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Influence of Nutrient Manipulation on Growth and Biochemical Constituent in *Anabaena variabilis* and *Nostoc muscorum* to Enhance Biodiesel Production

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Abstract: The present study aims to improve biomass and biochemical constituents, especially lipid production of *Anabaena variabilis* and *Nostoc muscorum* by formulating an optimal growth condition using various concentrations of nutrients (NO_3^-, PO_4^{3-} and CO_3^{2-}) for biodiesel production. The supplementation of the three nutrients by +50% showed the maximum dry weight and biomass productivity, while the macromolecule contents were varied. The depletion of N-NO_3^- by 50% N-NO_3^- showed the maximum lipid yield (146.67 mg L^{-1}) in *A. variabilis* and the maximum carbohydrate contents (285.33 mg L^{-1}) in *N. muscorum* with an increase of 35% and 30% over control of the synthetic medium, respectively. However, variation in P-PO_4^{3-} and C-CO_3^{2-} showed insignificant improving results for all biochemical compositions in both cyanobacteria. *A. variabilis* was the superior species for lipid and protein accumulation; however, *N. muscorum* showed the maximum carbohydrate content. Accordingly, *A. variabilis* was selected for biodiesel production. In *A. variabilis*, −50% N-NO_3^- resulted in 35% higher lipid productivity compared to the control. Furthermore, the fatty acid profile and biodiesel quality-related parameters have improved under this condition. This study has revealed the strategies to improve *A. variabilis* lipid productivity for biodiesel production for small-scale in vitro application in terms of fuel quality under low nitrate levels.

Keywords: biodiesel; biomass productivity; cyanobacteria; lipid productivity; nutrient manipulation

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1. Introduction

Food and energy sustainability are two of the world's most pressing issues for all governments. Food and fuel production must be sustainable, which requires the development of sustainable resources. On this basis, reliance on fossil fuels threatens the long-term viability of global fuel resources due to their depleting nature. It is important to realize the world's insatiable demand for energy and the fact that the world needs renewable energy sources in the future. Biodiesel has received much interest as a green and environmentally friendly fuel. Food crops such as soybean, sugar cane, sugar beet, and rapeseed are currently used to make biofuels [1]. However, using crop plants as a biofuel feedstock puts them in direct competition with food production for land and freshwater, posing important sustainability concerns [1].

Microalgae, including cyanobacteria, have been a great manufacturing resource in the energy industry to prevent competition with other economic sectors [2–4]. Biofuels made from microalgal, or cyanobacterial biomass tend to be a superior green choice to traditional alternatives. Microalgae can be used to produce a variety of renewable biofuels, including

biodiesel derived from microalgal oil and bioethanol, biomethanol and biobutanol formed by the anaerobic digestion of algal biomass [4], as well as biohydrogen and bioelectricity [2].

Cyanobacteria are an ecologically sound option for biofuel production because of their rapid development rate, higher photosynthetic potential, sustenance in the face of environmental conditions and involvement in global CO_2 mitigation [5]. Cyanobacterial lipids could be one of the promising feedstocks for biodiesel production [6,7]. The filamentous nitrogen-fixing cyanobacteria are especially attractive for producing biomasses and chemicals among the various groups of cyanobacteria. They can grow and synthesize all of their cellular components using sunlight, water, CO_2 and a few minerals [8,9].

Cyanobacteria have the ability to biosynthesize various byproducts, including lipids, carotenoids, carbohydrates, pigments, vitamins and aromatic hydrocarbons. Accumulated lipids in cyanobacterial thylakoid membranes are of particular interest and could be used as a lipid feedstock for biodiesel production [9,10]. Algal oils have characteristics comparable to those of vegetable oils and may be used to substitute fossil-fuel-derived products [4]. Cyanobacteria have the ability to accumulate total lipid production range from 7 to 30% of their biomass composition [11], and because of the ease of cell cultivation, lipid production, media adaptation and genetic modification, the use of these microorganisms as a source of lipids has increased [12–14]. However, the amount of produced lipids is insufficient for commercial applications. As a result, it is essential to focus on enhancing lipid yield per gram of dry weight.

