EFFECT OF NUTRITIONAL SUPPLEMENTATION ON GROWTH AND LIPID ACCUMULATION OF NATIVE MICROALGAL STRAIN OF MEGHALAYA

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***Abstract*:** Recently, Open Government Data Platform India's survey says that PM_{10} and $PM_{2.5}$ (PM-Particulate Matter with diameter of 10 microns and 2.5 micron) at the level of 150 micrograms (observed at familiar cities in India) which is six times higher than WHO's limit of 25 micrograms and WHO, reported that 3.7 million people deaths due to air pollution in recent years. Researches assumed that fuel and gases available up to 30 – 60 years. So, currently focusing on an alternative source of fuel which can solve air pollution and availability that is from Biological matter called "Biofuel" that is Eco friendly & Renewable sources. Biofuel from algae, yeast, bacteria have more attention than other commercial sources based on theoretical calculation, especially microalgae has even more attention than others but they have limitations such as when low-level lipid produced simultaneously increased in biomass at normal condition. In this study, we focused on to improve lipid production as well as biomass simultaneously. Significantly noticed an increased biomass in Mg supplement (NC-M1) (NC – Corresponding author; M1 & M5 – Co-author) is 170±5mg/L at concentration of 608µM where control is 135±8mg and also in Ca supplement produced 168±10mg/L at conc. 478µM where control 135±8mg/L. In other hand Lipid yield also increased in Nitrogen supplement (NC-M1) is 47±2mg at conc. 35.4mM where control is 27±1mg/L and Mg supplement is 52±5mg/L at conc. 342 µM where control is 48±2mg/L.

***Index Terms* -** Algae, Biofuel, Nutrition, supplementation, Lipid, Growth, neutral, yield, curve, production.

I. Introduction

Microalgae are simple, Eukaryotic or Prokaryotic, Unicellular, thallus, photosynthetic, generally called as phytoplankton in aquatic system. Algae are primary producers in global Aquatic system, it can synthesis energy by themselves through photosynthesis like plants, obtains light energy from sunlight and convert into chemical energy along with carbon sequestrate from the atmosphere into biomass via photosynthesis. Algae is the ancestor for plants and similar biochemical metabolisms exist in both [3, 1]. Even though algae and plants are not similar, the ultimate machinery such as photosynthesis, it made them correlated each other. Some of the algae have been used for environmental problems from hundreds of decades, including supplements and nutraceuticals [10]. Early 1940s, several new algal strains were identified as possible for fuel sources [4] because it has that capability to synthesize fuel precursors. Production at large scale population like photobioreactors, pond cultivation was practiced in the 1950s at USA, Germany and other countries [5,16]. An ASP (Aquatic species program) from U.S. DOE (Department of Energy) develops fuel to improve transportation fuel from microalgae on 1978, many algae and cyanobacteria were being produced commercially around the world and since last 20 years, algae are used as food to fulfil requirement of nutrition [12]. Microalgae evolved to be virtually found everywhere throughout the world, and their distributions, evolutionary histories [1] are repelled in extreme diverse metabolic capabilities in between strains [7]. These metabolisms generate large number of compounds with anthropogenic relevant substances including nutraceuticals e.g., carotenoids produced by *Dunaliella sp*. and *Haematococcus sp*. [4,5], the polyunsaturated fatty acids (PUFAs) can synthesis by various organisms [15], and the rich proteins as in the form of proteoglycan avail in whole-cell supplements. Eg., *Spirulina* and *Chlorella* sp. [16,11]. Some microalgae synthesize specific compounds like phycoerythrin, isoprenoid and other compounds that have been used at many areas [7]. Microalgae have been considering as alternative source of biofuel and recognized that some are producing variety of precursor of fuel products. Microalgae can accumulate large quantities of fatty acids as in the form of lipid while sequestering CO2, mostly neutral lipids in the form of triacylglycerol (TAG), which can be modified furtherly as FAMEs (Fatty acid methyl esters), the important component of biodiesel [9], via esterification or refined into some other fuel products [14], in other words total lipids (TL) and biomass from microalgae get convert into crude oil alternatively via thermochemical processes (hydrothermal liquefaction) [8]. Carbohydrates can be fermented into ethanol and some algae produces biohydrogen [2]. Along with their variety of products, microalgae have secured more attention as alternative fuel sources because algae have more benefits/favor than plants. Eg., cultivation period, time and cost effective and less manpower required. They can survive at hard environment like brackish/ saline water, so large quantity of fresh water can be saved [17].

II. MATERIALS AND METHODS:

2.1 Preparation of Culture medium

Many media are available commercially for algal cell culture but also some of media are popular and common for the culture. They are BBM (Bold basal medium), BG11, TAP11, etc. Here BBM medium was used for the entire experiment and culture, preparation of the medium followed as

Table 1 Composition of BBM medium.

S. No	Substances	Stock soln.	Working soln. (mL/L)
1	KH_2PO_2	17.5	1
2	$CaCl_2$	2.5	1
3	$MgSO_4.7H_2O$	7.5	1
4	$NaNO_3$	25	1
5	K_2HPO_4	7.5	1
6	NaCl	2.5	1
7	$Na_2EDTA.2H_2O$	1	1
8	$FeSO_4.7H_2O$	0.498	1
9	Trace Metal solution	-	1
10	H_3BO_3	1.15	0.7
11	Vitamins	-	1

Table 2 Composition of Trace elements

S. No	Trace metals	Volume (mg/0.1L)	Vitamins	Volume (per 0.1L)
1	H_3BO_3	280	Vitamin B_1	10g
2	$MnCl_2.4H_2O$	180	Vitamin H	2.5 mg
3	$ZnSO_4.7H_2O$	22	Vitamin B_{12}	1.5 mg
4	$NaMoO_4.5H_2O$	39	-	-
5	$CuSO_4.5H_2O$	7.9	-	-
6	$Co(NO_3)_2.6H_2O$	4.9	-	-

2.2 Isolation of algae and culture maintenance:

The Algal species were obtained from lab cultures (Neha chaurasia's lab at North Eastern Hill University, Shillong, India). Microalgae were cultured in the culture medium. The strains were allowed to settle down first then collected those strains from the bottom using pipette transferred to sterile Eppendorf tubes and were washed by centrifuging, mixing up to 5–6 times with sterile distilled water, then the cells were separated from colony, aggregation by adding sterile glass beads and vortexed. The action of the beads colliding into each other breaks up the algal clump, aggregation. The cells were then washed with BBM media then vortexed with glass beads well to disintegrate cells individually and finally inoculated into a culture flask with a defined amount of media in it.

