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Implications of Nitrogen Limitation and Sufficiency on the Acetyl Co-A Carboxylase Activity and Biodiesel Production from Marine *Chlorella Vulgaris* (JF894250)

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ABSTRACT

This study investigated implications of nitrogen limitation and sufficiency on the Acetyl Co-A carboxylase activity for stimulating lipid productivity in marine Chlorella vulgaris (JF894250). To identifying optimal nitrogen concentration the effect of N-limitation and N- sufficient conditions (0.36 to 142.86 mM) on growth and lipid accumulation of C. vulgaris was investigated. Maximum biomass production, specific growth rate of C. vulgaris was recorded (0.19 $gL^{-1}day$) during cultivation with an initial nitrate concentration of 142.86 mM. However, the maximum total lipid content was 113.46 mg/g of C. vulgaris on dry weight basis and neutral lipid was 61.29 µg eq triolein/ml in wet sample with 0.36 mM nitrate and the lipid productivity of 0.0094 $gL^{-1}day^{-1}$ was achieved with 0.36 mM nitrate. Correspondingly the lipid contents the fatty acids like C16:0, C18:0, C18:1 showed a clear effect viz. o viz. with N-limited condition. Hitherto the of ACCase contents of microalgae was strongly influenced by the variation of nitrogen concentration. The investigation also elucidated the direct response of acetyl co-A carboxylase (Accase) to N- limitation-sufficient conditions on the lipid accumulation in Chlorella vulgaris. Correspondingly the lipid content and the saturated fatty acids which contributes the major part of biodiesel was recorded a 90.42% of the total fatty acids under N-Limted conditions (0.36 Mm nitrate) while which showed a lucid expression of effect of which working hand in hand (vis o vis) of ACCase contents of microalgae was strongly influenced by the variation of nitrogen concentration.

Keywords: *Chlorella vulgaris*; Nitrogen limitation; Acetyl-CoA carboxylase; Lipid content; Biofuel

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INTRODUCTION

Microalgae have the remarkable ability to utilize non- arable land, saline water and the potential to transform sunlight and carbon di oxide into energy dense neutral lipids that could be readily transesterified into biodiesel [1]. Chlorophycean, microalgae belonging to the genus *Chlorella* have gained renewed attention as ideal feed stock for second generation biofuel production due to their ability to grow in diverse environment, to utilize multiple carbon source [2], to accumulate high biomass and lipid content (upto 55% of cellular dwt) with requisite fatty acid composition. Biodiesel is a low-value product from microalgae; hence lipid productivity should be done at a very low cost inorder to make it cost effective. The selected microalgal strain for biodiesel production should have the rare feature of high lipid content and high cell growth rate [3]. Lipid accumulation can be altered by temperature, light intensity, pH, salinity, mineral salts and nitrogen source. Nutrient stress induced alteration of biosynthetic pathways leading to high triacylglycerol (TAG) accumulation between 30 to 60% have been previously reported in microalgae [4] with most of the studies conducted in the model green algae *Chlamydomonas reinhardtii*. However, many aspects of TAG accumulation in response to nitrogen starvation and nitrogen replete condition remain

unresolved. Furthermore, it is intriguing to know whether what is observed in *Chlamydomonas* is conserved in other green algae with greater potential for outdoor, large-scale production of biofuels**.** Employment of nitrogen starvation usually causes a decrease in microalgal growth rate, thereby lowering the overall lipid productivity. Therefore, there is still an urgent need to develop an efficient lipid production strategy. Triacylglycerides (TAGs) are the dominant form of lipids postulated to be produced under stress conditions [3]. TAGs and polar lipids are synthesized from fatty acids. Fatty acid biosynthesis commences with the conversion of acetyl CoA to malonyl CoA through the enzyme acetyl CoA (ACCase). This reaction is the first step in fatty acid synthesis but its product serves as a universal precursor for various other compounds including biodiesel. The regulatory and rate-limiting role played by acetyl-CoA carboxylase makes this enzyme a powerful tool in a variety of biotechnological applications inclusive of engineering the microalgae for high biodiesel production. Very few studies [5,6] have reported on the variation in the biochemical mechanism of lipid synthesizing enzymes during nitrogen limitation. Hence, the present study focused on analysis of biomass, total protein, carbohydrate and fatty acid methyl ester (FAME) composition under nitrogen replete and limited condition and purification and characterization of ACCase under nitrogen limitation and sufficient conditions.

