

Effects of nitrogen, phosphorus, EDTA and sodium chloride on biomass and lipid accumulation of *Chaetomorpha aerea*

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ABSTRACT

The increase of total lipid and fatty acids production was studied under different nutrient stress conditions using the macroalga *Chaetomorpha aerea*. The effects of nitrogen, phosphorus, ethylene diamine tetra-acetate and sodium chloride on the growth and lipid accumulation were systematically investigated in laboratory conditions. The biomass was harvested at different stages of cultivation and assessed. The maximum changes of growth and lipid accumulation were observed in the exponential phase at different cultural conditions. The two-fold increase of total lipid was found in the order of $28.27 \pm 0.04\%$ (at 0.1 g/L nitrogen) > $27.30 \pm 0.37\%$ (at 0.5 g/L of phosphorus) > $25.86 \pm 0.77\%$ (at 0.05 g/L of EDTA) > $24.37 \pm 0.04\%$ (at 0.05 g/L NaCl) on 8th day of cultivation. The fatty acids were identified and quantified by gas chromatography mass spectrometry (GC-MS). The alga produces significantly high amount of monounsaturated fatty acid (MUFA) and saturated fatty acids (SFA) than the polyunsaturated fatty acids (PUFA) in different cultural conditions. The elevated levels of C16:1, C18:1 and C20:1 was identified under nitrate, phosphate and salt stress conditions, which are more suitable for biodiesel production.

KEYWORDS: Biodiesel, *Chaetomorpha aerea*, FAME, lipid, nutrient, stress

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INTRODUCTION

Nutrients are most widely used environmental factors used to stimulate the biochemical changes in algae and other plant communities. The light energy is converted to chemical energy in the form of carbohydrates, lipids and other bioactive compounds. From the past few decades microalgae are being considered as the most promising agent for lipid production technology.

Marine seaweeds or macroalgae are the excellent source of bioactive compounds like proteins, vitamins, carotenoids, dietary fibres and essential fatty acids. These essential fatty acids include both the saturated and unsaturated form. The lipid content of marine macroalgae is very low (1-5%) but under stress conditions they produce up to 30-40% [1]. The fatty acids of marine macroalgae generally have linear chains and even number of carbon atoms with one or more double bonds [2]. Macroalgae have been studied for long for the production of wide range of natural chemicals like agar, agarose, carrageenan and alginates. However, they have never been looked for lipids, as energy resource [1]. Microalgae have been considered for high lipid and

used for biodiesel application several times [3-6]. Stress induced changes in lipid and fatty acids profile have also been discussed for long [7-11]. But very few reports are available on macroalgal lipid and fatty acids [12-14]. The effect of nitrate and phosphates on lipidomic and other biochemical compositions of the macroalga *Ulva lactuca* were studied by Kumari *et al.* [15]. They investigated the changes in polar lipids, chlorophyll and protein content when they cultured the alga in artificial sea nutrient (ASW) medium supplemented with nitrate and phosphate. Biochemical composition of eighteen marine macroalgae belonging to Chlorophyta, Phaeophyta and Rhodophyta were studied from Okha coast, Gulf of Kutch, India [16]. They studied varied quantities of bioactive compounds like total lipid, protein, carbohydrate, phenol and amino acids. Seasonal variations in fatty acid compositions of 51 macroalgae were studied from Gulf of Mannar, Marine Biosphere Reserve of Southeast coast of India [17]. Comparative accounts of fatty acid compositions of three marine macroalgae were also studied from the coastal region of South India [18]. In our previous study we have reported the effect of nutrient and other abiotic stresses on growth and lipid accumulation in *Rhizoclonium africanum* [13].

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The effects of different nutrient stresses such as nitrogen, phosphorus, ethylene diamine tetra-acetate (EDTA) and sodium chloride (NaCl) on production of high lipid, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) in macroalgae has not been studied to date. In this study, our aim was to determine and compare the effects of different nutrient stresses on growth and lipid synthesis for biodiesel production from *C. aerea*.