2.3 Treatments on strains

Many chemical substances and their concentration (may be lack or excess of compound concentration) has their potential effect on every strain which leads to stress furtherly it promotes metabolic alteration, enzyme activity, changes in growth rate, changes in lipid accumulation so on. Based on the substance potential on strains were selected. Following substances were selected which

is most important for their growth, metabolism, protein synthesis for the supplementation, they are Nitrogen, Magnesium, Calcium, Phosphorus with different concentration along with control.

Table 3 Concentrations of Supplemented elements.

S. No	Volume (µL/L)	Nitrogen (mM/L)	Magnesium (µM/L)	Calcium (µM/L)	Phosphorous (µM/L)
1	1000 (Control)	17.6	304	238	228
2	1125	19.8	342	267.75	256.5
3	1250	22	380	297.5	285
4	1500	26.4	456	357	342
5	2000	35.4	608	476	456

2.4 Cell culture maintenance and preparation of growth curve

Strains were maintained up to 18 days (up to exponential phase) in optimum condition such as 25^{O} C ± 1^{O} C, manual shaking, light source up to 8 hours. 1mL of culture medium was taken in a sterile test tube on alternative days. The cell growth rate was measured by optical density at 680nm every alternate day up to 18 days using a spectrophotometer. Then growth curve was plotted with data by using Origin analytical software.

2.5 Lipid extraction

According to Zhu *et al.,* (2002) lipid extraction in the dry form with some modification carried out as wet instead of dry. Lipid extraction method was done by Bligh and Dyer (1959) to extract algal lipid. Cells were harvested by centrifugation at 6000 rpm for 4 min and washed trice with normal milliQ water. After that cells were kept for lyophilization using deep freeze drier to eliminate water from the samples. the samples were homogenised with help of mortar and pestle then lipid extracted using solvent, a mixture of chloroform: methanol (2:1, v/v). After homogenization, samples were collected into Eppendorf tubes and 2 mL H2O and 2 mL CH3OH was added to cellular mixture then shaken vigorously for 2 – 5 min and samples were centrifuged at 3000 rpm for 5 - 10 min. The lower phase was separated gently using a micropipette without shaking and isolated it from the solid phase. The solvent phase was taken to dry at water bath up to lipid getting dry at 50°C. The procedure was repeated until entire lipid was extraction. Finally, lipid weight was noted.

III. RESULT AND DISCUSSION:

3.1 Growth Behaviour and Biomass Yield under nutrient supplemented conditions

The growth curves of *Gracilaria emersonii* NC-M1 and *Chlorophyta* sp. NC-M5 were plotted under nutritional stress condition. The plotting was done based on the absorbance of Nitrogen, Magnesium, Calcium and phosphorus treated strains with control. Cell growth rate was varied based on each supplementation in the culture with varied concentration of nutrients. Supplemented culture has shown increased turbidity of cell suspension, the medium with supplementation of nitrogen, magnesium, calcium and phosphorus that support the cell growth compared to the control. Graphical data were plotted with absorbance of treated strain which shows the growth rate. The rate of algal growth is gradually increased in NC-M1 and NC-M5 among nutritional stress conditions after different concentration of nutrient supplemented (Fig. 1 and Fig. 2). Initial increase in nutrient concentration in medium leads increase in growth compared to control in all the nutrient supplemented condition. Among all the tested condition, Maximum increase in growth of *Gracilaria emersonii* NC-M1 was recorded under 476 µM Ca supplemented condition compared to control. At higher concentration of Phosphorous supplementation, reduced growth of *Gracilaria emersonii* NC-M1 was noticed. But in case of *Chlorophyta* sp. NC-M5, the trained of increasing growth was recorded as concentration of N increases in medium. Only under Ca (476 µM) supplemented condition was recorded with inhibition of growth.