MATERIALS AND METHODS

Identification of Isolated Strain

The phylogenetic identification of high lipid yielding strain of marine microalgae (isolated from coastal region of Pondicherry Sea) was done by sequencing its 18S rRNA. The genomic DNA was isolated from the micro algal cells by CTAB extraction method. The 18S rRNA sequences of the genomic DNA was amplified by PCR (AATCAACCTGACAAGGCAAC3′) and then sequenced using Applied Bio systems (3130 Genetic analyser).

Nurtient Medium and Experimental Design

The modified f/2 medium [7] was used in this study. The experiments were conducted at six different concentrations of sodium nitrate (0.36 mML^{-1} to 142.86 mML^{-1}). The gradient beginning from 0. 036 mML^{-1}, 0.71 mML^{-1}, 1.43 mML^{-1}, and 35.71 mML^{-1}, 71.43 mML^{-1}, and 142.86 mML^{-1}. The experiments were carried out in 2 L Erlenmeyer flasks. The medium and flasks were sterilized in an autoclave with 121℃ (250F) at 100 kPa (15 psi) above atmospheric pressure for 15 minutes in order to prevent any contamination during the early stages of growth. Each autotrophic batch cultivation was carried out in triplicate for 12 days at a continuous photon flux density of 70 $\mu Em^{-2} s^{-1}$ which was measured by using Digital Lux Meter (LX-101).

Cell Density

The cell density was measured at OD_{540} by using spectrophotometer (Unicam-UV300, UV- Vis double beam spectrophotometer) and the cell count was also monitored by counting the cells at 48 hr intervals using haemocytometer (Neubauer brand).

Detrmination of Biomass

Dry weight biomass:

Aliquots of algal suspension were filtered through preweighed, precombusted (100℃, 24 hrs) glass fiber filters (Whatman GF/C, 47 mm, nominal pore size 1.2 µm) and the filtered algal samples were washed with 20 ml 0.5 M ammonium bicarbonate. Triplicates for each group were treated in the same way. The filters were then dried at 95℃ to a constant weight, cooled down in a vacuum desiccator, and then weighed to obtain the dry weight [8].

Determination of ATP (adenosine tri phosphate) content:

The micro algal suspension was filtered through 25 mm Millipore GS filter (0.2 µm pore size) and the residue was extracted with a hot Tris buffer. The aliquot of 200 µl extract was added to 200 µl of firefly lantern luciferin and luciferase (Sigma Aldrich). The light emitted is proportionally equal to the amount of ATP extracted and the emission of light is measured using a luminometer (Berthold Sirius V2.2).The relative luminescence units (RLU's) obtained from the samples were correlated with the standard RLU's to obtain the amount of ATP in ng/ml. ATP in particulate material gives an insinuation of the biomass of the microalgae.

Neutral lipid estimation:
The fluorophore Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, C_{20} H_{18} N_2 O_2, Sigma aldrich) was used to determine neutral lipid in microalgal cells [9]. 3 µl of 1 $mgml^{-1}$ Nile red stock was added to 3 ml of algal sample. After Nile red addition, the fluorescence was measured at 475 nm excitation and 580 emission wavelengths using Hitachi F-2500 fluorescence spectrophotometer [10]. Sample fluorescence was standardized against triolein (1,2,3-trioleyl-*sn*-glycerol, $C_{57}H_{104}O_6$, Sigma Aldrich).

Microscopic analysis of nile red stained cells:
Nile red is used for the detection of intracellular lipid droplets by Epiflourescence microscope. In this study, 3 µl of dye (1 $mgml^{-1}$) has been added to 1 ml of cell suspension and then samples were viewed in Zeiss microscope [9].

Total lipid content:
Total lipid was estimated gravimetrically adopting the method by Folch et al. 100 mg of lyophilized algal biomass was lysed using probe ultra sonicator for 2 minutes repeatedly for five to six times, using a total of 30 ml of methanol: chloroform: water to achieve a ratio of (1:2:0.8; v/v/v). The supernatant was filtered through Whatman No. 41 coarse filter paper and the filtrate was collected into a separating funnel. Add $1/5^{th}$ part volume of water was added after vigorously mixing, the samples were kept overnight in the dark for clear phase separation at room temperature. The lower chloroform phase was collected in a pre- weighed clean beaker and allowed to dry to obtain the final weight of total lipid and this was further re-extracted for GC analysis.

