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Effect of Different Nitrogen Concentrationson the Growth, Proximate and Biochemical Composition of Freshwater Microalgae *Scenedesmus communis*

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ABSTRACT

The aim of this study was to improve the growth, proximate and biochemical composition of freshwater microalgae *Scenedesmus communis* cultured under different $NaNO_3$ concentration. Significantly ($p<0.05$) maximum cell density (11.6×10^6 cells/ml), biomass productivity (0.58 g/L), total chlorophyll, protein (23.58 % dry weight) and carbohydrate (27.26 % dry weight) were found in the highest concentration (18.5g/500ml) of $NaNO_3$ compared to control and lower concentration. On the contrary, lowest (6.5g/500ml) concentration of $NaNO_3$ resulted in significantly highest lipid 20.92% dry weight, specific growth rate (0.521 ± 0.01 day^{-1}), monounsaturated fatty acids (28.53 % of total fatty acids) and polyunsaturated fatty acids (67.37 % of total fatty acids). In case of carotenoid and total phycobiliprotein content, significantly ($p<0.05$) higher value of 6.43 mg/L and 6.14 mg/g, respectivelywas produced in control (12.5 g/500 ml $NaNO_3$) compared to other concentration of $NaNO_3$. Significantly ($p<0.05$) highest value of total saturated fatty acids was found (56.51 %) of total fatty acids in 15.5 g/500ml concentration of $NaNO_3$. Therefore, it could be concluded that high concentration of $NaNO_3$ boosted the growth and nutritional profile of *S. communis*. Moreover, lipid production and fatty acid profile was enriched by the lowest concentration of $NaNO_3$ showing economic and sustainability for commercially mass culture of this microalga.

1. Introduction:

Global demand for microalgae is increasing day-by-day since it drew attention as a green energy source of biofuel in the next generation. Based on this cue, a range of microalgae species are being cultured in hatcheries for various purposes, including human nutrition, biofuel production, aquaculture nutrition, manufacturer of pharmaceuticals, and cosmetics as well as biofertilizer production [Wijesekara et al., 2010]. As live feed, microalgae are mostly used in aquaculture food chain for various growth stages of crustacean, bivalve mollusk, some fish species, and zooplankton. Microalgae can easily survive in wastewater by absorbing nutrients, which reduces high cultivation cost. This offers eco-friendly, sustainable and energy-efficient biofuel production [Muller-Feuga, 2000]. *Scenedesmus* sp. has ability to grow well in diverse types of waste water [Toyub et al., 2008]. Having good amount of saturated and unsaturated fatty acids as well as all the essential amino acids, *Scenedesmus* sp. is highly potential for biodiesel and nutraceuticals production [Qu et al., 2019]. A better growth performance and biochemical composition of *S. obliquus* was found in Bold Basal Media than Bristol and 2.5% waste water media [Khatoon et al., 2017; Toyub et al., 2008]. It has also been reported that sodium nitrate has significant impact on the high specific growth rate of microalgaethan urea, ammonium carbonate and ammonium chloride [Podevin et al., 2015].

The economic viability of biodiesel produced from microalgae, as well as biomass productivity and lipid yields are important parameters that must be optimized. Alteration of cultivation techniques such as changing the nutritional elements of the growth medium, ambient conditions, and microalgae development phases can increase biomass productivity and composition of biomolecules, particularly fatty acid production in microalgae. Recently much work has been done on different microalgae species to enhance its growth performance, lipid production and enrich the nutrient composition. When a light source and CO_2 are available, nutrient limitation is one of the most effective triggers for increasing lipid accumulation in a single microalgal cell [Li et al., 2011]. As a component of high-value biological macromolecules such as proteins, chlorophylls, and DNA, nitrogen is a crucial macronutrient that affects the metabolism and, as a result, the growth and biochemical composition of microalgae. Adding or limiting nitrogen concentration has significant impact on the growth rate, as well as protein, lipid, and carbohydrate synthesis in microalgae. Photosynthetic activity and biochemical composition such as fatty acids, pigment, and protein are notably affected by nitrogen limitation, which is also effective to enhance the lipid content of microalgae. Nitrogen constraints also influence in conversion of free fatty acids into triacylglycerols (TAGs) over time, which is suitable microalgal lipid feedstock for biodiesel production [Nayak et al., 2019]. The addition of a limited amount of $NaNO_3$ to the microalgae culture

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medium on a daily basis improved algal growth, biochemical characteristics, and lipid synthesis [Zhang and Liu, 2016; Nayak et al., 2019]. The effects of different CO_2 and Fe^{3+} ion concentrations on the biomass yield, lipid productivity and accumulation, fatty acid profile, and the level of antioxidant compounds of *S. obliquus* were evaluated in N-9 medium [Abd El Baky et al., 2014]. Zarrinmehr et al. [2020] found that at sufficient nitrogen concentrations (72 mg/L), polyunsaturated fatty acids (PUFAs) increased five-fold, compared to nitrogen deprivation, where saturated fatty acids (SFAs) concentrations were higher than nitrogen sufficiency.

Therefore, to improve the growth, nutritional and biochemical composition, it is necessary to standardize the distribution of nutrients, particularly nitrogen for microalgae cultivation. Despite many studies on *Scenedesmus* sp., not focused work has been done on the effect of different nitrogen concentrations on the growth and biochemical composition of *S. communis*in Bold Basal media. This study aimed to explore various nitrogen concentrations which might enhance the growth, productivity, pigment, and proximate composition of freshwater *S. communis*.

2. Materials and Method

2.1. Microalgae collection and culture

The freshwater *S. communis* was collected from 'Live Feed Research Corner', Faculty of Fisheries, Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. The pure *S. communis*was cultured in slight modified Bold Basal Media [Stein, 1980] under controlled environment at 25.2 ± 0.7°C, 7.72 ± 0.17 pH, 4.53 ± 0.53 mg/L dissolved oxygen and 150 E m-2s-1 light intensity for 24 h.

