture conditions including nutrient limitation[1,2], high salinity[3,4], temperature range[5], light intensity[6,7] were followed in the past to maximize the lipid accumulation in micro-algae. Different physiological responses were elicited by algae under nutrient stress conditions[8]. Nutrient limitation while cultivating micro-algae promotes fatty acid biosynthesis by micro-algae. Micro-algae would accumulate cellular lipid under nutrient stress[9-16], which would be considered for commercial exploration towards production of biofuels. Among the nutrient stress, nitrogen limitation can enrich the micro-algal lipid content in large scale production. The concentrations of nitrogen in the culture alter the composition of total fatty acids in micro-algae[17].

The correlation between nutrient stress and lipid accumulation in both fresh water and marine micro-algae is widely reported but the accumulation of lipid droplets under nitrogen deficient conditions in *Chlorococcoposis minuta*, to the best of authors' knowledge has not been carried out before. This study investigated the effect of nitrogen sufficient and nitrogen deficient conditions (N+ and N-) in micro-algal biomass and lipid productivity to determine the response to nutrient stress by micro-algae.

MATERIALS AND METHODS

This study was carried out at Department of Biotechnology, Indian Academy Degree College- Autonomous, Bangalore, India between January-September, 2018.

Collection of micro-algae: Algal blooms were collected from fresh water lakes in and around Bangalore (Table 1) and the samples were stored in a refrigerator until use and formalin (1/10) was used for preservation.

Isolation and sub-culturing: Isolation of algal cultures was performed by serial transfer and cultivating at 20±1°C in a growth cabinet. The relative humidity was kept at 31±1% and 12:12 h light/dark cycle was followed for 15-20 days.

Table 1: Sample collection points

Collection point	Latitude/longitude coordinates
Bellandur lake	12°56'11.1" N 77°40'04.2" E
Benniganahalli lake	12°59'50.4" N 77°39'55.6" E
Madiwala lake	12°54'27.9" N 77°37'00.4" E
Ulsoor lake	12°59'01.8" N 77°37'11.6" E
Sankey tank	13°00'34.6" N 77°34'26.7" E
Hebbal lake	13°02'50.3" N 77°35'08.0" E

Aeration was done for 8 h daily using aquarium motor. Cultivation was done simultaneously in open systems by exposing the culture bottles to natural sunlight with aeration.

Identification of micro-algae: The grown algal cells were identified based on their morphology under micro-scope and the lipid containing species were selected for further studies.

DNA extraction: Microalgal cells were harvested from a culture by centrifugation and the total DNA was isolated using NucleoSpin® Microbial DNA column kit. The quality of isolated DNA was evaluated on 1.0% agarose gel.

Polymerase chain reaction: Amplification of the 18S rDNA region was conducted in a reaction mixture with a final volume of 20 µL that contained about 50 ng of template DNA and primers using the PCR Master Mix (Primers each-0.5 µM, *Taq* Buffer-1X, $MgCl_2$-1.5 mM, dNTP's-500 µM, *Taq* Polymerase-1U) and a Techne T.C 212 thermal cycler. 18S rDNA genes were amplified by polymerase chain reaction (PCR) with forward primer NS1 (5'-GTAGTCATATGCTTGTCTC -3') and reverse primer NS4 (5'- CTTCCGTCAATTCCTTTAAG -3'). The reaction consisted of initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 90 sec, with a final extension at 72°C for 5 min. A single discrete PCR amplicon band of ~1300 bp was observed when resolved on agarose. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with NS1 and NS4 primers using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer.

Sequencing: The purified DNA was sequenced at Eurofins Genomics, Bangalore. Nucleotide sequences were determined by automated sequence analysis using a Perkin Elmer/ABI Prism 3130 four capillary based DNA sequencer (Perkin Elmer, Foster City, CA) and Applied BioSystems BigDye Terminator version 3.1 (Perkin Elmer, Foster city, CA) and analyzed using ABI Sequencing Analysis 5.3.1 software.

Phylogenetic analysis: The 18s rDNA sequences obtained were compared against sequences in the GenBank nucleotide

collection through the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/). Phylogenetic trees were constructed using the neighbor joining method from The Molecular Evolutionary Genetics Analysis V5 (MEGA5) software after 1000 rounds of bootstrap resampling.