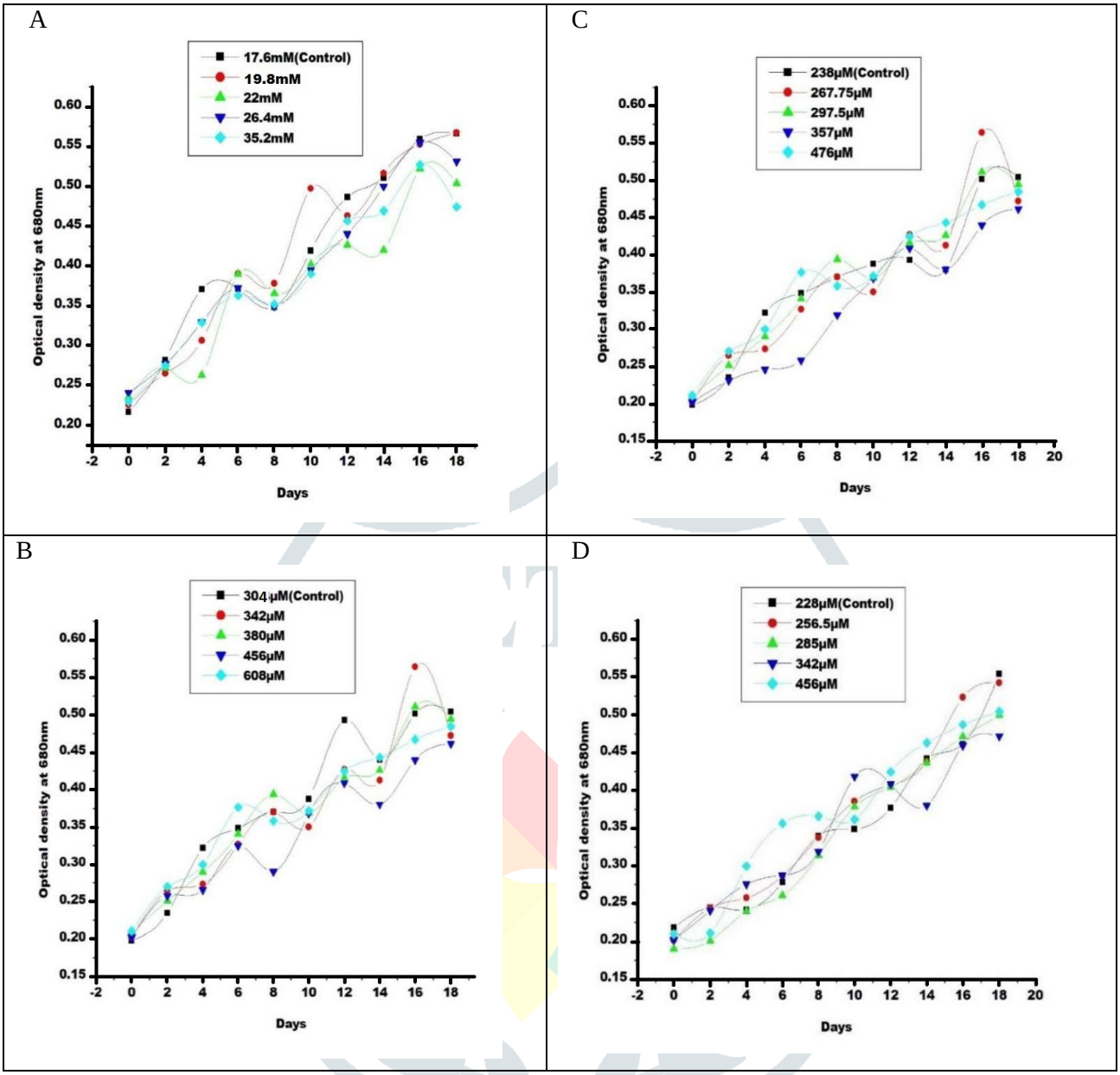

Figure 1 Growth curves of *Gracilaria emersonii* NC-M1 under nutrient supplemented condition: (A) Nitrogen supplementation (B) Magnesium supplementation (C) Calcium supplementation and (D) Phosphorous supplementation.