MATERIAL AND METHODS

Strain and Culture Condition

C. aerea was collected from the coastal zone of Indian Sundarbans (N 22°01.142, E 088°40.955) and isolated and cultivated in modified Bold Basal Medium [19] (Figure 1). The compositions of the modified BBM media was manipulated as NaNO_3 -0.25g; CaCl_2 -0.025g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.075g; K_2HPO_4 -0.075g; KH_2PO_4 -0.175g; NaCl-0.5g and dissolved in 1000 ml of double distilled water. The alga was grown at 20°C and 16:8 light-dark cycles with 135 rpm agitation in Eyla horizontal shaker-incubator (Eyla, Japan).

Measurement of Biomass Yield

For measurement of biomass, three sets of cultures were harvested at 2-day interval for thirty days from the date of inoculation. For harvesting, the cultures were washed with deionized water and blotted well with blotting paper. After proper air-drying, the biomass was transferred to pre-weighed vials for oven drying at 70°C to get a constant dry weight. The

gravimetric method was applied to calculate the biomass in terms of g/L [1].

Gravimetric Determination of Total Lipid

About 0.5g of dry algal biomass was taken and ground in mortar and pestle. The powdered sample was taken and mixed with 2ml of chloroform and 1ml of methanol [20]. The mixture was vortexed for 2-3 minutes and centrifuged at 8000g for 5 minutes at 20°C. Chloroform layer was collected carefully. The process was repeated 2-3 times and all the collected chloroform was mixed together and evaporated using rotary evaporator at room temperature. After getting the lipid residue, it was dried in oven at 60°C and weighed with electronic balance.

$\% \text{ of lipid in dry biomass} = \frac{\text{final weight of the lipid sample after drying}}{\text{weight of the dry biomass}} \times 100$

Preparation of Fatty Acid Methyl Ester (FAME)

Transesterification method was used to prepare fatty acid methyl esters. After extraction, the lipid samples were taken in a 10 ml screw-cap borosil glass tube and transesterification reagent methanolic hydrochloric acid (1:4 v/v) was added. The solution was then kept in a borosil glass beaker containing double distilled water and heated for 6-8 hours at 70°C. The solution was then cooled down and to avoid particulate matters it was centrifuged at 10,000 rpm for 10 minutes. The FAME extract was then transferred to Gas chromatography-mass spectrometry (GC-MS) auto-sample vials for analysis [11].

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was performed using FAME and detection was made with an Agilent 6890N Gas Chromatograph connected to an Agilent 5973 Mass Selective Detector at 70 eV (m/z 50- 550; source at 230°C and quadrupole at 150°C) in the electron impact mode with a HP-5 ms capillary column (30 m \times 0.25 mm i.d. \times 0.25 μm film thickness). The temperature of the oven was set at 160°C for 2 min and then increased by 5°C/min to 300°C. The flow rate of the carrier gas (helium) was set to 1.0 mL/min. With split ratio of 50:1, the inlet temperature was maintained at 300°C. Mass spectrometric fragmentation was used to determine structural assignments. It was confirmed by comparing fragmentation patterns and retention times of authentic compounds. On the basis of retention time identification of individual fatty acids was done [12-13].

Statistical Analysis

Statistical analysis was performed in triplicate ($n=3$) and data expressed as mean \pm standard deviation (SD) with standard error (SE) bars. Significant differences ($p < 0.05$) between means in terms of biomass, lipid and fatty acids were obtained through one-way analysis of variance (ANOVA) and Tukey's test.