Several studies demonstrated that light intensity, light wavelength, pH, temperature and nutrient availability could affect microalgal growth rates, biomass productivity, and lipid yield [15–19]. The nitrogen, phosphorus and carbon concentrations in the culturing system, in particular, are thought to be determinants of biomass and lipid productivity [15,16,20–23]. As a result, taking these parameters into account when manipulating the culture environment may aid in overcoming the barriers of lower biomass productivity, thereby triggering the desired product yield. Few reports available in this context show a positive outcome but only in fragmentary form. Moreover, there is currently a lot of research on single-celled microalgae such as *Spirulina* and *Synechococcus*, but the smaller-sized single-celled microalgae are susceptible to rotifers and other protozoans and harvesting costs are relatively high [24]. As a result, the development of filamentous microalgae with great industrial applicability, such as filamentous cyanobacteria, is expected to solve these issues.

In light of the above facts, this study aimed to examine the effects of supplementing macronutrients (NO_3^-, PO_4^{3-} and C-CO_3^{2-}) on the biomass, protein, carbohydrate and lipid content and fatty acid profile of two filamentous heterocystous cyanobacteria, namely, *Anabaena variabilis* and *Nostoc muscorium*. The cultivation of the cyanobacteria was done in the synthetic medium under autotrophic conditions to screen the best treatment condition in the context of biomass and lipid productivity and biodiesel quality.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

The tested species, *Nostoc muscorum* and *Anabaena variabilis* were obtained from the culture collection of Phycology Research Unit, Botany Department, Faculty of Science, Tanta University, Egypt. Each strain was inoculated and cultivated in 100 mL of Allen's medium [25] consisting of $NaNO_3$ (1.5 g L^{-1}), $K_2HPO_4{\cdot}3H_2O$ (0.039 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.075 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.027 g L^{-1}), Na_2CO_3 (0.02 g L^{-1}), $Na_2SiO_3{\cdot}7H_2O$ (0.058 g L^{-1}), Ferric Citrate (0.006 g L^{-1}), Citric acid (0.006 g L^{-1}), EDTA salt (0.001 g L^{-1}) and 1mL trace element solution (H_3Bo_3 (2.86 g L^{-1}), $MnCl_4{\cdot}4H_2O$ (1.18 g L^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.222 g L^{-1}), $NaMoO_4{\cdot}2H_2O$ (0.391 g L^{-1}), $CuSO_4{\cdot}5H_2O$ (0.019 g L^{-1}) and Co $(NO_3){\cdot}2H_2O$ (0.0494 g L^{-1}). The cultures were incubated under the continuous fluorescent light of 300 µmolm^{-2} s^{-1} at 30 ± 2 °C and continuously supplied with sterilized filtered air with a flow rate of 0.05 vvm to prevent biomass clinging to the flask's bottom.

2.2. Determination of Growth

The cyanobacteria were grown in triplicate batch cultures using a 1-L Erlenmeyer flask containing 500 mL of Allen's medium at an initial optical density (OD_{750}) of ~0.05 and using inoculum in the exponential growth phase for 24 days. Algal growth was measured every couple of days by determining the cellular dry weight (CDW) as described in our previous work [26]. The algal pellet was washed three times using sterilized distilled water to remove traces of growth medium [27], followed by drying the harvested biomass at 60 °C until consistent weight. The specific growth rate was calculated during the exponential growth phase. Specific algal growth rate (μ) was calculated using the following Equation (1):

$$\mu = \frac{\ln CDW_e \ \ln CDW_0}{\Delta T} \quad (1)$$

where CDW_0 and CDW_e are a cellular dry weight at the beginning and the end of the exponential growth phase, respectively, and ΔT is the difference in time.