Total protein content:
100 mg of lyophilized sample was weighed and 1 ml of 1 N sodium hydroxide was added and the cells were lysed by a probe ultra sonicator for 2 minutes repeatedly for five to six times and centrifuged at 3000 rpm for 10 minutes and the supernatant was taken for protein estimation using Lowry et al. BSA as standard [11]. The intensity of the colour was measured spectrophotometrically at 660 nm.

Total carbohydrate content:
Total carbohydrate was estimated following the phenol-sulphuric method. 100 mg of lyophilized sample was homogenised in 1 ml of distilled water. To the 1 ml of the algal biomass suspension was mixed with 1 ml of cold 5% phenol and 5 ml of concentrated sulphuric acid was added to develop colour. The intensity of the colour was measured spectrophotometrically at 490 nm.

Gas chromatography of Fatty Acid Methyl Esthers (FAME)
Total lipids from the algal biomass were transesterified using the method suggested by. The column used was a BPX 70 of 30 m × 0.32 m × 0.25 µm (SGE International Private Ltd) and a Flame ionization detector was used. The carrier gas used was Nitrogen (30 ml/min) and the mobile phase was Hydrogen (35 ml/min). The startup oven temperature was 140°C for 5 min and was then increased at a rate of 4°C/min to achieve the final temperature of 240°C and held for 20 min. The Chromatograms of standard (FAME mix C4 - C24, Sigma Aldrich) and the samples were compared to characterize the composition of fatty acid methyl esters.

Determination of Nitrate in Sea Water
The molar concentrations of nitrate nitrogen utilized by the microalgae in the control, nitrogen limitation and nitrogen sufficient studies were estimated by the method suggested by Grasshoff et al. [12]. To 50 mL volume of the filtered sea water sample 1 mL aliquot of the sulphanilamide was added and mixed. After a reaction time of about 1 min, 1 mL of the N-1-naphthy1-ethylendiamine dihydrochloride reagent was added, mixed and a reaction time of 15 min was imparted. Absorbance was measured spectrophotometrically at wavelength of 540 nm.

Purification and Characterization of Acetyl CoA Carboxylase
Preparation of cell free extract:
The known amount of lyophilized cultures were suspended in 30 ml of extraction buffer (100 mM MES pH 6.5, 10 mM K-Citrate, 2 mM Dithiothreitol, 2 mM EDTA, 100 mM $NaHCO_3$ and 200 µM PMSF) and disrupted by physical means, i.e., with the help of pestle and mortar in the presence of ice. Then the pressate was then centrifuged at 34,000 g for 15 min at 4°C. The supernatant was used for further purification steps. Cell disruption and all subsequent purification steps were carried out at 4°C.

Ammonium sulphate fractionation:
Ammonium sulphate was added to the cell free extract kept in ice bath, with slow stirring to yield 30% saturated solution which was then centrifuged at 34,000 g for 10 min. The precipitate was discarded and the supernatant was subjected to 80% saturation. The precipitate obtained was dissolved in 3 ml of extraction buffer and then dialysed for 4 hrs at 4ºC against extraction buffer.

Affinity chromatography:
The avidin-agarose (Sigma Aldrich) was poured in to an appropriate column and washed with 5 to 10 column volumes of wash buffer (100 mM MES buffer pH 6.5, 100 mM KCl, 2 mM Dithiothreitol). Then the sample containing biotinylated protein was applied to the column. Acetyl CoA Carboxylase was eluted from the column using elution buffer(100 mM MES buffer pH 6.5, 100 mM KCl, 2 mM Dithiothreitol, 0.5 mg biotin/ml) as described [6,13].

SDS-PAGE:
The purified enzyme samples were then electrophoresed along with protein marker to know the purity as well as the molecular weight of the purified enzyme.