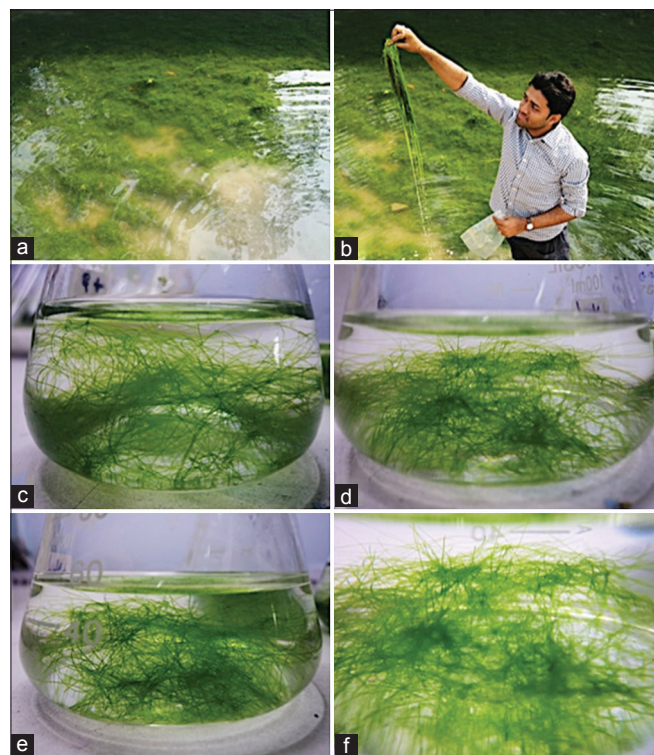


Figure 1: a-b) *C. aerea* collection from sampling site, c) Nitrogen induced culture, d) Phosphorus induced culture, e) EDTA induced culture, f) NaCl induced culture

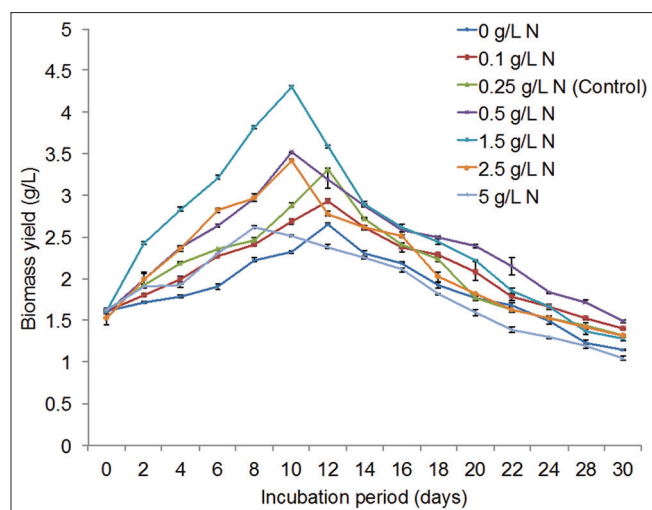


Figure 2: Biomass yield of *C. aerea* at different concentrations of nitrogen (N)

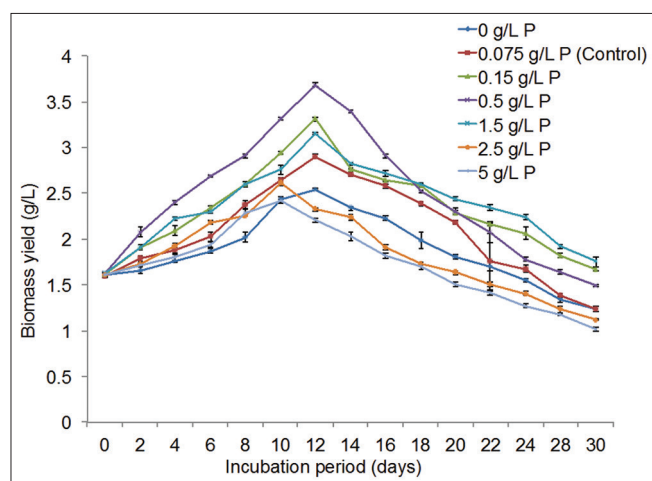


Figure 3: Biomass yield of *C. aerea* at different concentrations of phosphorus (P)

RESULTS

Biomass Yield

The effects of nitrogen, phosphorus, EDTA and NaCl on biomass yield is summarized in Figures 2-5. At the initial stage of cultivation, alga showed very slow growth but with time it changed significantly. The exponential growth period was observed at 8-10 days of cultivation. Interestingly after day 14, the growth pattern was arrested with increasing concentrations of nutrients in the culture. Untreated culture of *C. aerea* showed highest biomass yield of 3.31 ± 0.03 g/L at 12 days of cultivation. The alga showed maximal biomass of 4.30 ± 0.03 g/L at 1.5 g/L of nitrogen after 10 days incubation period. On day 12, maximal biomass was harvested as 3.68 ± 0.03 g/L at 0.5 g/L phosphorus. Filamentous chlorophytes showed positive growth with increasing concentration of EDTA. The highest biomass in the alga was obtained as 3.27 ± 0.02 g/L at 0.08 g/L of EDTA after 10 days incubation. At very low