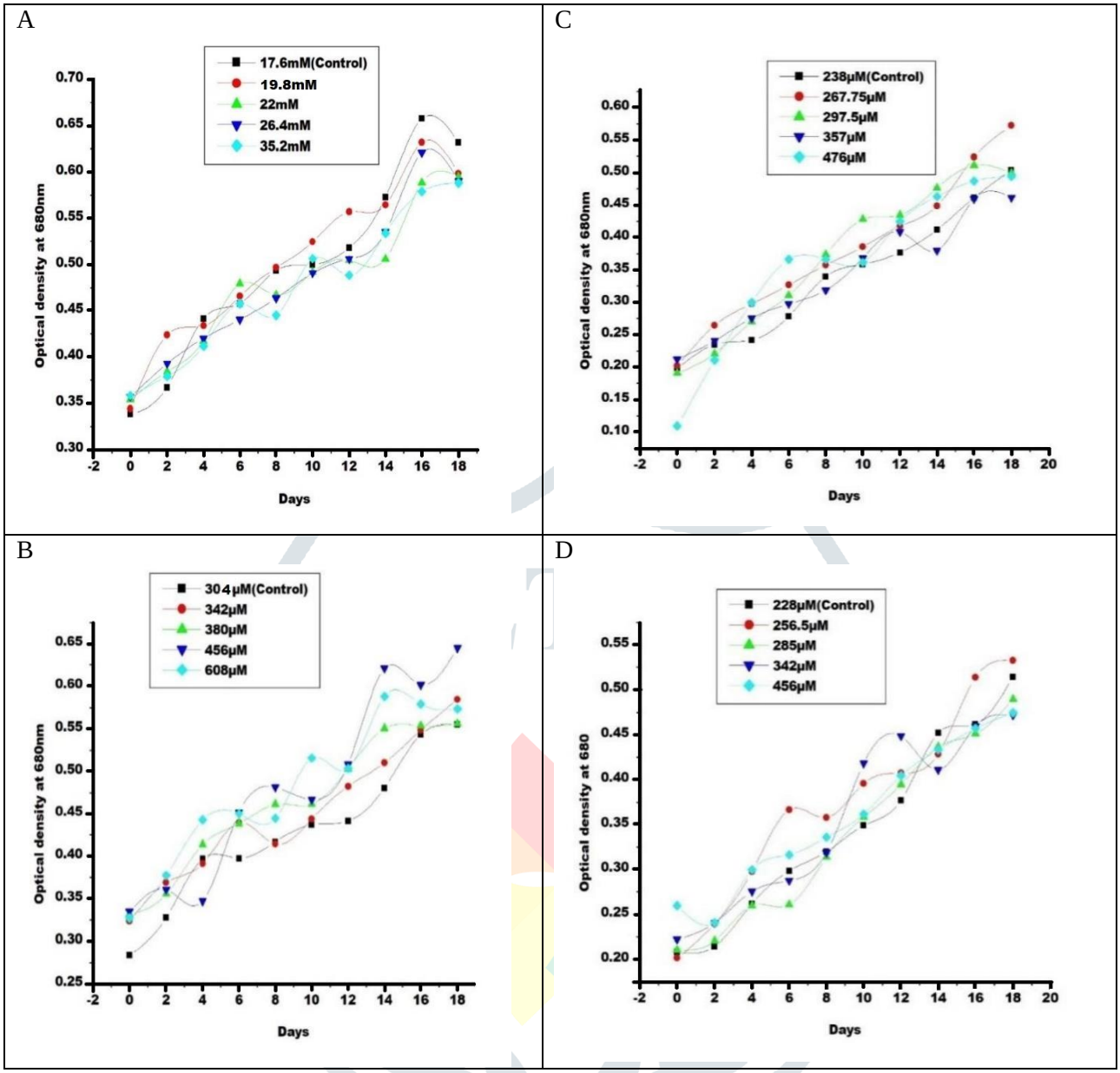

Figure 2 Growth curves of *Chlorophyta* sp. NC-M5 under nutrient supplemented condition: (A) Nitrogen supplementation (B) Magnesium supplementation (C) Calcium supplementation and (D) Phosphorous supplementation.

3.2 Effect of Nutritional supplementation on Biomass

Initial increase in nutrient concentration in medium leads increase in growth compared to control in all the nutrient supplemented condition. Among all the tested condition, Maximum increase in biomass yield of *Gracilaria emersonii* NC-M1 (170±5 mg/L) was recorded under 608 µM Mg supplemented condition compared to control (135±8 mg/L) (Fig. 3). The supplementation of Ca showed 2nd highest increase in biomass yield i.e. 168±10 mg/L compared to control (135±8 mg/L) at concentration of 478µM. At 19.8 mM concentration of nitrogen, slight increase in biomass yield was recorder (160±4mg/L) compared to control. At higher concentration of Phosphorous supplementation, reduced growth of *Gracilaria emersonii* NC-M1 was noticed. But in case of *Chlorophyta* sp. NC-M5, the trained of increasing biomass yield was recorded as concentration of N increases in medium. Only under Ca (476 µM) supplemented condition, the reduction in biomass yield was recorded (Fig. 4).

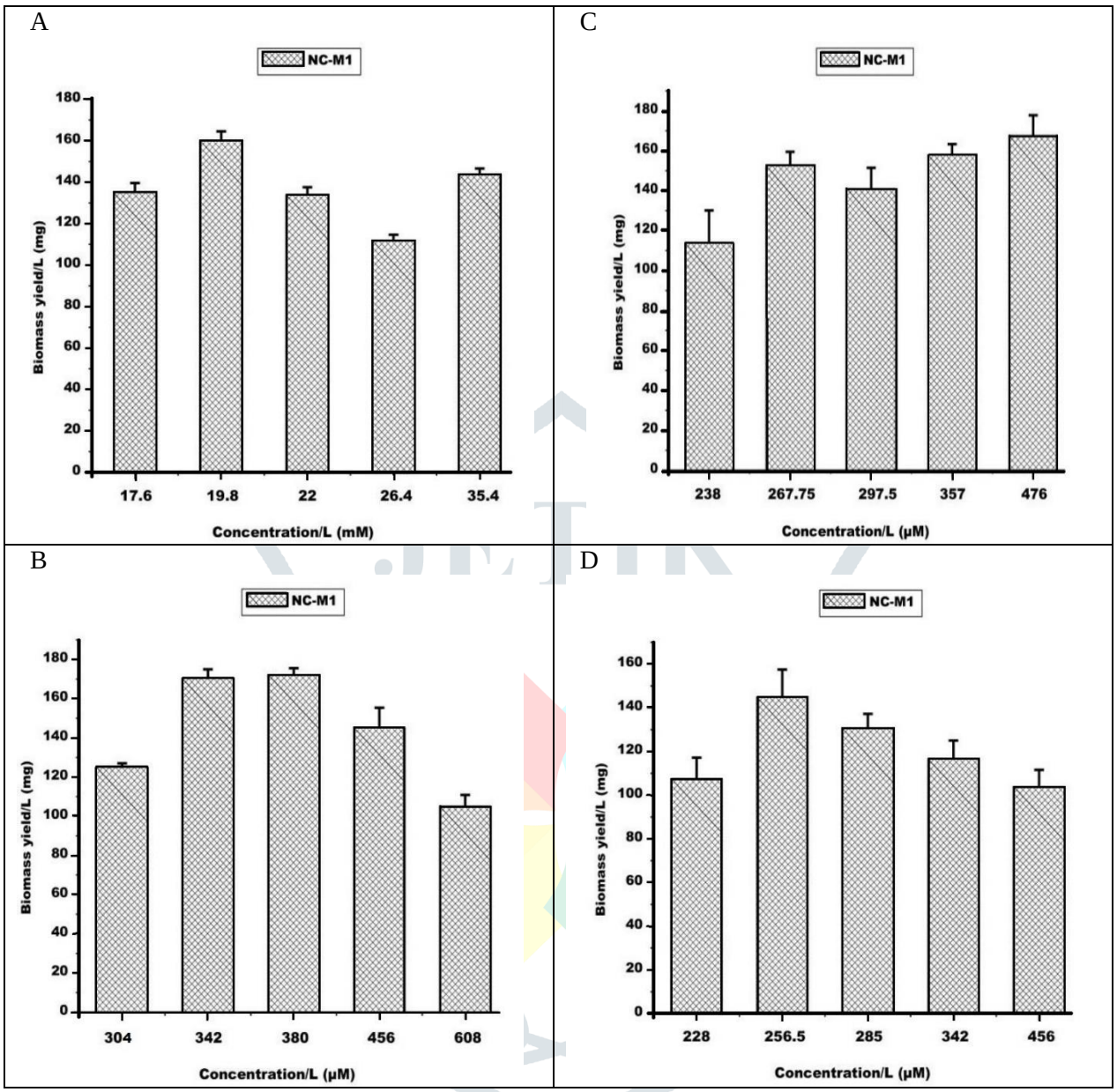

Figure 3 Biomass yield of *Gracilaria emersonii* NC-M1 under nutrient supplemented condition: (A) Nitrogen supplementation (B) Magnesium supplementation (C) Calcium supplementation and (D) Phosphorous supplementation.