Assay for Acetyl CoA Carboxylase Activity
Acetyl-CoA carboxylase (ACCase) was purified to near homogeneity, characterized and its specific activity was quantified using radiolabelled ^{14}C assay. The affinity eluted enzyme samples from the control, nitrogen limited and nitrogen sufficient cells were assayed to elucidate the activity of ACCase by the incorporation of ^{14}C bicarbonate into acid and heat stable product of the enzymatic reaction (malonyl Co-A) using the procedure of Roessler, 1990. The assay buffer contained 100 mM Tricine buffer (pH 8.2), 0.5 mM acetyl Co- A, 1 mM ATP, 2 mM $MgCl_2$, 10 mM KCl, and 10 mM [^{14}C] $NaHCO_3$ (Specific activity = 11.1 MBq/mmol). The reaction commenced by the addition of enzyme and terminated after 10 min at 30°C by the addition of 0.5 ml of 2 N HCl. Then it was transferred to a scintillation vial and heated at 70°C until dry. The residue was then dissolved in 0.3 ml of 2 N HCl and scintillation cocktail was subsequently added, the ^{14}C count was taken using Wallac 1409 DSA Liquid Scintillation counter. In order to correct for nonspecific radioactivity, control assays were also carried out without acetyl-CoA. Under standard assay condition, one unit of activity is defined as the amount of enzyme required to catalyze the formation of 1 µmol of malonyl CoA per minute and per mg.

RESULTS AND DISCUSSION

Effects of Initial Nitrogen Concentration on Cell Growth and Lipid Production of *C. vulgaris* (NIOT-74)
Chlorella vulgaris (NIOT 74) was grown in original nitrogen concentration of f/2 medium (7.14 mM) as control and 1/5 (1.43 mM), 1/10 (0.71 mM), and 1/20 (0.36 mM) as nitrogen limited concentrations and 5X (35.71 mM), 10X (71.43 mM) and 20X (142.86 mM) as nitrogen replete concentrations. Figure 1 shows that when nitrogen concentration was decreased from 7.14 mM to 0.36 mM the biomass production declined from significantly ($P<0.05$) from 0.89 ± 0.02 gL^{-1} to 0.44 ± 0.02 gL^{-1}, whereas lipid productivity increased from 5.88 $mg.L^{-1}.d^{-1}$ to 9.45 $mg.L^{-1}.d^{-1}$. Highest lipid productivity also occurred at the lowest nitrogen concentration (0.36 mM). Conversely, the biomass production increased with the increase in nitrogen concentration upto 71.43 mM beyond which there was decline in biomass production. Maximum biomass production 1.20 ± 0.005 $g.L^{-1}$. The cell count values and specific growth rate mirrored the biomass production. Correspondingly the ATP production increased with the nitrogen concentration upto 71.43 mM beyond which there was a decline (Figure 2). Nitrogen limited conditions showed a significant reduction in biomass production ($P<0.05$), whereas an appreciable increase was recorded in nitrogen sufficient condition. Thus it is indicated that nitrogen has a strong influence on the growth of the marine microalgae *Chlorella vulgaris*.

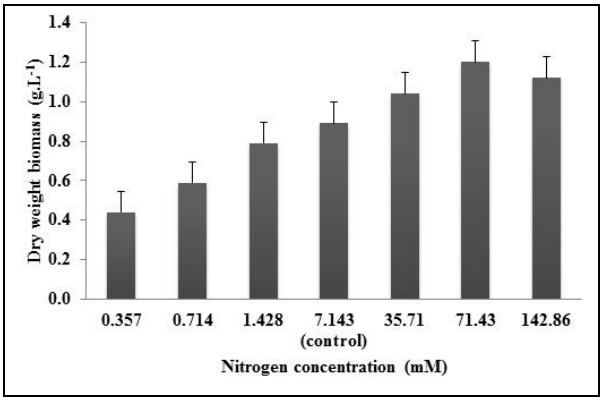

Figure 1: Dry weight biomass production in *Chlorella vulgaris (NIOT-74)* grown in f/2 medium with different initial nitrogen concentration. Values are mean ± SD