Biomass productivity (*BP*) was calculated according to Essa et al. [28], as shown in Equation (2):

$$BP = \frac{CDW_e - CDW_0}{\Delta T} \quad (2)$$

where CDW_0 and CDW_e are the weights of dry biomass at the beginning and the end of the exponential growth phase, respectively, and ΔT is the difference in time.

2.3. Effect of Nutrients Manipulation

The sub- or supra-optimal effects of the essential nutrients ($NaNO_3$, $K_2HPO_4{\cdot}3H_2O$ and Na_2CO_3) on the growth and biochemical components of carbohydrates, proteins and lipids of the two tested cyanobacteria (*Nostoc muscorum* and *Anabaena variabilis*) were studied using four concentrations of these nutrients (Table 1). The experiment was performed using 10 cultivation media (3× replicates), 1 for control and 9 for each nutrient concentration in an individual factor experiment.

Table 1. The concentrations of the different nutritional composition of Allen's medium (mg L^{-1}).

Nutrients	Zero%	−50%	Control	+50%
$NaNO_3$	0	750	1500	2250
$K_2HPO_4{\cdot}3H_2O$	0	19.5	39	58.5
Na_2CO_3	0	10	20	30

2.4. Determination of Total Lipid for the Isolated Species

At the late exponential phase, 10 mL culture was centrifuged at 3000 rpm for 15 min. The supernatant was discarded, and the algal pellet was used for the estimation of biochemical components. Protein and carbohydrates were estimated by alkali hydrolyzing of algal pellet using 1 N NaOH at 90 °C for 2 h [29]. The micro phenol-sulfuric acid method was used to determine the total carbohydrates content [30,31] by mixing 50 μL of the hydrolyzed sample with 30 μL of 5% phenol, and then, 150 μL of 98% H_2SO_4 was added and placed in a water bath for 5 min at 90 °C. After cooling, the absorption was measured at 490 nm using a spectrophotometer (Shimadzu UV-2401PC, Kyoto, Japan) against a blank of deionized water. D-glucose was used as a standard to calculate carbohydrate content.

The Lowry method [32] was used to estimate total soluble protein content in the hydrolyzed sample by mixing the hydrolyzed sample with an equivalent volume of thiobarbituric acid 0.67%. The mixture was placed in a boiling water bath for 15 min, and the absorbance was measured at 535 nm and transformed to protein concentrations using bovine serum albumin as a standard reference.

Total lipids were extracted according to the Folch method [33] by mixing a specific amount of algal pellet in chloroform: methanol (2:1, *v/v*) and incubation overnight with shaking at room temperature (27 ± 3 °C). The suspension was centrifuged for 10 min at

5000 rpm, the supernatant was collected and the residual was extracted two more times. The solvent was evaporated, and total lipid content was estimated gravimetrically. Lipid productivity (*LP*) was calculated according to Equation (3), as discussed earlier [28]:

$$LP = \frac{LC_e - LC_0}{\Delta T} \quad (3)$$

where LC_0 and LC_e are the lipids content at the beginning and the end of a batch run, respectively, and ΔT is the difference in time.

2.5. Estimation of Fatty Acid Methyl Esters

The organic phase was collected after lipid extraction to prepare fatty acid methylated ethers (FAMEs), according to Lepage and Roy [34]. FAMEs were analyzed using gas chromatography after the solvent had evaporated. FAMEs were analyzed using a Hewlett-Paackard 5880 gas chromatograph equipped with the mass selective detector and coupled to a HP 3990A integrator. Ultra 2 (cross-linked 5% phenyl methyl silicon 25 m length × 0.2 mm diameter × 0.33 µm film thickness) column was used and helium gas was used as a carrier. The program was heated to 200 °C for 1 min, and the temperature of the detector was 300 °C. The column temperature was held at 80 °C for 2 min, then gradually increased to a maximum of 215 °C. One µL of FAMEs sample was injected in splitless mode. The helium gas velocity was kept constant at 1 mL min^{-1}.