2.2 Experimental Design

There were four treatments of different nitrogen ($NaNO_3$) concentration as 6.5g/500ml (T1), 9.5g/500ml (T2), 12.5g/500ml (control), 15.5g/500ml(T3), and 18.5g/500ml (T4) according to 24% up and down from control. This experiment was divided into two parts: firstly, analysis of growth curves and secondly, analysis of pigment and proximate composition. To analyze the growth curve, 2% *S. communis* seeds (~ 10^5 cells/ml) from pure stock were inoculated in 300ml culture media of each treatmentin a 500 ml conical flask. Growth parameters such as cell density, optical density, biomass, and chlorophyll (a, b) were measured everyday to explore the stationary phase up to 13 days of the culture period. Afterward, mass culture was continued in a 2 - L volume Erlenmeyer flask along with 2% *S. communis* seed inoculation till stationary phase for each treatment. Then the harvested microalgae through a centrifuge (Hitachi* High-speed Refrigerated Centrifuge, Himac CR 21g-II) was dried at 40°C temperature using a hot air oven, and subsequently, preserved at refrigerator for following pigments, proximate, and fatty acid analysis.

2.3. Determination of Growth Curve

2.3.1. Determination of Cell Density

Microalgal cells were counted every day with a Hemacytometer throughout the growth curve experiment according to Lavens and Sorgeloos [1996].

2.3.2. Determination of Optical Density

Daily absorbance change at 600 nm wavelength was measured using a Nano Drop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany) where treatment wise culture media was used as the blanks.

2.3.3. Determination of Biomass

Biomass was estimated through slight modification of procedure described by Ratha et al. [2016]. After rinsing with 10 ml distilled water and drying for 4 hours 100°C in a Hot Air Oven (LNO- 150, LABNICS Equipment, USA), followed by 15-20 minutes desiccation, filter papers (47 mm Whatman® GF/C) were weighed. Vacuum filtration was used to filter 1.0 ml microalgae sample from each culture through pre-weighed filter paper. The filter paper with biomass was then rinsed three times with 10 mL distilled water before being oven-dried for another four hours at 100°C, followed by 15-20 minutes of desiccation. Filter paper with biomass was also weighed. The dry biomass concentration was then estimated by dividing the difference between the pre and post-filtration weights of the dried filter paper by the filtered volume.

2.4. Specific Growth Rate and Cell Doubling Time Estimation

The Specific Growth Rate, μ (day^{-1}) and cell doubling time of cultured microalgae was calculated according to Xu et al. [2016].

SGR, μ (day^{-1}) = $\ln(C_2 - C_1)/(t_2 - t_1)$

Where,

$C_2 - C_1$ = Difference in Cells/ml between the end and beginning of the experiment; $t_2 - t_1$ = Time interval between final and initial days

From the above equation, the cell doubling time of the culture was calculated as days required for duplication using the following formula:

t_d (day) = 0.693/SGR

2.5. Productivity Estimation

For each treatment, volumetric [Green, et al., 1995], areal [Ugwu et al., 2008], and lipid [Benemann and Tillett, 1987] productivities were estimated using dry biomass and percentage of lipid at the stationary phase of microalgae cultures.

Volumetric productivity, VP (g L^{-1} day^{-1}) = $(X_n - X_0)/N$

Where, X_n = Final biomass (g/L), X_0 = Initial Biomass (g/L) and N = Culture days

Areal productivity, AP (g $cm^{-2}day^{-1}$) = (VP x V)/A

Where, VP = Volumetric Productivity (g L^{-1} day^{-1}), V = Total volume of the culture (L),

A = surface area occupied ground (cm^2).

Lipid productivity, LP (mg L^{-1} day^{-1}) = VP x (% lipid/100)

Where, VP = Volumetric productivity of the culture, % lipid = Percentage of lipid content as dry weight basis.

2.6. Determination of Pigments

2.6.1. Extraction of Microalgae for Chlorophyll Determination

Microalgae sample was extracted according to Jenkins [1982] with few modifications for chlorophyll determination. A 1.0 ml of microalgae sample from the culture was transferred to the filter (47 mm Ø Whatman® GF/C glass microfiber filter papers) after 1.0 ml of 1% $MgCO_3$ vacuum filtration. Then the filter paper was submerged into 5.0 ml of 90% acetone for 2 minutes and then ground for 1 minute by a tissue homogenizer to make well mixed suspension. After recurring this procedure twice, centrifuge tubes were refrigerated (4°C) for 1 h in the dark, thereafter, centrifuged at 3000 x g for 10 minutes.

2.6.1.1.Determination of Chlorophyll a and b (Dichromatic method)

The clean extract was measured absorbance at 750, 664, 647, and 630 nm wavelengths, where OD at 750 nm was used as a turbidity correction factor and subtracted from the other wavelengths' absorbance. The concentrations of chlorophyll a and b in the acetone extract were calculated by the following equations [Jeffrey and Humphrey, 1975]:

C_a (mg/L) = 11.85 (OD 664) - 1.54 (OD 647) - 0.08 (OD 630)

C_b (mg/L) = 21.03 (OD 647) - 5.43 (OD 664) - 2.66 (OD 630)

After that, the amount of pigment per unit volume was calculated as follows:

Chlorophyll a, mg/m^3 = (Ca × extractvolume, L)/ volume of sample, m^3

2.6.2. Determination of Carotenoids

At the stationary phase of each culture 1.0 ml microalgae sample was extracted and carotenoid amount was calculated following Khatoon et al. [2020].

2.6.3. Extraction of Phycobiliproteins

S. communis powder (40 mg) was soaked in 10 ml phosphate buffer solution (pH 7.0; 0.1M), vortex mixed thoroughly, and stored at 4°C for 24 h. After centrifuging at 6000 x g for 10 minutes, the supernatant was collected and absorbance was measured using a NanoDrop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany) at wavelengths of 562, 615, and 652 nm, with phosphate buffer as a blank [Siegelman and Kycia,1978].