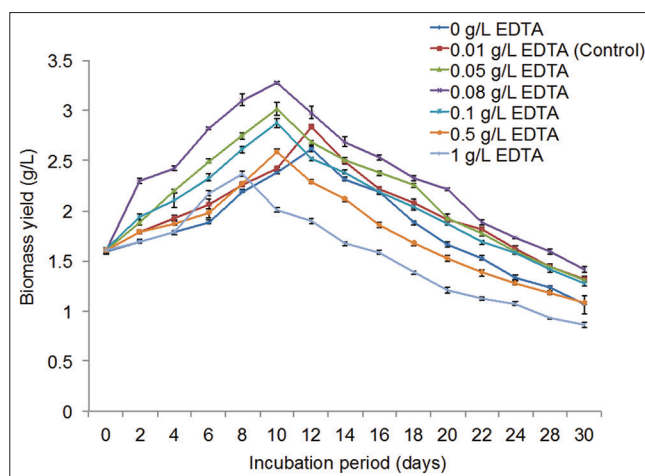


Figure 4: Biomass yield of *C. aerea* at different concentrations of EDTA

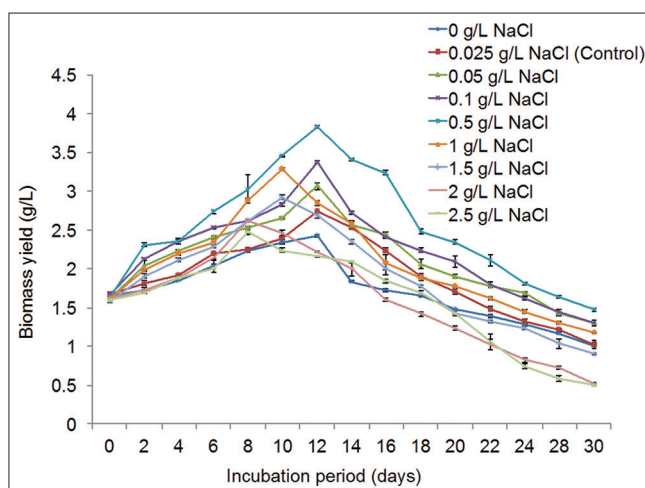


Figure 5: Biomass yield of *C. aerea* at different concentrations of NaCl

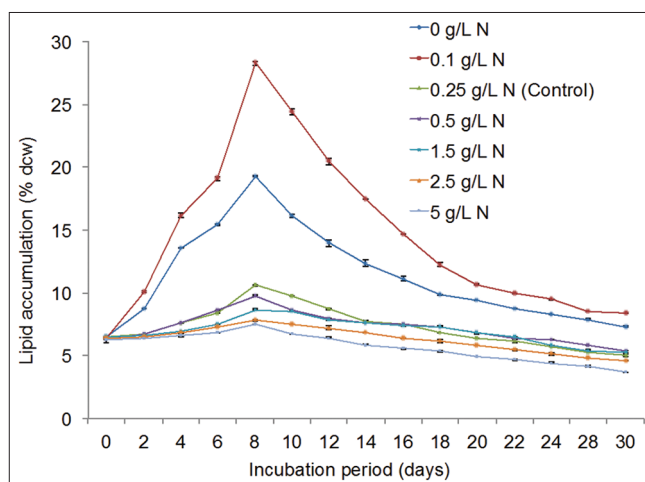


Figure 6: Lipid accumulation of *C. aerea* at different concentrations of nitrogen (N)

concentration of NaCl (0.5 g/L), the highest biomass of the alga was obtained as 3.83 ± 0.03 g/L after 12 days of incubation.