Nutrient stress: In order to determine the genetic variations that lead to lipid production by the micro-algae, nutrient stress was selected. Two sets of experiments were carried out in which Bristol's basal medium as a control and Bristol's medium without nitrogen source was used for experiments. The microalgal cells were inoculated into two sets and cultivated for a period of 15 days. The culture was kept at 25±2°C under a continuous cool white fluorescent light illumination at 100 μmol m^{-2} sec^{-1}. After stipulated time period, the algal cells were separated from the culture broth by centrifugation at 2000 rpm for 5 min and analyzed for growth and lipid productivity.

Biomass yield: Biomass (g L^{-1}) of micro-algae was determined by measuring the optical density of samples at 600 nm (OD_{600}) using UV-Vis spectrophotometer. Biomass concentration was then calculated by multiplying OD_{600} values with 0.6, a pre-determined conversion factor obtained by plotting OD_{600} versus dry cell weight (DCW). The biomass yield was calculated from the following equation:

$$\text{Biomass yield (g L}^{-1}) = (B_t\text{-}B_0)\times\text{Volume of culture}$$

where, B_t is the biomass concentration at the end of cultivation period (T_t) and B_0 is the initial biomass concentration at the beginning of the cultivation period (T_0).

Total lipid estimation: Lipid extraction from dried algal cells were carried out by chloroform: methanol extraction method[18]. The total lipid content of dry weight was calculated using the following Equation:

$$\text{Lipid content (\%)} = \frac{m_2\text{-}m_0}{m_1}\times 100$$

where, m_1 is the weight of the dried algal cells, m_0 is the weight of the empty new screw cap tube and m_2 is the weight of the new screw cap tube with the dried lipid.

Confocal microscopy: The distribution of intracellular neutral lipid bodies in microalgal stained cells was studied by confocal microscopy. After staining with Nile red (10 μg mL^{-1}), the slides were prepared using 10% glycerin (v/v) and observed under confocal microscope (Confocal Zeiss LSM880) equipped with Airyscan. The excitation wavelength was set to 530 nm and the emission wavelength at 604 nm for both neutral and polar lipids. The fluorescence measurements to oil content was converted using the results from the internal standards.

RESULTS

Identification of micro-algae: The amplification of 18S rDNA genes with the primers NS1 and NS4 resulted in 1300 bp fragments visualized in agarose gel. Nucleic acid sequences of the 18S rDNA genes were compared with those available in the GenBank database using NCBI/BLAST to search for related sequences. All of the determined sequences corresponded to known species with a high sequence similarity. According to BLAST analysis of corresponding sequences, it was indicated that micro-algae strain was closely related to *Chlorococcopsis minuta* based on sequence similarities (Fig. 1).

Biomass and Lipid content under nutrient stress: In order to determine the effect of nutrient deprivation in micro-algae, media without nitrogen source was used to produce nutrient stress and media with nitrogen source was used as control. The results revealed that biomass content was higher in non-stressed micro-algae (N+ media) with a yield of 12.1 g L^{-1}, whereas the total lipid content was 7.2% (Fig. 2). In the case of micro-algae grown in nutrient limited conditions (N- media), the biomass content was lower with the yield of 8.4 g L^{-1} and the total lipid content was 11.7%.

Lipid droplet synthesis under N deprivation: To investigate the lipid droplet formation in *C. minuta* under nitrogen deprived conditions, the cells were visually assayed using confocal microscopy after incubation with Nile red. The confocal imaging of lipid droplets stained by Nile red correlated with the lipid content experiments. The number of lipid droplets was increased in N deficient (N-) conditions when compared to N sufficient (N+) conditions (Fig. 3, 4).