2.6.3.1. Spectrophotometric Estimation of Phycobiliproteins

The amount of phycocyanin, phycoerythrin and allophycocyanin in the sample was determined using the extinction coefficients and the following formulae [Siegelman and Kycia, 1978]:

Phycocyanin (mg/ml) = $\{A_{615}-(0.474\times A_{652})\}/5.34$

Allophycocyanin (mg/ml) = $\{A_{652}-(0.208\times A_{615})\}/5.09$

Phycoerythrin (mg/ml) = $\{A_{562}-(2.41\times PC)-(0.849\times APC)\}/9.62$

The following formulas were used to compute total phycocyanin, phycoerythrin, and allophycocyanin (mg/g) [Silveira et al., 2007]:

Phycobiliprotein(mg/g) = (Pigment concentration × V) / DB

Here, V= solvent volume, DB= Dried biomass

The sum of the phycocyanin, phycoerythrin, and allophycocyanin contents in dried microalgae biomass was used to compute total phycobiliproteins (mg/g).

2.7. Determination of Proximate Compositions

2.7.1. Protein Determination

Protein was determined by spectrophotometric method according to Lowry et al. [1951] with few alterations. Each sample contained 5.0 mg of oven-dried microalgae biomass, which was used to make a 25 ml well mixed (by tissue homogenizer) solution with distilled water. For each sample, 0.5 ml of the 25 ml solution was taken and 50 ml of Reactive 2 (20 g of Na_2CO_3 in 100 ml of 0.1N NaOH) and 1 ml of Reactive 1 (1 % NP tartrate) were combined with the microalgae solution. After that, 0.5 ml of the mixture was mixed with 0.5 ml of 1N NaOH and maintained at 100°C for 5 minutes in a hot water bath. The samples were then chilled in a cold water bath before being added 2.5 ml of the produced mixed reagent and maintained for 10 minutes. Following that, 0.5 ml of Folin reagent was added to the mixture and then kept in a dark place for 30 minutes. At a wavelength of 750 nm, the absorbance of the mixture was measured using a Nano Drop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany). To create a calibration graph, a stock solution of 2000 µg/L of standard (albumin) was created, and a series of standards (20 µg/L, 40 µg/L, 80 µg/L, 100 µg/L, and 200 µg/L) were made from the stock solution. For the standard series, the same procedures as for protein analysis were used; a calibration line was constructed according to absorbance, and the protein content of each sample was estimated appropriately.

2.7.2. Lipid Determination

Lipid was determined according to Bligh and Dyer [1959] and [Folch et al., 1957]. Each sample's aluminum dish was labeled and weighed as the starting weight. Then, with distilled water, 50 mg of each sample was placed in a centrifuge tube and diluted into a 5x volume. The material was then homogenized with 3 ml 2:1 methanol: chloroform (v/v) using a tissue homogenizer. After that, all of the tubes were centrifuged for 4 minutes at 1000 rpm at 4°C, and the supernatants were pipetted into clean tubes and placed in ice. 3 ml 1:2 methanol: chloroform (v/v) was mixed homogeneously with the sample once more. The tubes were then centrifuged again using the previously described method, and the supernatants were transferred to the prior tubes of supernatants.1.5 ml of 0.9 % NaCl was vortex mixed into this combined supernatant, and the tubes were stored at 4°C for 1 h. The tubes were centrifuged at 1000 x g for 10 minutes at 4°C after 1 h. The top layer of methanol and chloroform was discarded, while the bottom layer was transferred to the aluminum dish that had been prepared previously. The solvent was then evaporated in a hot air oven at 40°C (LNO- 150, LABNICS Equipment, USA). The aluminum dishes were then weighed to get the final weight. Finally, the initial weight was subtracted from the final weight to get the lipid weight in the samples. Lipid content was calculated by the following formula:

Lipid (% dry weight) = lipid weight/sample weight

2.7.3. Carbohydrate Determination

Carbohydrate was determined according to Dubois et al. [1956]. A 5.0 mg oven-dried biomass sample was used to make a 25 ml well mixed (tissue homogenizer) solution with distilled water for each sample. After that, 1.0 ml of the 25 ml solution was taken for each sample, followed by 1 ml of 5 % phenol solution and 5.0 ml of sulfuric acid. The samples were then placed in a cold water bath for a period of time. When the mixture become cool, absorbance of the solution was taken at 488 nm wavelength using Nano Drop Spectrophotometer (NanoPlus, WAVE ANALYTICS D-82362 Weilheim, Germany) to estimate carbohydrate. To build a calibration graph, a 1000 µg/L standard (glucose) stock solution was prepared, and then a series of standards at various dilutions (20 µg/L, 40 µg/L, 60 µg/L, 100 µg/L, and 140 µg/L) were made from the stock solution. For the standard series, the same procedures as for carbohydrate analysis were used; a standard graph was constructed based on the standard values obtained from the absorbance, and the carbohydrate content for each sample was calculated appropriately.

2.8. Fatty Acid Analysis

Fatty acids were determined by "Two steps transesterification (2TE)" method after slight modification according to Griffiths et al. [2010]. At first, lipid was extracted by Digital Soxhlet Apparatus (FOOD ALYTRD 40) after mixing 70 ml diethyl ether into 500 mg microalgae powder. After extraction, solvent was removed at 60°c by Hot Air Oven. 1.5 ml methanolic NaOH was added into lipid extract and mixed at 80°C by sonicator for 5 minutes. After cooling at room temperature, 2.0 ml BF3 methanol was added into the mixture and sonicated for 30 minutes at 80°C. After cooling tubes at room temperature, 1.0 ml isooctane and 5.0 ml saturated NaCl was mixed through well shaking. The fatty-acid methyl-esters (FAMEs) containing upper organic layer was transferred to a new tube and 1.0 ml sample was taken into vial for analysis of fatty acid methyl-esters by Gas Chromatography Mass Spectrophotometry (GC-2020 plus, SHIMADZU, Japan). A capillary column (length 30m, internal diameter 0.25 mm, film thickness 0.15 ìm, phase ratio 250) was used to separate FAME. Helium was used as the carrier gas, with a flow rate of 1.42 ml/min. The temperature program for the column was as follows: 180° to 280°C at 5°C/min, then held at 280°C. By comparing retention time to a standard, FAMEs were identified (FAME mix C8-C24; Sigma-Aldrich; Germany).