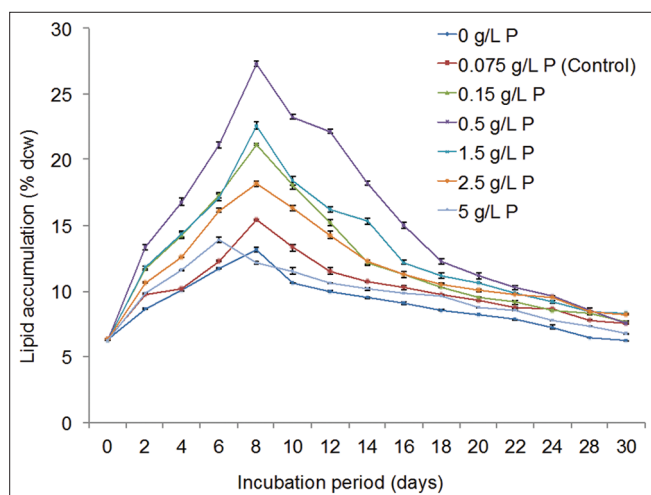


Figure 7: Lipid accumulation of *C. aerea* at different concentrations of phosphorus (P)

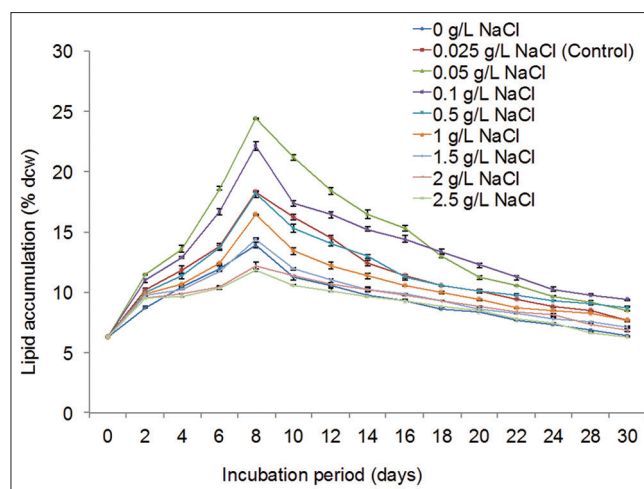


Figure 9: Lipid accumulation of *C. aerea* at different concentrations of NaCl

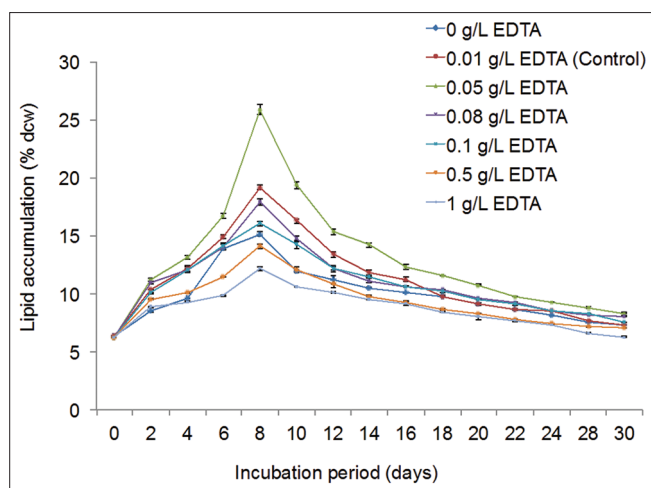


Figure 8: Lipid accumulation of *C. aerea* at different concentrations of EDTA

Total Lipid Accumulation

Figures 6-9 summarize the total lipid accumulation under different concentrations of nitrogen, phosphorus, EDTA and NaCl. The results clearly demonstrated that the lipid accumulation in the macroalga increased drastically with the concentrations of nutrients on 10th day of cultivation. The optimum lipid accumulation for the alga was noticed on day 8 of cultivation. In control condition, the highest lipid accumulation in *C. aerea* was $10.57 \pm 0.05\%$ on 8th day of cultivation. At 0.1 g/L of nitrogen, *C. aerea* showed the maximum amount of total lipid accumulated as $28.27 \pm 0.04\%$ after 8 days of incubation. On day 8, the highest lipid accumulated in *C. aerea* was calculated as $27.30 \pm 0.37\%$ at 0.5 g/L of phosphorus. The alga showed maximum lipid accumulation of up to $25.86 \pm 0.77\%$ (on 8th day) at 0.05 g/L of EDTA. On day 8, the alga showed highest lipid accumulation of $24.37 \pm 0.04\%$ at 0.05 g/L NaCl. The stationary phase for lipid accumulation was noticed after day 18 onwards.