2.6. Evaluation of Biodiesel Properties

The quality of the produced biodiesel was assessed from FAMEs by calculating the main chemical and physical properties, including the average degree of unsaturation (ADU%), iodine value (IV, $gI_2 \cdot 100\ g^{-1}$ oil), cetane number (CN), kinematic viscosity (υi, $mm^2\ s^{-1}$), Specific gravity (ρ), Cloud point (CP, °C), and Higher Heating Value (HHV, MJ kg^{-1}) were calculated using Equations (4)–(10), as discussed by Krzemińska et al. [35] and Hoekman et al. [36]. The values of different parameters obtained were compared with the international standards ASTM D-6751 and EN-14214 to estimate the biodiesel properties produced from the cyanobacterial biomass and calculated as follows:

$$ADU = \sum N \times Mf \quad (4)$$

where N is the number of carbon–carbon double bonds in FA, and Mf is the mass fraction of each FA.

$$\upsilon i = 0.6313 \times ADU + 5.2065 \quad (5)$$

$$\rho = 0.0055 \times ADU + 0.8726 \quad (6)$$

$$CP = -3.356 \times ADU + 19.994 \quad (7)$$

$$CN = -6.6684 \times ADU + 62.876 \quad (8)$$

$$IV = 74.373 \times ADU + 12.71 \quad (9)$$

$$HHV = 1.7601 \times ADU + 38.534 \quad (10)$$

2.7. Statistical Analysis

All experiments were done at least in triplicates and data were expressed as mean ± standard deviation (SD). The individual means were examined for significance using one-way analysis of variance (ANOVA) and post-hoc Duncan Multiple Range Test (DMRT) at the probability level of $p < 0.05$ using the Statistical Package for Social Sciences (SPSS) statistics software version 23 (IBM, Armonk, NY, USA)

3. Results and Discussion

3.1. Growth Curve

Figure 1 shows the growth curve of *A. variabilis* and *N. muscorum* cultivated in Allen's medium for 24 days. The exponential growth phase started after two days of incubation and ended on the 14th day for *A. variabilis* and on the 16th day for *N. muscorum*. The maximum biomasses were 0.69 g L^{-1} and 0.64 g L^{-1} for *A. variabilis* and *N. muscorum*, respectively, followed by a decline in the relative growth and stationary phase. Thus, the biochemical composition and productivities were calculated on these days of incubation. *A. variabilis* showed the highest growth rate of 0.15 mg d^{-1}compared to *N. muscorum* (0.11 mg d^{-1}).