2.9. Statistical Analysis

MS Excel was used to determine the mean of three replications' values, standard deviation and standard error. IBM SPSS (v. 26.0) was used for statistical analysis. Significant difference between the treatments in terms of growth parameters, productivity, pigments, and proximate composition was evaluated with One Way ANOVA. Duncan test was applied to determine the homogenous subsets (alpha = 0.05).

3. Result and Discussion

Microalgae growth, biochemical properties as well as culture duration was significantly affected with different $NaNO_3$ concentrations in the culture media. Nitrogen's effect on the growth of *S. communis* has been shown clearly in Figure 1 and Figure 2 based on growth parameters such as cell density, optical density, biomass, chlorophyll a and b, which varied significantly ($p < 0.05$) from day-1 to day-12 whether there were no significant difference in day-0 ($p > 0.05$). Lag phase remained for day-0 and exponential phase began from day-1 where stationary phases significantly varied among the treatments. In case of five treatments such as 6.5 g/500ml $NaNO_3$ (T1), 9.5 g/500ml $NaNO_3$ (T2), 12.5 g/500ml (control), 15.5 g/500ml $NaNO_3$ (T3), and 18.5 g/500 ml $NaNO_3$ (T4), stationary phases were found on day-7, 8, 9, 10, 11, respectively according to all growth parameters. Cell growth had dropped after completing the stationary phase.

Figure 1a showed that the significantly ($p < 0.05$) highest cell density 1.16×10^7 cells/ml was obtained under T4 among all other treatments and the lowest cell density 6.41×10^6 cells/ml was obtained under T1 at their stationary phase. On the other hand, T2, C and T3 resulted cell density in a respective way such as 7.08×10^6, 9.34×10^6 and 1.02×10^7 cells/ml. Initial cell density was around 1.65×10^5 cells/ml for all the treatments. Similar to this result, Zarrinmehr et al. [2020] reported that minimum and maximum cell concentration of *Isochrysis galbana* were achieved in 36 and 144 mg/L $NaNO_3$ respectively in Walne's medium. It can be justified as higher nitrogen concentration promotes the algal biomass production, but lower nitrogen concentration has remarkable effect on retarding microalgae growth [Zarrinmehr et al., 2020].

A regular change in optical density over the cultivation period till stationary phases for all the treatments has been shown in Figure 1b, where minimum 0.585 ± 0.001and maximum 0.835 ± 0.001 absorbance was observed under T1 and T4 at their stationary phases on day-7 and day-11. Biomass had also varied significantly ($p < 0.05$) according to the nitrogen concentration (Figure 1c). The lowest (0.33 ± 0.003 g/L) and highest (0.58 ± 0.004 g/L) biomass were produced under T1 and T4, respectively on day-7 and day-11. 0.38 ± 0.001, 0.44 ± 0.001 and 0.52 ± 0.003 g/L biomass were obtained consecutively from T2, C and T3 at their stationary phase.Yang et al. [2008]reported that biomass production in *Chlamydomonas reinhardtii* was subdued up to 31.7% under nitrogen deficiency as nitrogen limited conditions of microalgae culture has led to a significant decrease in the cell density, biomass productivity and chlorophyll synthesis.

A gradual rise was observed in chlorophyll production from the lower nitrogenous treatment to the higher nitrogenous treatment due to continuous increment of cell density. Significant ($p < 0.05$) change in chlorophyll a and bhas also been shown in Figure 2(a, b) according to the treatments; moreover, values of chlorophyll a and b have significant strong correlation ($R^2 = 0.97$; $p<0.05$) between them. Chlorophyll a values were amplified successively 667.47 ± 9.2 to 1100.6 ± 5.7 mg/L and chlorophyll b values were 248.33 ± 3.37 to 494.3 ± 1.5 mg/L fromminimum to maximum nitrogenous treatment. Zarrinmehr et al. [2020] also found that chlorophyll a and chlorophyll b reduced with diminishing nitrogen concentration from 144 to 0 mg/L. Zhang and Liu [2016] observed that total chlorophyll content in *I. galbana* decreased from 5.9 to 3.9 mg/L