Changes of Fatty Acid Composition

The fatty acid composition variations due to the different concentrations of nitrogen, phosphorus, EDTA and NaCl in the culture are listed in Table 1. Highest lipid containing cells from control and stress cultures were taken for FAME analysis and are presented in Table 1. A total of seventeen fatty acids were obtained of which nine belong to saturated fatty acids, three to monounsaturated fatty acids and remaining were polyunsaturated fatty acids. The MUFA in this study increased when the concentrations of nitrogen was decreased. Similarly the MUFA was increased with increased concentrations of phosphorus, EDTA and NaCl in the culture. Interestingly the PUFA content was decreased with alterations of nutrients in the culture. The saturated fatty acids were increased in phosphorus and NaCl stress but decreased in nitrogen and EDTA stress. A significant increase of C15:0, C16:0, C16:1, C20:0, C20:1 and C22:0 were found in all treated conditions. In the filamentous alga, C17:0 was increased in phosphorus, EDTA and NaCl treated cells except nitrogen. Simultaneously, the decrease of C18:2 and C18:3 in nitrogen and EDTA treated conditions were studied. Furthermore, the absence of these fatty acids in phosphorus and NaCl treated cells were also observed. The absence of C20:3 in nitrogen, phosphorus and NaCl treated cells and C20:4 in nitrogen and EDTA treated cells were observed. Lignoceric acid (C24:0) was decreased in nitrogen-starved cells whereas it increased in other nutritional stresses. Overall the productions of palmitic and palmitoleic acids were enhanced under nutrient stresses than the control. Phosphorus treatment showed better results for stearic acids and oleic acids than other fatty acids. EDTA stress resulted increase in palmitic, palmitoleic and stearic acids in the macroalga, whereas only palmitic and palmitoleic acids were increased under NaCl stress. Arachidic and paullinic acids were significantly increased in high lipid containing cells cultivated at 0.05 g/L EDTA and NaCl respectively.

DISCUSSION

Environmental changes altered the biochemical composition as well as physiological changes including lipid accumulation in

Table 1: FAME compositions of *C. aerea*

Fatty acids	Fatty acid compositions (%)				
	Control	0.1 g/L N	0.5 g/L P	0.05 g/L EDTA	0.05 g/L NaCl
Lauric (12:0)	0.95±0.35	1.55±0.21 ^a	ND	0.75±0.21 ^c	1.75±0.21 ^{ab}
Myristic (14:0)	9.40±0.28 ^{ab}	6.90±0.28	9.40±0.28 ^b	0.50±0.28	5.10±0.28 ^{bc}
Pentadecylic (15:0)	0.75±0.21	1.70±0.28 ^{ab}	1.50±0.28 ^c	1.25±0.35 ^b	1.45±0.35
Palmitic (16:0)	49.10±0.28	51.00±0.42 ^a	47.65±0.21 ^{ab}	43.00±0.42 ^b	50.45±0.35 ^a
Palmitoleic (16:1)	8.5±0.28	10.5±0.28 ^c	12.35±0.35 ^{bc}	9.75±0.21 ^{ab}	12.65±0.35 ^a
Margaric (17:0)	0.70±0.14 ^c	0.60±0.28	1.00±0.28 ^a	0.75±0.21	1.05±0.35 ^b
Stearic (18:0)	4.65±0.21	3.70±0.28	6.35±0.21 ^{bc}	8.65±0.21 ^{ab}	4.40±0.28
Oleic (18:1)	16.95±0.63	18.45±0.35 ^b	17.75±0.21 ^a	16.60±0.42	15.60±0.14 ^c
Linoleic (18:2)	4.65±0.63 ^a	1.40±0.42	ND	1.00±0.42 ^{bc}	ND
α-linoleic (18:3)	1.50±0.42	0.70±0.28 ^{ab}	-	1.95±0.21 ^{bc}	-
Arachidic (20:0)	0.60±0.28 ^c	0.60±0.14 ^a	0.60±0.28 ^{ab}	1.45±0.35 ^a	1.40±0.28 ^c
Paullinic (20:1)	0.35±0.07	0.70±0.28	0.40±0.28	2.20±0.28 ^{ab}	1.05±0.49 ^b
Dihomo-γ-linolenic (20:3)	0.25±0.21 ^{bc}	ND	ND	0.45±0.35	ND
Arachidonic (20:4)	0.40±0.28 ^c	ND	0.20±0.14	ND	1.00±0.42 ^{ab}
Eicosapentaenoic (20:5)	ND	0.45±0.35 ^{bc}	0.50±0.28	ND	0.50±0.28 ^c
Behenic (22:0)	0.50±0.28	0.65±0.35 ^{ab}	0.75±0.07	0.50±0.28	1.70±0.28 ^c
Lignoceric (24:0)	0.65±0.35	0.35±0.35	0.75±0.21	1.50±0.28 ^{ab}	1.35±0.35 ^{bc}
SFA	67.30±0.26	67.05±0.28	68.00±0.22	58.35±0.28	68.65±0.30
MUFA	25.80±0.32	29.65±0.30	30.50±0.28	28.55±0.30	29.30±0.32
PUFA	6.80±0.38	2.55±0.35	0.70±0.21	3.40±0.32	1.50±0.35