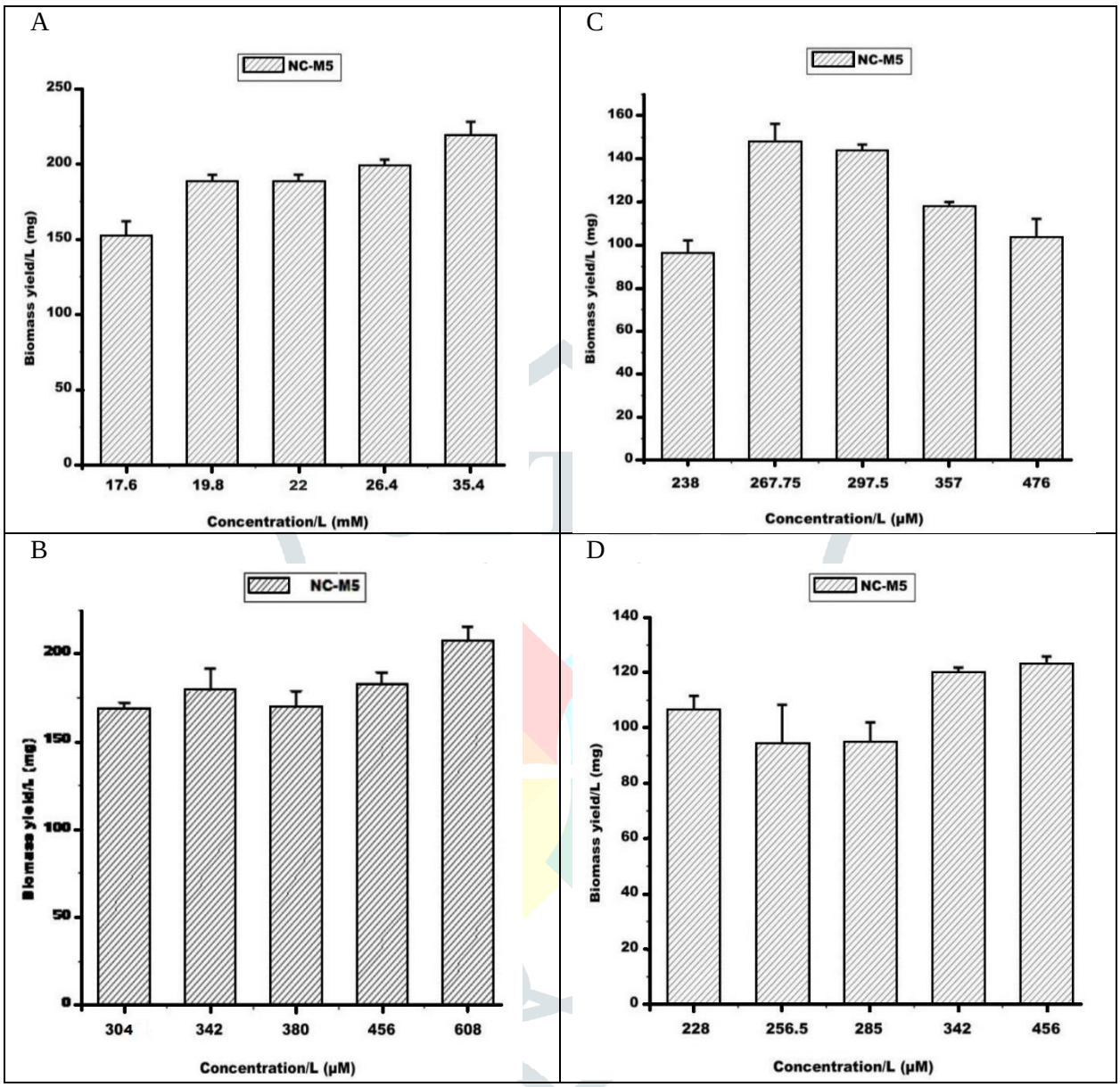

Figure 4 Biomass yield of *Chlorophyta* sp. NC-M5 under nutrient supplemented condition: (A) Nitrogen supplementation (B) Magnesium supplementation (C) Calcium supplementation and (D) Phosphorous supplementation.

3.3 Effect of nutrient supplementation on Lipid Content

Figure 5 and 6 illustrate the nutrient supplementation stress on lipid yield on *Gracilaria emersonii* NC-M1 and *Chlorophyta* sp. NC-M5. *Gracilaria emersonii* NC-M1 was found with highest increase in lipid yield (47±2 mg/L) at nitrogen supplement (35.4 mM) condition compared to control (27±1mg/L). While maximum increased in lipid yield (52±5 mg/L) of *Chlorophyta* sp. NC-M5 was recorded under Mg supplemented (342µM) condition compared to control (48±2mg/L). Both the supplemented condition showed the lipid accumulation significantly 47 ±2 mg/L at 35.4mM concentration of nitrogen (NC-M1) and magnesium showed 45.66 ±4.04 mg/L at 456µM and for calcium 43± 10.14 mg/L at 267.75µM, 39.13 mg ± 7.27mg, for phosphorus 29.86mg ± 3.94 mg at 285µM, 36.33mg ± 3.78 mg at 342µM, 30mg ± 2mg and also lipid increased in NC-M5 for nitrogen showed 46.66 ± 2.08 mg at 35.2mM, 48.66mg ± 0.57mg and calcium at 476µM showed 27 mg ± 6.245 mg increased lipid content. The cultures treated with lower concentrations showed insignificant lipid productivity when compared with control. The cultures with higher nitrogen concentration showed higher lipid productivity. In this study, Nitrogen supplement with two species i.e. *Gracilaria emersonii* and *Chlorophyta* sp. surplus in nitrogen at concentrations of only 19.8mM, 22mM compared to the control at 17.6mM showed a

dramatic change in the lipid productivity, it is seen that the productivity is insignificant or negligible compared to that of the control.