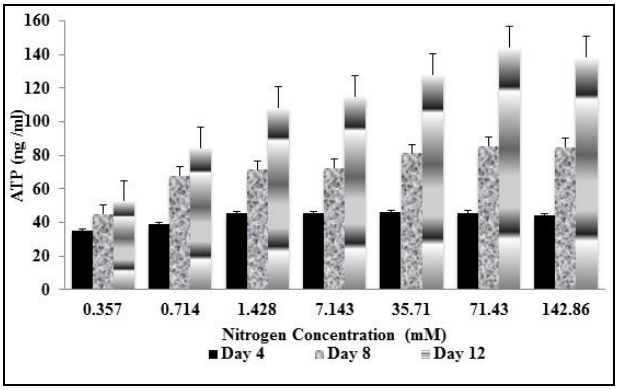

Figure 2: ATP production in *Chlorella vulgaris (NIOT-74)* grown in f/2 medium with different initial nitrogen concentration values are mean ± SD

The present results suggest that the optimal nitrogen concentration for cell growth as well as lipid accumulation was 1.43 mM. The optimal initial value was high enough to support sufficient growth and low enough to permit rapid depletion of nitrogen so that adequate lipid productivity and biomass productivity is achieved in a single strategy. To circumvent the problem of decreasing biomass yield during nitrogen limitation, a commonly recommended countermeasure proposed by Dorval et al. is to use a two stage cultivation strategy, dedicating the first stage for micro algal growth in nitrogen rich medium and the second stage for lipid accumulation under nitrogen limitation or other physiological stress. But in the two stage operation algal biomass production is normally reduced greatly after its transfer to nitrogen limited or nitrogen free media with a concomitant decrease in lipid productivity [14]. Hence, as revealed by the results single stage nitrogen limitation using optimal nitrogen concentration seems to be more efficient for stimulating lipid productivity in marine *C. vulgaris*. Similar single stage lipid productivity stimulating Potassium nitrate concentration of 0.313 g L^{-1} has been advocated by Yeh and Cheng for freshwater *C. vulgaris*. The optimal nitrate concentration advocated by these researchers is higher than the optimal concentration (0.2 g.L^{-1}) obtained during the present study. From the economy point of view this is a desirable trait to reckon with.

Effects of Initial Nitrogen Concentration on the Fatty Acid Profile of *C. vulgaris* (NIOT-74)

The initial nitrogen concentration of the culture media has also been reported to affect the fatty acid profile of microalgal species. Table 1 elucidates the change in fatty acid profile of marine *C. vulgaris* as a function of initial nitrogen concentration of the culture media. When nitrogen concentration was decreased from 7.14 mM to 0.36 mM the the fatty acid species C16:0 increased significantly fom 9.59% to 43.53% ($P<0.05$). In the nitrogen replete condition when the nitrogen concentration was increased from 7.14 to 142.86 mM C16:0 decreased from 9.59% to 8.47%. There was also an insignificant increase in C18:1 and C18:2 with the decrease in nitrogen concentration of culture media. The neutral lipid content was observed by differences in fluorochromatic properties with nile red dye using epiflourescence microscope revealed an increasing volume of golden yellow fluorescence of cytoplasmic neutral lipid per cell volume when cultures were grown at suboptimal doses of nitrate nitrogen, whereas it decreased when cultures were grown at higher doses of nitrate nitrogen (Figure 2). The result of fluorescence spectrophotometer readings also mirrored similar trend (Figure 3).

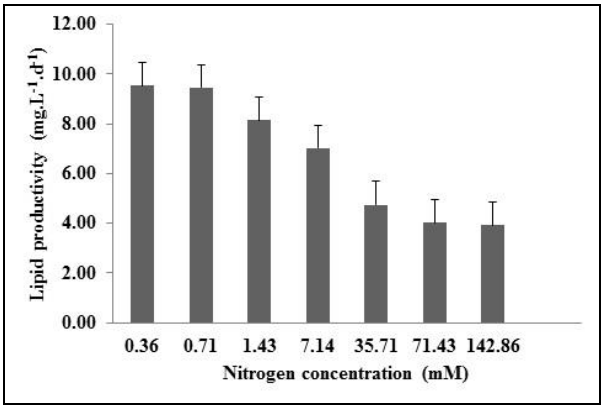

Figure 3: Lipid productivity in *Chlorella vulgaris (NIOT-74)* grown in f/2 medium with different initial nitrogen concentration values are mean ± SD