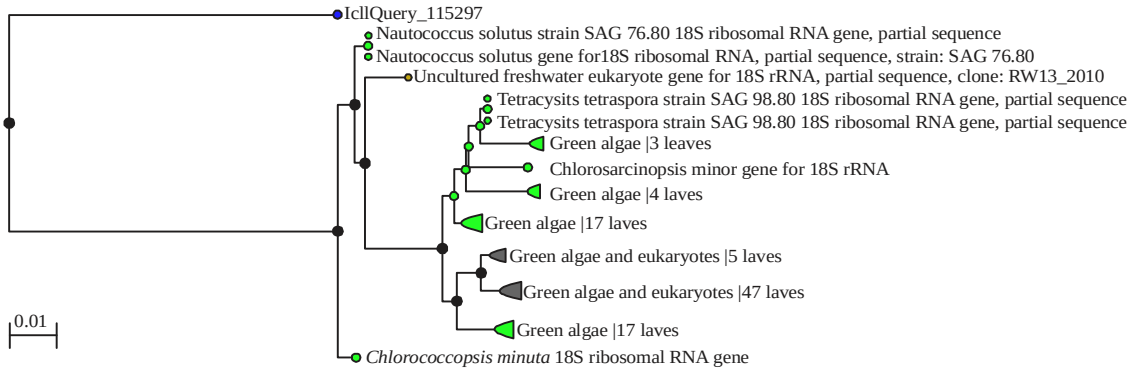

Fig. 1: Dendrogram constructed by the Neighbor-joining method

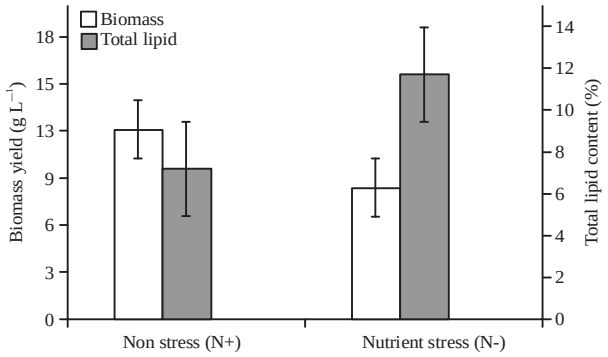

Fig. 2: Variations in biomass yield and total lipid content of micro-algae under N sufficient (N+) and N deficient (N-) conditions

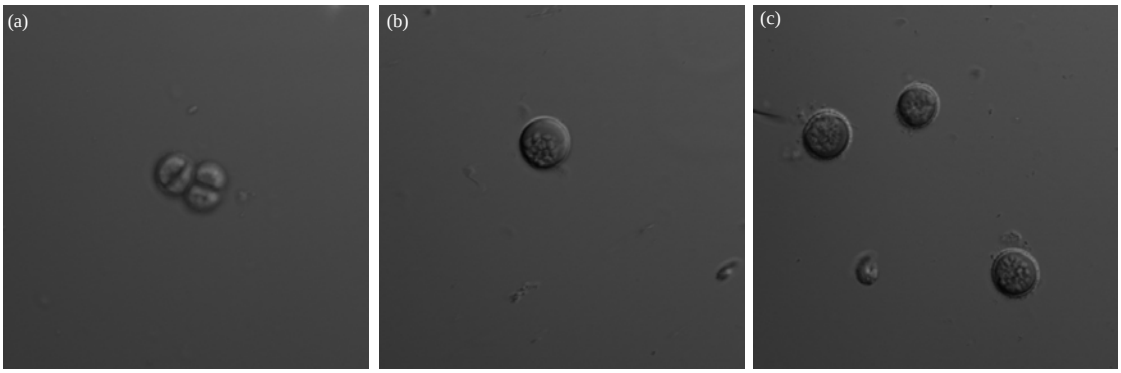

Fig. 3(a-c): Confocal imaging of *C. minuta* cells grown under N sufficient (N+) conditions for a period of 15 days, (a) 2nd day of cultivation, (b) 5th day of cultivation and (c) 15th day of cultivation indicates absence of lipid droplets
Scale bars represent 10 µm

DISCUSSION

In the present study, the total lipid content of *C. minuta* under nutrient stress was 62.5% higher than cells grown under non-stress conditions. Similar results were obtained in *Chlorella* sp., where the lipid content was 70% higher when the micro-algae were cultivated in nitrogen deficient medium[19]. Micro-algae grown under optimal conditions produce higher biomass while micro-algae grown in nutrient starvation accumulate high levels of neutral lipids[20]. However,