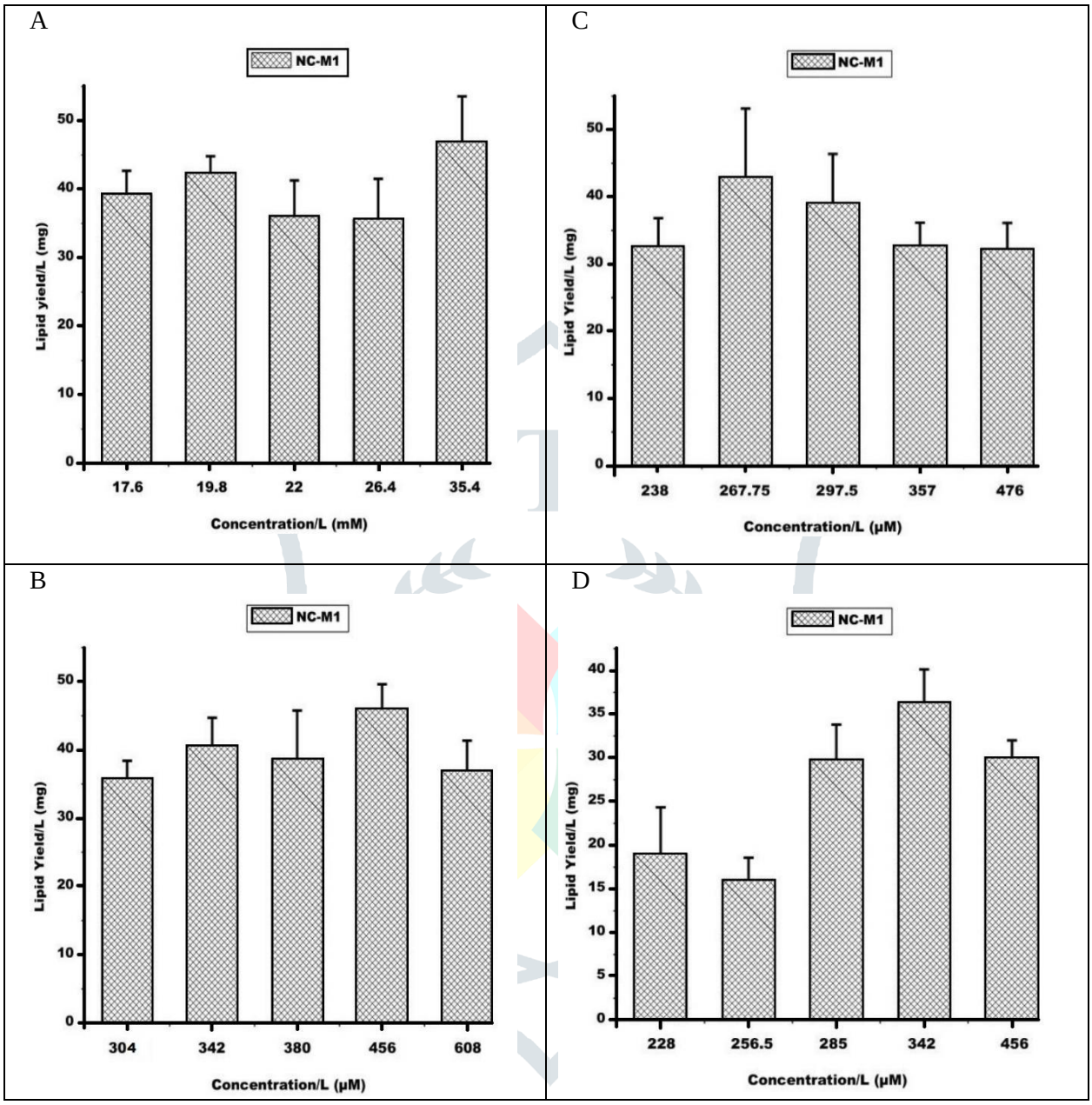

Figure 5. Lipid yield of *Gracilaria emersonii* NC-M1 under nutrient supplemented condition: (A) Nitrogen supplementation (B) Magnesium supplementation (C) Calcium supplementation and (D) Phosphorous supplementation.