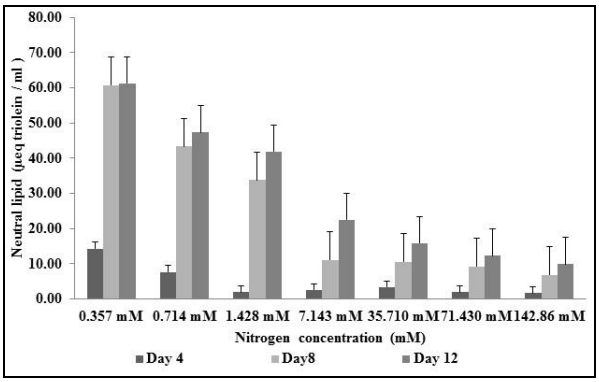

Figure 4: The neutral lipid of *Chlorella vulgaris* estimated by Nile Red Fluorescence (NIOT-74) under different initial nitrogen concentration values are mean ± SD

The total lipid content determined gravimetrically also revealed an increasing trend in lipid accumulation (9 to 20%) for nitrogen limited cultures and a decrease trend (7 to 5%) observed for nitrogen sufficient cultures**.** These results are similar to the observations in *Isochrysis galbana* by Rosseler [13] and Attilio Converti et al. [15]. However, the protein content showed a reverse trend; i.e., cellular protein content decreased from 71 to 52% for nitrogen limited cultures and increases from 71 to 73% for nitrogen sufficient cultures. The carbohydrate content during nitrogen limitation increased slightly for1.43 mM and 0.71 mM from the control condition but decreased in 0.36 mM and increased for 35.71 mM and levelled at 71.43 mM and 142.86 mM**.** It has been well documented that nitrogen limitation leads to decreased protein production, increased carbon storage compounds (triglycerides/carbohydrate) accumulation and a decreased chlorophyll-a content [16,17]. Various studies have also shown that nitrogen limitation has a pronounced effect on the biochemical composition of green microalgae and could result accumulation of lipids as a means of storage under nutrient limitation (Figure 4).

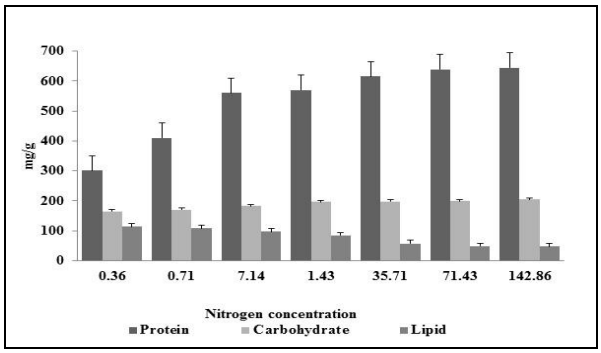

Figure 5: Protein, lipid and crabohydratecontent in *Chlorella vulgaris (NIOT-74)* grown in f/2 medium with different initial nitrogen concentration values are mean ± SD

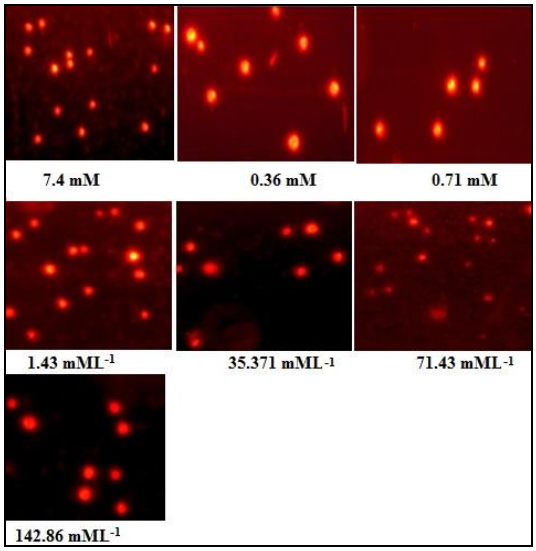

Figure 6: Nile red staining and observation using epifluorosence microscope under 40X magnification during nitrogen limitation experiment, the golden yellow colour indicates oil content of this algae inside the cell bodies

When nitrogen is depleted in the medium the cellular nitrogen in enzymes and essential cell structures is apparently utilized and any CO_2 fixed is then converted into carbohydrates or lipids rather than into proteins. This was demonstrated in *Chlorella vulgiaris*, in which lipid content reached a maximum i.e. 113.35 mg/gm at 0.36 mM nitrogen concentration.