Values expressed as mean ± SD (n=3). Superscript letters indicate significant differences ($p < 0.05$) for each treatment (ND- Not detected)

micro- and macroalgae. As shown in Figures 2-5 there is a slight decrease in the growth during the first day of inoculation, but the growth increased in the later stages of culture. From this, it was assumed that at the time of inoculation the cells began to adapt themselves to the new environment and assimilated almost no nutrients, which might suppress the cell division but promote the lipid productivity [21]. The similar observation was found in the report done by Valenzuela-Espinoza *et al.* [22]. The biomass yield in the present study was found to be maximum (4.30 ± 0.03 g/L) at 1.5 g/L of nitrogen on 10th day of cultivation. Hence the biomass with sodium nitrate (NaNO₃) as nitrogen source was significantly higher than others like phosphate, EDTA and NaCl. Similarly, the maximum lipid accumulation in the macroalgal was $28.27 \pm 0.04\%$ on day 8, which was almost 3 times better than the control. From the above study it was confirmed that the alteration of nutrients were effective on growth of macroalgae and lipid accumulation. Similar results were obtained in previous works [23-26]. In the present study different nutritional combinations were applied on the green macroalga *C. aerea* and it was reported that highest lipid accumulation takes place at limited nitrogen. Reitan *et al.* cultured the microalgae at different culture media lacking nitrogen and observed the increase of lipid [27]. Nitrogen limited condition resulted increase of total lipid on day 17 in *Chlorella vulgaris* [28]. In our previous study, marine macroalga *Rhizoclonium africanum* showed maximum growth and lipid production at limited nitrogen [13]. Most of the works on biomass and lipid accumulation had been done using unicellular microalga [6, 28-32], while very few reports are available on filamentous macroalga [12, 14, 33]. In the present study different concentrations of four nutrients like nitrogen, phosphorus, EDTA and NaCl were introduced in the culture medium to study the growth pattern in terms of biomass yield and lipid accumulation in marine macroalga *C. aerea*.

The biosynthesis of lipid along with carbohydrate and protein was involved with many enzymatic reactions [21]. In the photosynthetic reaction the light energy is converted to

chemical energy in the form of adenosine tri-phosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), which could then be used to convert CO₂ and glyceraldehyde 3-phosphate (G₃P) in the dark reaction. The G₃P is the primary precursor for producing carbohydrates in the form of glucose and starch through a series of enzymatic reactions. The biosynthesis of lipid was done through a series of enzymatic reactions where glucose is converted to pyruvate (glycolysis). The pyruvate is the primary precursor, which forms acetyl CoA by the enzyme acetyl CoA carboxylase (ACCase) and the acetyl CoA is converted to malonyl CoA [34]. The nutrients may alter the formation of lipid biosynthesis through chemical changes. The fatty acids are formed from malonyl CoA (Figure 10). The lipid accumulation and fatty acid synthesis within the cell is due to the activation of ACCase enzyme [35-37]. The ACCase activity was regulated by the alkaline pH, NADPH and Mg²⁺ [35]. The alkaline optimum pH, increased NADPH and Mg²⁺ within the chloroplast stroma enhanced the ACCase activity. The nutrient deficient conditions changed the alkaline pH, NADPH and Mg²⁺, which enhanced the activity of ACCase. The active ACCase then catalyzed the reaction of acetyl CoA to malonyl CoA and thus large quantity of fatty acids were formed. When photosynthesis proceeds, the chlorophyll within the chloroplast absorbs light and efflux protons (H⁺) from the stroma to the thylakoids. This results the increase of stromal pH from 7 to 8. Then Mg²⁺ flowed into the stroma along with the H⁺ leading to an accumulation of Mg²⁺. When light interacts with the chlorophyll, the water splits to form electrons and these electrons are transported through the electron transport chain (ETC) to form NADPH with the help of enzyme Ferredoxin NADP reductase (FNR). This leads to the accumulation of stromal NADPH. This indicated that the biosynthesis of lipid is enhanced due to the influence of light and chlorophyll and other factors like alkaline pH, NADPH, Mg²⁺ and activity of ACCase.