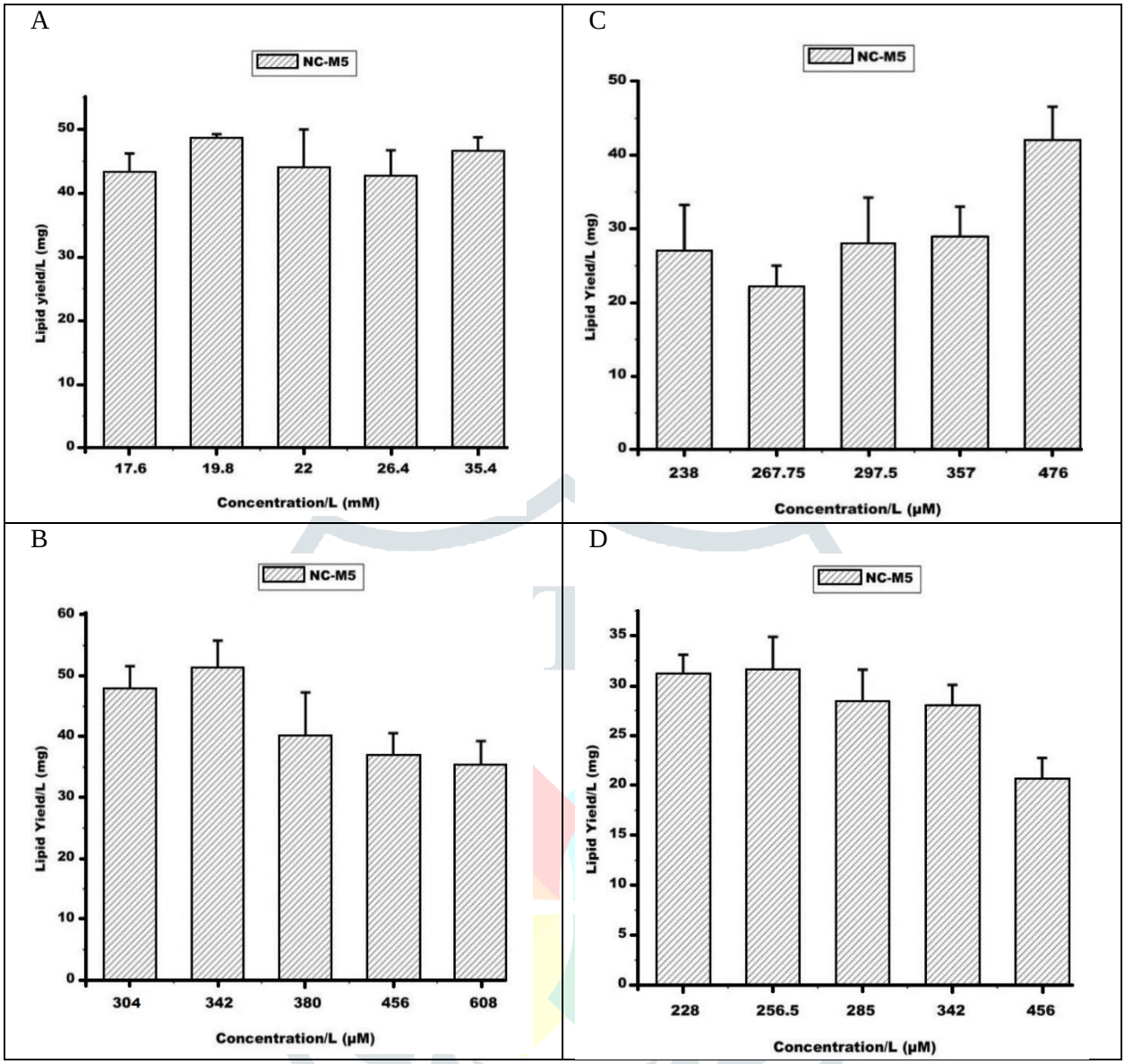

Figure 6 Lipid yield of *Chlorophyta* sp. NC-M5 under nutrient supplemented condition: (A) Nitrogen supplementation (B) Magnesium supplementation (C) Calcium supplementation and (D) Phosphorous supplementation.

3.4 Discussion

Nitrogen, Magnesium, Calcium and Phosphorus is an essential component to algae metabolism and also osmosis, that creates mild hypertonic environment so, that could affect biochemical pathways. Nutritional supplement can decrease or increase the level of lipid accumulation, biomass synthesis and cell division (Fredeen *et al.*, 1990; Jacob and Lawlor 1993; Lippemeier *et al.*, 2003). Although there were some studies shown that the effect of essential substances like nitrogen, calcium, magnesium and phosphorus to algae alters the lipid storage, it is varied and specifically depending on the algal species (Siron *et al.*, 1989; Reitan *et al.*, 1994; Zhao *et al.*, 2009). In this study, *Gracilaria emersonii*, under nitrogen supplementation (26.4mM and 35.2mM) shown increased lipid content when compare with control (Fig.2). The increase in lipid content mainly attributed by supplementation of nitrogen have shown. Magnesium shows some higher lipid accumulation at 456 and 608µM concentration. But phosphorus and calcium treatment were not shown such a lipid accumulation but biomass yield increased. For *Chlorophyta* sp. Calcium and phosphorus have increased lipid content and yield at 476 and 256.5µM concentration. Neutral and polar lipids are found higher in microalgae. Neutral lipids made of triacylglycerols (TAGs) and esters. Neutral lipids have been identified as the primary form of stored carbon in microalgae (Deng *et al.*, 2010). (Xuxiong *et al.*, 2012) found that the supplementation of nitrogen on specific species, its shown increased lipid content significantly similar to *Gracilaria emersonii*. (Liang et al., 2012) reported that the phospholipid content decreased while glycolipid increased under less concentration of phosphorus. Li *et al.*, 2006, suggested that galactolipid was accumulated to compensate for the loss of phospholipids during low concentration of phosphorus. Digalactosyldiacyl glycerol could be synthesized from diacylglycerol that results from phospholipids hydrolysis and/or from de novo synthesis. Depend on nutrient availability, it has been shown to cause significant changes in the lipid biosynthesis of algae (Reitan *et al.*, 1994; Sato *et al.*, 2000). However, Guschina *et al.*, 2003) found only minor alterations of fatty