Fatty Acid Methyl Esters (FAME) Composition

The process by which the increased lipid synthesis is induced and the drastic changes which occur in the fatty acid fraction with increasing limitation of nitrogen is of significant interest in basic studies on lipid metabolism. The FAME composition different concentrations of nitrate nitrogen show significant variations. The palmitic acid methyl ester (C16:0) which is mostly associated with triacylglycerol increased under nitrogen limitation conditions and proportionately decreased under nitrogen sufficient conditions**.** Ahlgren et al. [18] found an increase in triglycerides under nitrogen limitation for 24 species including cyanobacteria, green algae and flagellates.

Table 1: Fatty acid composition of *Chlorella vulgaris* at different nitrogen concentrations during nitrogen limitation and nitrogen sufficient condition during the experiment

	Control 7.14 mM	**0.36 mM**	**0.71 mM**	**1.43 mM**	**35.71 mM**	**71.43 mM**	**142.86 mM**
C4 : 0	0.13	0	0.23	0.03	0.04	0.1	0.02
C6 : 0	0.23	0.64	1.07	0.09	0.15	1.05	0.09
C8 : 0	0.34	0.02	0.11	0.03	0	1.11	0.08
C10 : 0	0.43	0.09	0.21	0.38	0.14	0.82	0.2
C11 : 0	1.04	33.04	33.55	0.39	13.43	1.73	0.05
C12 : 0	22.62	2.06	3.16	26.61	2.48	21.03	27.08
C13 : 0	3.36	0	0.28	3.59	0	2.7	0
C14 : 0	5.81	1.18	1.66	2.93	0.78	5.04	1.72
C14 : 1	0.78	0.14	0.26	1.33	1.12	0.84	0
C15 : 0	6.1	1.26	2.13	3.86	6.1	7.19	2.22
C15 : 1	3.02	0	4.36	3.21	4.63	3.68	0
C16 : 0	9.59	43.53	31.7	24.84	8.47	9.07	34.01
C16 : 1	18.56	4.77	6.57	12.24	20.45	15.53	9.72
C17 : 0	0.11	0.03	0.13	0.76	0.03	0.23	0.04
C17 : 1	18.17	4.22	5 .91	11.37	29.7	20.54	18.12
C18 : 0	5.78	3.48	5.92	5.34	12.2	6.13	6.97
C18 : 1 n 9 t	0.07	1.23	1.16	0.51	0.02	0.13	0.12
C18 : 1 n 9 c	0	0.41	0.57	0.16	0	0.05	0.03
C18 : 2 n 6 t	0.16	0.08	0.18	0.06	0.02	0.11	0
C18 : 2 n 6 c	0.04	0	0.08	0.03	0	0.06	0
C20 : 0	0.04	0.19	0.32	0.03	0.04	0.04	0

C18 : 3 n 6	0.3	0.2	0.27	0.22	0.03	0.29	0.07
C20 : 1	0.18	0.09	0.22	0.09	0	0.16	0.03
C18 : 3 n 3	0.28	0.23	0.45	0.16	0.02	0.14	0.07
C21 : 0	0.03	0.23	0.3	0	0.04	0.04	0
C20 : 2	0.32	0.04	0.17	0.42	0	0.26	0.06
C22 : 0	0.05	0.09	0.07	0.47	0	0.03	0
C20 : 3 n 6	0	0.67	0.52	0.02	0.03	0.01	0
C22 : 1 n 9	0.4	0.19	0.26	0.32	0	0.2	0.04
C20 : 3 n 3	0.12	0.26	0.32	0.18	0.03	0.08	0.02
C20 : 4 n 6	0.28	0.19	0.31	0.24	0	0.36	0.05
C23 : 0	0.2	0.36	0.38	0.15	0	0.12	0.01
C22 : 2	0.2	0.25	0.26	0.21	0.01	0.16	0.05
C24 : 0	0.21	0.25	0.88	0.15	0.01	0.23	0.03
C20 : 5 n 3	0.37	0.25	0.48	0.11	0	0.16	0.02
C24 : 1	0.36	0.28	0.35	0.22	0	0.35	0.06
C22 : 6 n 3	0.3	0.05	1.11	0.24	0	0.22	0.04

Richardson et al. [19] also showed an increased triglyceride content under nitrogen limited condition when compared with nitrogen sufficient cultures. Thus the present results indicated that nitrogen limitation will be useful in enhancing lipid accumulation as well as to increase the triglyceride content (essential for biodiesel).