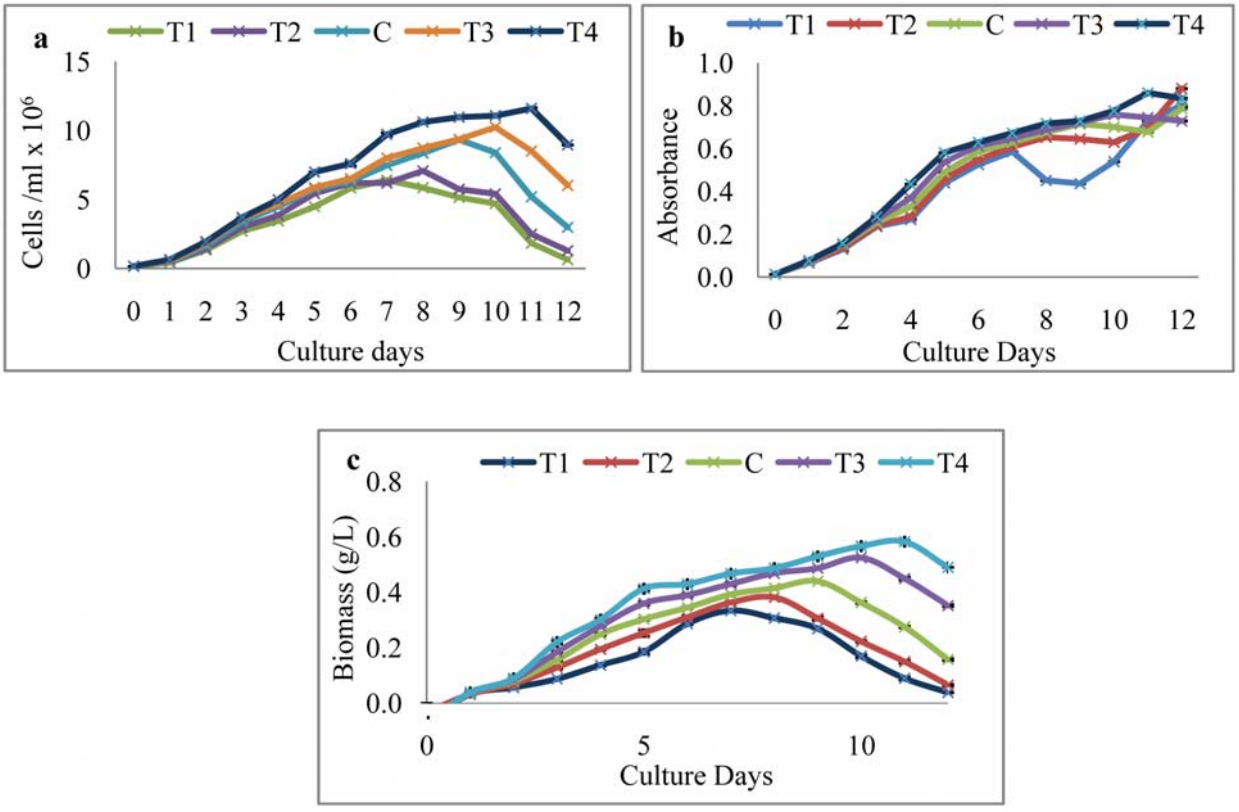

Figure 1.(a) Cell density (cells/mlx10^6), (b) Optical density (Abs) and (c) Biomass (g/L dry weight) of freshwater *S. communis* cultured in different nitrogen concentrations. Values are mean ± SE.

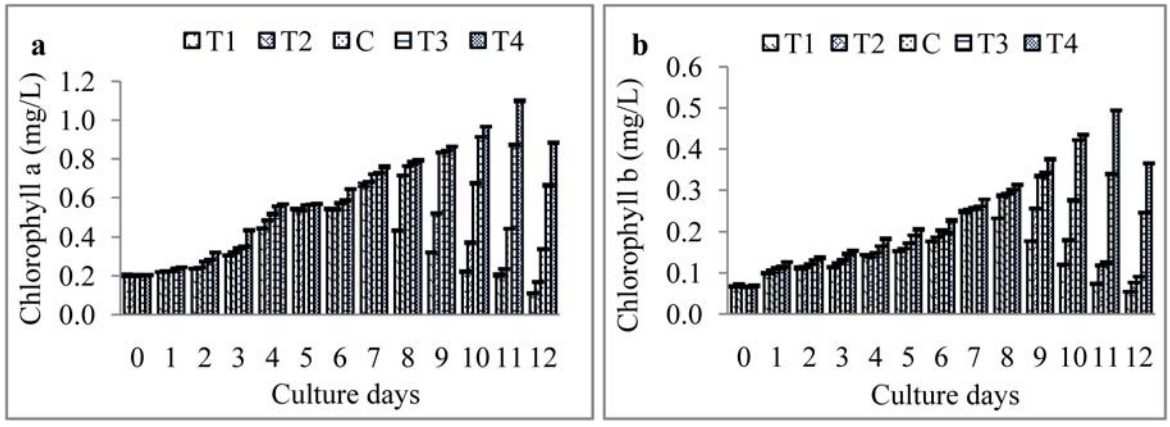

Figure 2. (a) Chlorophyll a content (mg/L) and (b) Chlorophyll b content (mg/L) of freshwater *S.communis* cultured in different nitrogen concentrations. Values are mean ± SE.

under nitrogen deprivation. Nitrogen starvation lessens efficiency of photo system II activity and hampers the photosynthetic pigment synthesis as a restriction factor [Zhang and Liu, 2016; Xie et al., 2017]. Among these growth parameters, there have strong correlation ($p < 0.05$) between cell density and optical density, biomass, chlorophyll a, chlorophyll b (R^2e = 0.94) for all the treatments.

Figure 3 showed that the highest amount of carotenoid (6.43 ± 0.46 mg/L) was produced under control treatment and lowest carotenoid content was obtained 3.81 ± 0.15 mg/L from T 2.4.75 ± 0.4 mg/L carotenoid was produced under the least nitrogen concentration 6.5g/ 500 ml $NaNO_3$ which is considerably higher amount as nitrogen limitation influences carotenoid production in the cell [Juneja et al., 2013]. Change in nitrogen concentration from standard concentration of Bold Basal media may cause stress on cell metabolism which interferes on carotenoid accumulation [Juneja et al., 2013].

The mean value of phycobiliprotein, phycocyanin, phycoerythrin and alophycocyanin of all the treatments are presented in Table1. Total phycobiliprotein was significantly ($p < 0.05$) highest under control

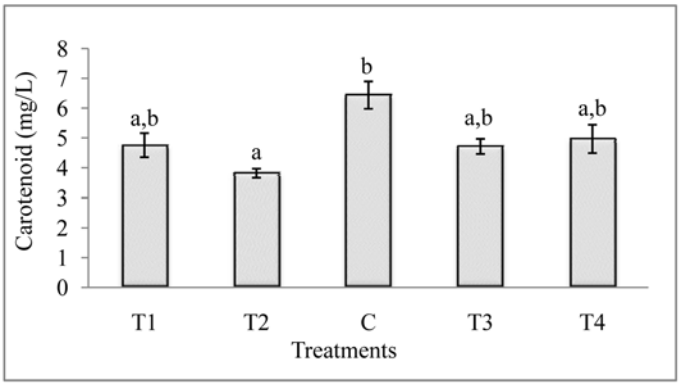

Figure 3. Carotenoid content (mg/L) of freshwater *S. communis* cultured in different nitrogen concentrations. Values are mean ± SE.