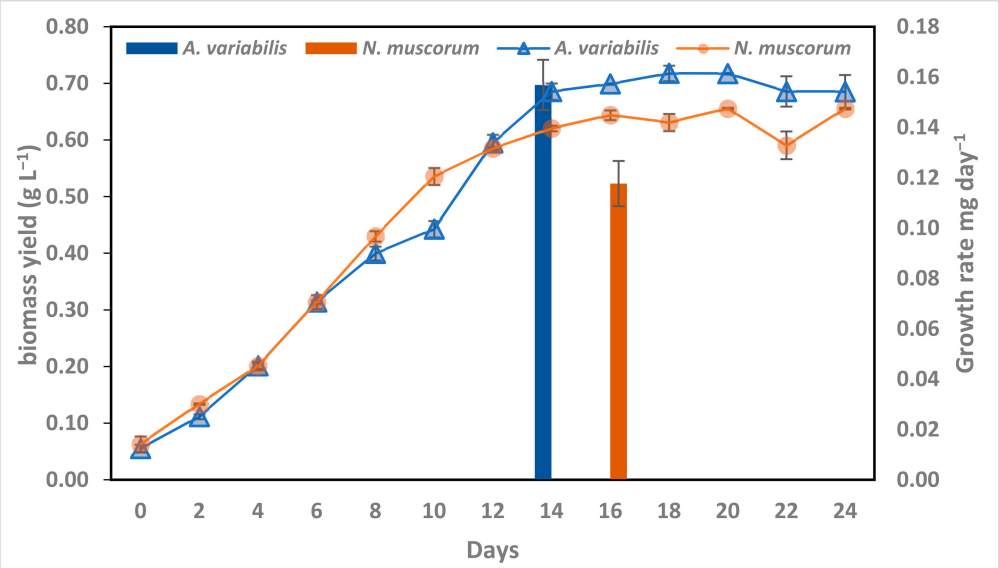

Figure 1. The growth curve of *A. variabilis* and *N. muscorum* showing the dry weight (g L^{-1}) and growth rate mg d^{-1}.

3.2. Biomass and Biomass Productivities under Different Nutrient Concentrations

Biomass yield and productivity are essential parameters for biofuel production since they indicate the rate at which the cyanobacterial strains grow [9]. The most important species for significant biomass production are those with faster growth rates and greater biomass productivity [21,37]. These characteristics include selective advantages such as lower contamination from most competitive organisms, a high biomass yield and a substantial reduction in production expenses during large-scale cultivation [38].

The biomass and biomass productivity of two cyanobacterial isolates were monitored at the 14th and 16th days of cultivation for *A. variabilis* and *N. muscorum*, respectively (Table 2). The highest dry weight and biomass productivity were observed at high nitrate concentrations (+50%), followed by the control. The depletion of the nitrogen source reduced the biomass and its productivity, especially at 0% N-NO_3^- of both species. *A. variabilis* showed the maximum biomass and biomass productivity at +50% N-NO_3^- (0.79 g L^{-1} and 0.042 g L^{-1} d^{-1}, respectively) with an increase of 29% and 15% compared to the control, respectively. While reducing nitrate concentration reduces the biomass and biomass productivity than control. *N. muscorum* showed the same response to different N-NO_3^- concentrations where +50% N-NO_3^- showed the maximum biomass yield and biomass productivity but with slightly low values compared to *A. variabilis* (Table 2).

Table 2. Biomass and lipid production of *A. variabilis* and *N. muscorum* grown in different nutrient concentrations.

Nutrients	Treatments	*A. variabilis*		*N. muscorum*	
		CDW (g L^{-1})	**BP (mg g^{-1} d^{-1})**	**CDW (gL^{-1})**	**BP (mg g^{-1} d^{-1})**
	Control	**0.69 ± 0.026 ^{c}**	**0.031 ± 0.003 ^{c}**	**0.64 ± 0.006 ^{c}**	**0.029 ± 0.001 ^{c}**
Nitrate	0%	0.31 ± 0.016 ^{f}	0.010 ± 0.001 ^{f}	0.30 ± 0.002 ^{g}	0.008 ± 0.002 ^{h}
	(−)50%	0.56 ± 0.007 ^{d}	0.025 ± 0.003 ^{de}	0.56 ± 0.003 ^{de}	0.021 ± 0.003 ^{e}
	(+)50%	0.79 ± 0.026 ^{a}	0.042 ± 0.004 ^{a}	0.74 ± 0.002 ^{a}	0.034 ± 0.002 ^{a}
Phosphate	0%	0.55 ± 0.003 ^{de}	0.025 ± 0.003 ^{de}	0.53 ± 0.003 ^{ef}	0.019 ± 0.002 ^{f}
	(−)50%	0.56 ± 0.011 ^{d}	0.026 ± 0.003 ^{d}	0.55 ± 0.002	0.021 ± 0.003 ^{e}
	(+)50%	0.74 ± 0.023 ^{b}	0.035 ± 0.003 ^{b}	0.68 ± 0.005 ^{b}	0.032 ± 0.001 ^{b}
Carbonate	0%	0.51 ± 0.012 ^{e}	0.022 ± 0.002 ^{e}	0.51 ± 0.001 ^{f}	0.017 ± 0.002 ^{g}
	(−)50%	0.57 ± 0.042 ^{d}	0.027 ± 0.003 ^{d}	0.58 ± 0.002 ^{d}	0.023 ± 0.003 ^{de}
	(+)50%	0.75 ± 0.013 ^{b}	0.039 ± 0.004 ^{a}	0.66 ± 0.004 ^{b}	0.032 ± 0.000 ^{b}
F-value		468.564 **	181.82 **	671.088 *	458.538 *