Utilization of Sodium Nitrate

Though the sea water (used for experiment) contains 20.39 µM of nitrate and 3.26 µM of inorganic phosphate, this amount is inadequate for growing microalgae to attain reasonable biomass. It was observed that almost 97-99% of the nitrate has been utilized by the cultures grown at both nitrogen limited and nitrogen sufficient conditions. but the utilization of inorganic phosphate is almost reduced to 64%, 79% and 92% for 0.36 mM, 0.71 mM and 1.43 mML^{-1} respectively and 95-96% utilized for control and nitrogen sufficient cultures.

Purification and Characterization of Acetyl-CoA Carboxylase

An attempt to elucidate the biochemical mechanisms that are responsible for the variation in the activity of the enzyme ACCase (a potential regulatory enzyme in lipid biosynthesis pathway), in control, nitrogen limited and nitrogen sufficient conditions. ACCase was purified from the cultures of control, nitrogen limited, nitrogen sufficient conditions and it showed a single band of molecular weight 150 kDa (Figures 5 and 6)**.**

While purifying ACCase, it was observed that the total protein content decreased during each purification step and the affinity eluted samples alone were subjected to radioactivity assay (^{14}C assay using Scintillation counter) to determine the *in vitro* activity of the enzyme in order to elucidate the biochemical mechanism that is responsible for the variations in the regulation of lipid accumulation in *Chlorella vulgaris*. It was observed that the specific activity of ACCase decreases under nitrogen limitation and proportionately increases under nitrogen sufficient conditions (Tables 1 and 2); this illustrates that during nitrogen limitation the enzyme ACCase is utilized more for enhancing lipid accumulation. This enzyme may be accountable for the faster accumulation of lipids than proteins in *Chlorella vulgaris*, i.e. allocating cellular carbon into the lipid biosynthetic pathway. The formation of malonyl CoA via the carboxylation of acetyl CoA by ACCase is considered as a rate limiting step and is regulated by various biochemical mechanisms including nitrogen limitation as discussed by Livne and Sukenik [20].

Table 2: Purification of ACCase from *Chlorella vulgaris* (Jf894250) and the *in vitro* activity of affinity purified ACCase

	Total protein (mg)			
Nitrate nitrogen concentration (mML^{-1})	**Crude extract**	**Ammonium sulphate fractionation**	**Eluted fraction from affinity chromatography**	**Specific activity (Units/mg protein)**
7.14	60.75	12.08	2.53	57.73
0.36	30.75	6.98	1.7	43.15
0.71	41.25	7.95	1.97	48.92
1.43	47.25	9.29	2.44	51.61
35.71	81.75	18.2	2.57	66.27
71.43	87.01	21.67	2.59	75.31
142.86	106.5	24.31	2.64	83.97

Roessler and Livne et al. also reported that nitrogen starvation in microalgae forced an increase in the cellular lipid content, variations in lipid composition. Reduction in the *in vitro* activity of ACCase, on the contrary, recovery from

starvation was characterized by decreased lipid content, increase in the *in vitro* activity and cellular abundance of ACCase [13,20].

CONCLUSION

The growth response, dry weight biomass and lipid production in *Chlorella vulgaris (JF894250)* were significantly influenced by nitrogen limitation and sufficient conditions. The total lipid content revealed an increasing trend in nitrogen limited cultures and a decreasing trend in nitrogen sufficient cultures whereas protein content showed a reverse trend. Gas chromatographic analysis of FAME revealed that the content of palmitic acid methyl ester (C16:0) associated with triacylglycerol increased dramatically under nitrogen limiting conditions. The specific activity of acetyl co-enzyme carboxylase reduced under nitrogen limiting conditions indicating that ACCase is responsible for allocating cellular carbon into the lipid biosynthetic pathway, which inturn had enabled *Chlorella vulgaris (JF894250)* to enhance lipid when grown at nitrogen limiting condition. The marine Microalgae *Chlorella vulgaris (JF894250)* seems to be very promising source of triglycerides for biodiesel production under single stage nitrogen limitation strategy using optimal nitrogen concentration for stimulating lipid productivity.

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