Table 1: Phycocyanin, alophycocyanin, phycoerythrin and total phycobiliprotein content (mg/g) of freshwater microalgae *S. communis* cultured in different nitrogen concentrations. Values are mean ± SE.

Treatments	Phycocyanin(mg/g)	Alophycocyanin (mg/g)	Phycoerythrin (mg/g)	Total Phycobiliprotein (mg/g)
T1	1.656 ± 0.016^{d}	3.145 ± 0.049^{c}	1.108 ± 0.079^{b}	5.909 ± 0.014^{c}
T2	1.187 ± 0.096^{b}	1.984 ± 0.009^{a}	0.777 ± 0.023^{a,b}	3.947 ± 0.081^{b}
C	1.544 ± 0.055^{c,d}	2.771 ± 0.003^{b,c}	1.82 ± 0.149^{c}	6.136 ± 0.201^{c}
T3	1.211 ± 0.062^{b,c}	2.503 ± 0.097^{b}	0.84 ± 0.067^{a,b}	4.554 ± 0.226
T4	0.681 ± 0.05^{a}	1.622 ± 0.122^{a}	0.492 ± 0.055^{a}	2.795 ± 0.227^{a}

treatment and the lowest in T4. Almost similar amount of phycobiliprotein was recorded in T1 and C which is contradictory with Zhao et al. [2017] who found that nitrogen deficiency led to the decline of photosynthetic performance by reducing production of photosynthetic pigments such as chlorophyll and phycobiliproteins. Phycocyanin and alophycocyanin were highest in T1, but highest amount of phycoerythrin was obtained in control treatment. Shifting nitrogen concentration from standard concentration interferes in enzymatic activity of pigment production and photosynthesis [Juneja et al., 2013]. As phycobiliprotein is an accessory pigment for light harvesting in photosynthesis, lower chlorophyll production and photosynthetic activity tends to increase in phycobiliprotein in T1 compared to standard nitrogen concentration.

Change in productivity, specific growth rate and cell doubling time of *S. communis* under different treatments has been shown in Table 2. Volumetric and areal productivity among the treatments are not significantly different ($p > 0.05$), but the lipid productivity, SGR and cell doubling time have significant difference among the treatments ($p < 0.05$). Volumetric ($0.054 \pm 0.005 gL^{-1}day^{-1}$) and areal productivity ($5.41 \pm 0.52 mg\ cm^{-2}day^{-1}$) was highest in high nitrogen concentration T4. The findings of the current result was supported by Zhu et al. [2014] who demonstrated that maximum biomass productivity was obtained in higher nitrogen concentration.Lipid productivity was highest $10.56 \pm 2.35\ mgL^{-1}\ day^{-1}$ in very low nitrogen concentration (T1) and lowest $6.712 \pm 0.64\ mgL^{-1}day^{-1}$ in control.

Nitrogen stress has positive response in case of lipid production, as change in standard nitrogen concentration may cause hectic culture environment that result in higher lipid production rate. Compared to adequate nitrogenous medium, two fold lipids were produced in nitrogen deficient culture conditions [Pruvost et al., 2011; Jia et al. 2015]. Maximum specific growth rate was obtained in least nitrogen concentration, T1 and minimum in highest nitrogen concentration, T4. Cell doubling time was reduced in lower nitrogen concentration and increased with the higher nitrogen concentration. Table 2 shows positive relation between specific growth rate and cell doubling time as lower cell doubling time aided in higher specific growth rate. Like the present result, Saha et al. [2013] stated that cell growth rate was greater in cultures subjected to lower nitrogen concentration.

Figure 4 presents the effect of gradient concentration of nitrogen on the protein, lipid and carbohydrate content of *S. communis*. There was highly significant difference in protein, lipid and carbohydrate content of *S. communis* according to nitrogen stress ($p < 0.05$). Gradual increment of proteinproduction was found from lower nitrogenous treatment to higher nitrogenous treatment. Maximum protein content 23.58 ± 0.31 % dry weight was produced under T4 and minimum 18.53 ± 0.16 % dry weight was produced under T1. Compared to the control treatment (13.27 ± 0.24 % dry weight), lipid percentage was significantly higher in nitrogen stress treatments such as T1 (20.92 ± 0.98 % dry weight), T4 (15.87 ± 0.54 % dry weight), T2 (14.98 ± 0.41 % dry weight) and T3 (14.81 ± 0.2 % dry weight).

Protein percentage increased gradually according toincrement of nitrogen concentration as nitrogen is a major component for the biosynthesis of nitrogenous organic compounds like protein, chlorophyll and nucleic acids [Simionato et al., 2013]. Menegol et al. [2017] attained 45% higher protein content (138 mg g^{-1}) in *Heterochlorellaluteo viridis* cultivated with 60 mgL^{-1} $N\text{-}NO_3$ nitrogen concentration than 12 mg L^{-1} $N\text{-}NO_3$. Jia et al. [2015] reported similar findings to the present study that two fold lipid was produced by nitrogen deficient culture condition than nitrogen sufficient medium. As storage products lipid and carbohydrate accumulation in microalgae is caused by nitrogen starvation, which is also accompanied by degradation of cellular nitrogenous compounds, such as nucleic acid, proteins, and photosynthetic pigments, Chl a, Chl b, and carotenoids (Pruvost et al., 2011). Due to lack of sufficient nitrogen concentration in microalgae culture, most of the carbon fixed in photosynthesis is used in the lipid synthesis instead of protein. This change in lipid metabolic pathway influences the accumulation of neutral lipid including triacylglycerides (TAGs) in the cytoplasm of microalgae as a source of carbon and energy [Li et al., 2011].