acids in Coccomyxa mucigena, *C. peltigeravariolosae*, *Trebouxia aggregata* and *T. erici*. The present results suggest that no obvious change of unsaturated fatty acid composition occurred in *Gracilaria emersonii*. under supplementation. This leads to an accumulation of carbon, which might be stored in the form of TAG that is rich in saturated fatty acid (Elly and Alexander 2011). Rise in lipid content was also observed under increased concentration of calcium. Calcium plays a significant role in plant growth and development, including signal transduction pathogenic resistance and stress-tolerance; increased biomass yield and chlorophyll synthesis was also observed. (Nguemezi, 2010). The myriad processes in which the bivalent ion of this metal participates is large and involves nearly all aspects of plant growth and development. (Hepler, 2005). Calcium has been found to be essential for growth of Chlorella sp. (Manuel, 1944). Calcium starvation although registered a partial decrease in biomass yield, the lipid pool showed a profound rise. In general, under nutrient limited conditions, the growth of the algae slow down, and there is a reduction in the requirement for the synthesis of new membrane compounds. However, fatty acid biosynthesis has not been interrupted as photosynthesis continues. Therefore, the cell deposits these fatty acids in the form of triacylglycerols. (*Sharma et al.,* 2012) Furthermore, under normal culture condition, the two major components generated by photosynthesis are ATP and NADPH, which are used for producing biomass. As cell growth and proliferation are hampered under nutrient limitations, regeneration of NADP+ has been achieved by consuming NADPH for fatty acid biosynthesis. (Thompson *et al.,* 1996). It has been suggested that production of lipids might be a protective measure against cell injury under osmotic stress. (Duan, 2012) This hypothesis can be extended to infer that production of excess lipids might be an emergency response of microalgae to several kinds of stresses, including deficiency or surplus of vital nutrients such as calcium, when accumulation of organic molecules with high calorific values has to be prioritized over growth. (Taiz *et al.*, 2010). In the present study, lipid accumulation was increased at elevated level of NaCl. This is advantageous for the large-scale outdoor cultures as there are always colossal chances of contamination by other algae/microbes. Thus, NaCl can act as a controlling agent, and reduce the chance of invading microbes to spoil the mass cultures. The fatty acid composition of algae can vary both quantitatively and qualitatively with its physiological status and culture conditions, and the properties of biodiesel are mainly determined by its fatty acid esters. (Knothe *et al.,* 2005) The unsaturated bonds are vulnerable to oxidation during storage and this factor lowers the acceptability of any oil for biodiesel production. (Behrens *et al.,* 1996).

IV. CONCLUSION

Numerous research studies imply at nutrients depleted conditions, microalgae change their biosynthetic pathways to improve production of lipid accumulation. However, they are unable to synthesis excess quantity of biomass and lipid each other at the same time. But the nutritional supplemented stress on lipid accumulation of algae is not well reflected. Hence, to enhance biomass and lipid yield, nutrient (N, Mg, Ca, P) supplementation study on *Gracilaria emersonii* NC-M1and *Chlorophyta* sp. NC-M5 have been performed. Our results showed the maximum increase in biomass yield for *Gracilaria emersonii* NC-M1 under Mg supplemented condition (608µM) while maximum increased in lipid yield (47±6) was noticed under N supplemented (35.4mM) condition. Furthermore, FAME profile influences the properties of biodiesel for example kinematic viscosity, saponification and iodine values, cetane number, storage quality and the higher heating value. This demands further study for large scale production under the above selected conditions.

V. ACKNOWLEDGEMENT:

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VI. ABBREVIATION:

1. NC-M1 & NC-M5 – NC represents Neha Chaurasia (corresponding author abb); M1 and M5 – Madan (Co-author abb).
2. WHO – World Health Organisation.
3. ASP – Aquatic Species Program.
4. DOE – Department of Energy.
5. FAME – Fatty Acid Methyl Ester.
6. BBM – Bold Basal Medium.

VII. DECLARATION:

7.1 Ethics approval

Not applicable

7.2 Consent for Publication

Not Applicable

7.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

7.4 Competing Interest

The authors declare that they have no competing interests

7.5 Funding

Not applicable.

Author Information

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VIII.BIBLIOGRAPHY

1. Andersen RA (2013) The microalgal cell. In: Richmond A, Hu Q (eds) Handbook of microalgal culture: applied phycology and biotechnology, 2nd edn. Wiley, Oxford, pp 1–20.
2. Barreiro DL, Prins W, Ronsse F, Brilman W (2013) Hydrothermal liquefaction (HTL) of microalgae for biofuel production: state of the art review and future prospects. In: 20th Eur Biomass Conf. vol 53 pp 113–127.
3. Borowitzka MA (2013) (eds) Algae for Biofuels and Energy- Develop in Appl Phycol, springer.DOI.10.1007/978-94-007-5479-9_1.
4. Borowitzka MA (2013a) High-value products from microalgae—their development and commercialisation. J Appl Phycol 25:743–756.
5. Borowitzka MA (2013b) Energy from microalgae: a short history. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Houten, pp 1–15.
6. Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008) Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr Opin Biotechnol 19:235–240. doi:10. 1016/j.copbio.2008.05.007.
7. Falcon LI, Magallo ´n S, Castillo A (2010) Dating the cyanobacterial ancestor of the chloroplast. ISME J 4:777–783.
8. Fehling J, Stoecker D, Baldauf SL, Falkowski PG, Knoll AH (2007) Photosynthesis and the eukaryote tree of life. In: Falkowski PG, Knoll AH (eds) The evolution of primary producers in the sea. Academic Press, New York, pp 76–107.
9. Goers M, Schumann R, Hepperle D, Karsten U (2010) Quality analysis of commercial Chlorella products used as dietary supplement in human nutrition. J Appl Phycol 22:265–276.
10. Hossain ABM, Salleh A, Boyce AN, Chowdhury P, Naqiuddin M (2008) Biodiesel fuel production from algae as renewable energy. Am J Biochem Biotechnol 4:250–254.
11. Khan Z, Bhadouria P, Bisen PS (2005) Nutritional and therapeutic potential of spirulina. Curr Pharm Biotechnol 6:373–379.
12. Kiple KF, Ornelas KC (2000) The Cambridge world history of food. Cambridge University Press, Cambridge.
13. Open Government Data Platform India; webpage: https://community.data.gov.in/automobiles-and-pollution-in-india/
14. Pienkos PT, Darzins A (2009) The promise and challenges of microalgal-derived biofuels. Biofuels Bioprod Biorefin 3:431–440.
15. Radakovits R, Jinkerson RE, Darzins A, Posewitz MC (2010) Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell 9:486–501.
16. Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for single cell oil production. Rec Adv Lip Metab Relat Disord 86:807–815.
17. Tamiya H (1957) Mass culture of algae. Annu Rev Plant Physiol 8:309–334.