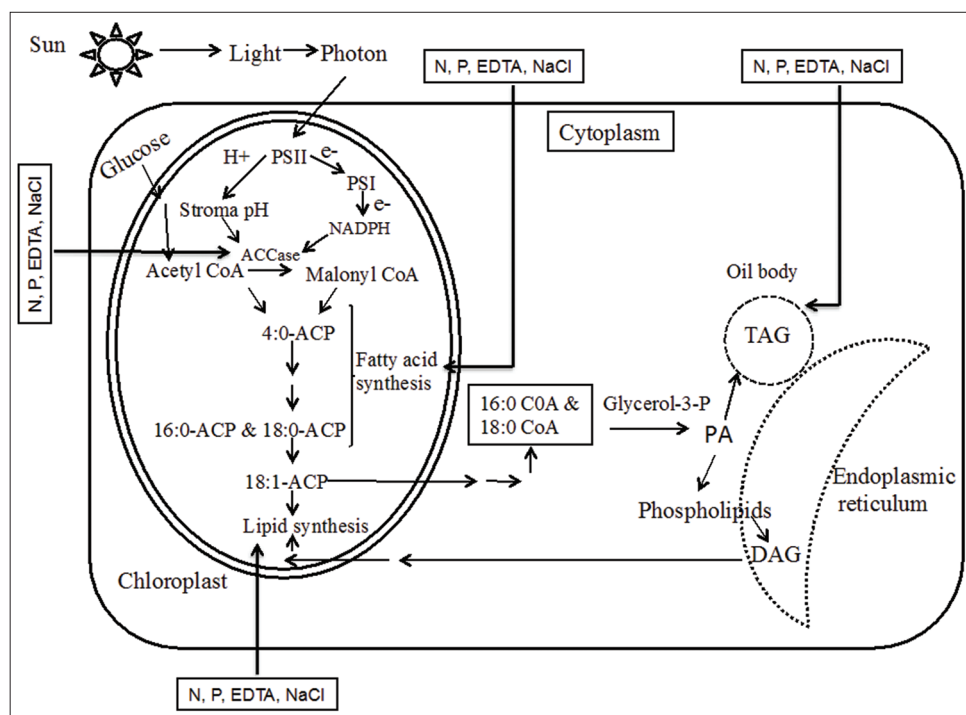


Figure 10: Lipid and fatty acid biosynthetic pathway under stress

The nutrient deficiency enhanced the production of Chlorophyll content including chlorophyll a, which might provide the more alkaline pH, Mg^{2+} and NADPH to enhance ACCase activity. However more lipid accumulation takes place in nutrient limitation than the nutrient starvation. Sukenik and Livne has reported that the nitrogen limitation leads to a reduction of cellular abundance of ACCase with a decrease in the activity of the enzyme, but cell division almost ceased ensuing in the accumulation of lipid [37]. Therefore, it suggested that the optimal cultivation conditions including nutrient deficiency and accumulation lead to high biomass and high lipid productivity for biodiesel production.

CONCLUSION

From the above study it can be concluded that marine macroalga *Chaetomorpha aerea* showed extensive growth and lipid accumulation under nitrogen, phosphorus, EDTA and NaCl stress. The FAME composition also indicated its potentiality towards biodiesel application.

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