The highest 27.26 ± 1.31% dry weight carbohydrate was produced under T4 and lowest 17.48 ± 1.44 % dry weight in T1. More carbohydrate was produced under cultivation with higher nitrogen concentration and gradual decrease was found in lower nitrogen concentration except T2. A previous study also found equivalent outcome with this present study that is lower carbohydrate yield (18.75 ± 0.59%) in *Tetradesmus obliquus* was obtained after 14 days at nitrogen stress condition, compared to the control (22.04 ± 0.61%). Nitrogen stress condition induced the accumulation of carbohydrate at early stage but started to reduce on day 4 when the carbon shifted towards lipid production [Nadzir et al., 2021]. Li et al. [2011]stated that, after 2 days of nitrogen stress condition, the photosynthetically assimilated carbon flux of *Pseudochlorococcum* sp. was turned into fatty acid and neutral lipid synthesis in lieu of carbohydrate

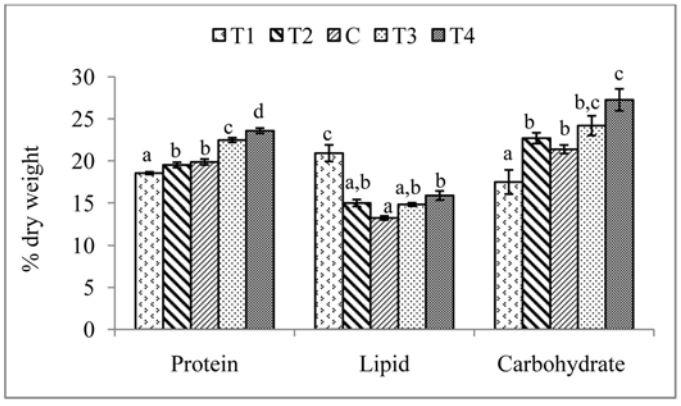

Figure 4. Proximate composition (% dry weight) of freshwater *S. communis* cultured in different nitrogen concentrations. Values are mean ± SE.

Table 2: Change in productivity of *S. communis*cultured in different nitrogen concentrations. Values are mean ± SE.

Parameters	T1	T2	C	T3	T4
Volumetric Productivity (g L^{-1} day^{-1})	0.05 ± 0.01^{a}	0.05 ± 0.01^{a}	0.050.01^{a}	0.05 ± 0.01^{a}	0.05 ± 0.00^{a}
Areal Productivity (mg cm^{-2} day^{-1})	5.01 ± 0.77^{a}	4.62 ± 0.66^{a}	5.07 ± 0.58^{a}	5.38 ± 0.49^{a}	5.41 ± 0.52^{a}
Lipid Productivity(mg L^{-1} day^{-1})	10.56 ± 2.36^{b}	6.89 ± 0.76^{a}	6.71 ± 0.65^{a}	7.96 ± 0.68^{a,b}	8.58 ± 0.88^{a,b}
Specific Growth Rate, μ (day^{-1})	0.52 ± 0.01^{d}	0.47 ± 0.00^{c}	0.45 ± 0.0^{c}	0.41 ± 0.01^{b}	0.38 ± 0.01^{a}
Cell doubling time, td (day)	1.33 ± 0.03^{a}	1.48 ± 0.0^{b}	1.54 ± 0.05^{b}	1.69 ± 0.03^{c}	1.80 ± 0.03^{d}

synthesis.

The result of fatty acid methyl esters (FAMEs) composition analysis with GCMS is given in following Table 3. There 21 compounds were identified as fatty acid methyl esters consisting of 9 saturated fatty acids, 5 monounsaturated fatty acids and 7 poly unsaturated fatty acids.

Figure 5 shows that poly unsaturated fatty acids (PUFAs) percentage and n-6 fatty acids percentage were highest in T1 as 67.37 ± 0.72% and 63.81 ± 0.21% of total fatty acids, followed by T2 which were 61.27 ± 3.29% and 55.03 ± 2.78% of total fatty acids respectively.The lowest PUFAs and n-6 fatty acids percentage were found under T3, but T4 resulted comparatively higher than C.

Highly unsaturated fatty acids (HUFAs) were highest (6.67 ± 0.52% of total fatty acids) in T4 and lowest (0.94 ± 0.18% of total fatty acids) in T2. There was significant difference among the treatments for n-3 fatty acids ($p < 0.05$) which was highest 12.5 ± 1.2% of total fatty acids in control treatment, on the other side, lowest 3.6 ± 0.5% of total fatty acids in T1. Mono unsaturated fatty acids (MUFAs) were maximum 28.53 ± 2.94% of total fatty acids under T1, whereas minimum 9.27 ± 0.54% of total fatty acids under T2. In T4 (26 ± 1.45% of total fatty acids), MUFAs percentage was comparatively higher than C (22.31 ± 1.14% of total fatty acids) and T3 (20.1 ± 1.19% of total fatty acids). The highest value of total saturated fatty acids (SAFA) was found 56.51 ± 1.92% of total fatty acids in T3; contrarily lowest concentration was 8.68 ± 1.19% of total fatty acids in T1. Higher nitrogen concentration compared to standard concentration increased production of saturated fatty acids which are potential for biofuel production.

In support of the present study, Kudahettige et al. [2018] also found that the relative percentage of unsaturated fatty acids were higher in *S. dimorphus* than saturated fatty acids in the culture media of lowest nitrogen content. Zarrinmehr et al. [2020] found that poly unsaturated fatty acids content of *I. galbana* decreased into 41.48% of total fatty acids in sufficient nutrient concentration compared to lower nitrogen concentration. Microalgae growing under nutrient stress conditions could change its metabolic strategies and biochemical composition [Juneja et al., 2013].