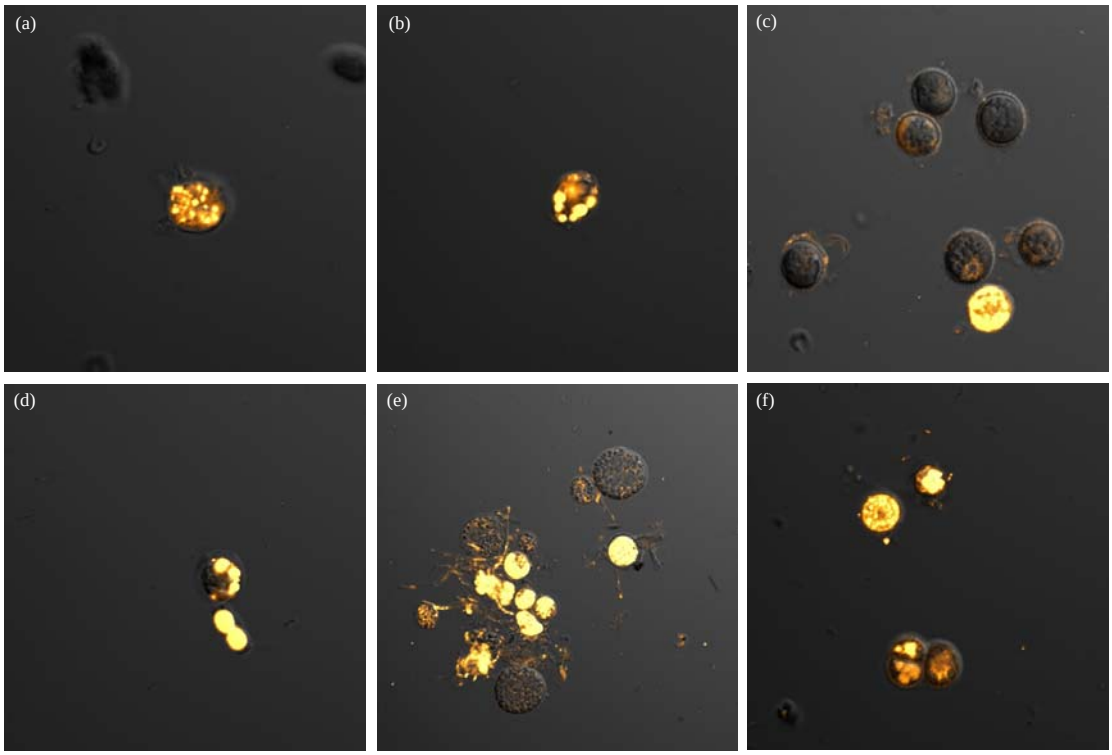

Fig. 4(a-f): Confocal imaging of *C. minuta* cells grown under N deficient (N-) conditions for a period of 15 days, (a) 4th day of cultivation, (b) 6th day of cultivation, (c) 8th day of cultivation, (d) 10th day of cultivation, (e) 12th day of cultivation and (f) 15th day of cultivation indicates lipid droplet accumulation in cells as yellow fluorescence stained by Nile red. Scale bars represent 10 µm

the reduction in biomass content under nutrient limited conditions can be countered by other culture conditions. In a study using *Chlamydomonas reinhardtii* with respect to lipid metabolism under phototrophic and mixotrophic conditions, increased cellular lipid content with highest lipid content (42%) under nitrogen limitation with the addition of acetate was observed[21]. Similar results were reported in which addition of sodium acetate along with nitrogen and phosphorous deficiency increased both the biomass and total fatty acid yield (150.1%) than normal culture conditions[22]. In another study, addition of urea has balanced both lipid and biomass accumulation in *Chlorella* sp.[19].

Stress in micro-algae leads to increased lipid content and can decrease the biomass due to inhibited cell division[23,24]. Nutrient stress resulted in accumulation of triacylglycerols in micro-algae which could be utilized for the production of biofuel[25]. The absence of one or other macro-nutrients in the culture medium affects the cellular homeostasis and oleaginous micro-algae accumulate more lipids under nutrients stress conditions[26-28], which is due to the decreased cellular proliferation rate and photo synthetic activity[29]. Increase in the lipid content of micro-algae in the absence of nitrogen was indicated in previous studies[9,30,31]. Findings of this study revealed that the nitrogen starvation can result in the accumulation of lipid productivity in *C. minuta*.

CONCLUSION

In this study, the effect of nutrient deprivation on biomass and lipid productivity of *Chlorococcopsis minuta* by means of removing nitrogen source from the growth medium was determined. The findings revealed that the presence of nitrogen source in the growth media increased the total biomass yield but resulted in lower lipid content in the cells. On the contrary, the absence of nitrogen source has increased the lipid content indicating the nutrient stress mechanisms for higher lipid productivity in micro-algae.

SIGNIFICANCE STATEMENT

This study explored that the increase in lipid content of *C. minuta* as confirmed by accumulation of more lipid droplets under nutrient stress conditions indicated the positive effect of nitrogen limitation in the growth medium.

The results can be beneficial in terms of increased lipid productivity in *C. minuta* through nitrogen deprivation. The scope for future research involves identification of nitrogen assimilation-associated genes and their expression under nutrient stress (nitrogen starvation) conditions.

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