Table 3: Fatty acids content (% of the total fatty acids) of *S. communis*cultured in different nitrogen concentrations. Values are mean ± SE

Carbon	Fatty acid	T1	T2	C	T3	T4
Saturated fatty acid						
C8:0	Octanoic acid	0.011± 0.01	0.08±0.00	0.01±0.01	0.018±0.02	0.019±0.01
C10:0	Decanoic acid	0.008±0.00	0.01±0.01	0.002±0.00	0.002±0.00	0.002±0.00
C12:0	Lauric acid	0.18±0.04	1.13±0.2	0.01±0.00	0.01±0.00	0.02±0.00
C13:0	Tridecanoic acid	0.18±0.04	0.87±0.11	0.04±0.01	0.16±0.06	0.03±0.01
C14:0	Myristic acid	0.51±0.08	0.01±0.00	0.012±0.01	0.06±0.00	0.07±0.01
C16:0	Palmitic acid	3.04±0.19	3.54±0.8	8±0.70	7.3±0.27	7.2±0.42
C18:0	Stearic acid	1.91±0.32	7.2±10	4.72±1.02	5.75±0.08	5.4±0.40
C20:0	Arachidic acid	1.33±0.30	5.7±0.73	12.84±1.22	11.96±1.37	4.32±0.25
C17:0	Heptadeca-noic acid	0.172±0.03	0.07±0.02	0.04±0.02	0.1±0.01	0.08±0.00
C21:0	Heneicosa-noic acid	1.131±0.07	11±1.00	21±1.04	31.14±0.08	25.58±0.60
C22:0	Behenic acid	0.22±0.11	——-	0.2±0.10	0.1±0.05	0.16±0.01
Monounsaturated fatty acid						
C16:1	Palmitoleic acid	1.68±0.07	7.68±0.20	18.78±0.63	13.27±0.58	18.32±0.78
C18:1	Oleic acid	2.1±0.80	1.32±0.30	0.16±0.06	2±0.40	0.014±0.00
C20:1	cis-11-Eicosenoic acid	0.28±0.01	0.112±0.02	0.014±0.00	0.01±0.002	0.016±0.01
C22:1	Erucic acid	24.48±2.06	0.134±0.01	3.34±0.44	4.82±0.21	7.63±0.70
C24:1	Nervonic acid	0.002±0.00	0.02±0.01	0.01±0.00	0.013±0.00	0.004±0.00
Polyunsaturated fatty acid						
C18:2n-6	Linoleic acid	62.23±0.09	54.81±2.70	15.91±0.94	13.87±0.004	18.13±0.84
C20:3n-6	Eicosatrienoic acid	1.54±0.104	0.024±0.02	2.41±0.11	1.16±0.64	2.05±0.19
C20:4n-6	Arachidonic acid	0.04±0.02	0.2±0.07	0.08±0.04	0.044±0.001	0.62±0.13
C18:3n-3	Linolenic acid	1.4±0.37	5.52±0.42	8.81±0.55	7.49±0.03	6.3±0.01
C20:5n-3	Eicosapenta-noic acid	1.45±0.00	0.37±0.02	3.6±0.65	3±0.34	3.89±0.17
C22:5n-3	Docosa-pentaenoic acid	0.007±0.00	0.28±0.06	0.09±0.005	0.045±0.02	0.094±0.03
C22:6n-3	Docosa-hexaenoic acid	0.69±0.13	0.06±0.01	——-	——-	——-

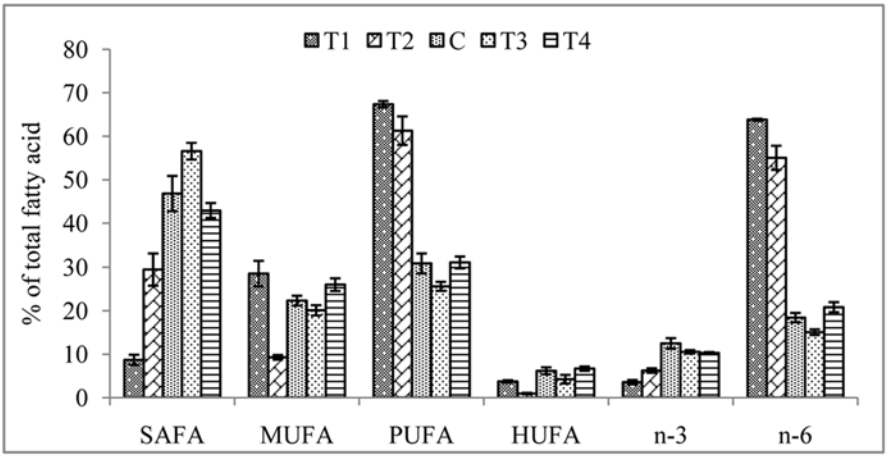

Figure 5. Fatty acids' composition (% total fatty acids) of freshwater *S. communis* cultured in different nitrogen concentrations Values are mean ± SE.*{SAFA- Saturated Fatty Acids, MUFA- Mono Unsaturated Fatty Acids, PUFA- Poly Unsaturated Fatty Acids, HUFA- Highly Unsaturated Fatty Acids, n3- Omega 3 Fatty Acids, n6- Omega 6 Fatty Acids.}

4. Conclusion:

The response of *S. communis* under different nitrogen concentration revealed that higher nitrogen concentration had greater impact on increment of biomass productivity, photosynthetic chlorophyll concentration, protein, carbohydrate and saturated fatty acid percentage. Although lower nitrogen concentration had significant effect on enhancing lipid, monounsaturated and polyunsaturated fatty acid percentage as well as trivial increment of carotenoid and phycobilliprotein content. The results of this study will be helpful to boost the production and improve the nutraceutical as well as pharmaceutical attributes of the freshwater microalgae *S. communis* through mass cultivation with appropriate nitrogen concentration. Based on the findings, it is possible to improve the nutritional profile of the fish diet formulated by *S. communis* and explore the antimicrobial properties to develop drug. Therefore, it can be concluded that this result will enrich the potentiality of aquaculture industry, biofuel production worldwide and pave the way for future research as well.

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