Different letters in each column indicate significant differences at $p < 0.05$ using Duncan's multiple range test. * Significant at $p < 0.05$ and ** significant at $p < 0.01$.

The maximal biomass yield at higher N-NO_3^- concentrations can be attributed to the key role of nitrogen in photosynthesis [39] and in the synthesis of several structural and functional molecules such as proteins, enzymes and nucleic acid [21]. Thus, nitrogen availability is one of the most important factors in determining cyanobacterial growth. In the current study, the results showed that the difference in N-NO_3^- concentrations affected the growth of both cyanophytes significantly at $p < 0.05$. These findings agreed with Kim et al. [40], who showed that NH_4^+ and NO^{3-} promoted cell growth initially. Several studies related to the growth of cyanobacteria proposed increased biomass production at high nitrogen levels, which is represented here at +50% N-NO_3^- for both species [20]. Sarkar et al. [41] demonstrated that *Anabaena circinalis* and *Cylindrospermopsis raciborskii* biomass increased with increasing nitrogen concentration. This finding may be due to the fact that increased nitrogen concentration enhances CO_2 assimilation through photosynthesis, resulting in the improvement in algae biomass [42]. On the other hand, lessening the nitrogen level was a limiting factor in *Chlorella vulgaris* growth [43]. The same trend was observed in a cyanobacterium, *Synechococcus* spp. where dry weight did not increase significantly at low nitrate concentrations, compared to control and high nitrate concentration [9,20].

Regarding phosphate manipulation, the maximum dry weight and biomass productivity of both species were detected at +50% P-PO_4^{3-}, while the depletion of phosphorus lessens biomass to the lowest value among treatments. *A. variabilis* was the leading species with a biomass of 0.74 g L^{-1} and a biomass productivity of 0.04 g L^{-1} d^{-1} at +50% P-PO_4^{3-} (Table 2). Meanwhile 0% P-PO_4^{3-} showed the lowest biomass and biomass productivity. These results were in accordance with Ernst et al. [20], who stated that *Synechococcus* spp. under a low phosphate concentration (0.3 mg L^{-1} K_2HPO_4) showed the lowest biomass relative to high concentrations. Sarkar et al. [41] demonstrated that phosphorus supplementation boosts biomass production in *A. circinalis*. On the contrary, the depletion of P-PO_4^{3-} by −50% showed the highest biomass and productivity in green alga *Micractinium reisseri* [21]. These differences could be because some microalgae can accumulate phosphorus in the form of polyphosphate granules in their cells to utilize them under phosphorus depletion conditions [44]. This decrease in biomass yield could be attributed to physiological and morphological modifications in the cell caused by the nutrient-depleted condition, which result in the termination of cell division followed by cell lysis and death [45].

Carbon impact is not less important than the effect of nitrogen and phosphorus on the growth and metabolism of algal cells. Since atmospheric CO_2 has a lower water solubility than inorganic carbon sources such as $NaHCO_3$ and $NaCO_3$, supplying the cultivation medium with an inorganic carbon source can be an effective way to achieve increased

biomass and the desired output [46]. However, the degree to which carbonate is used varies between organisms due to differences in their growth and biochemical compounds [47,48]. In the current study, +50% carbonate induced the dry weight and biomass productivity of both species, while 0% carbonate reduces biomass to the minimum value among treatments. *A. variabilis* showed the highest dry weight and biomass productivity of 0.75 g L^{-1} and 0.04 g L^{-1} d^{-1} at +50% C-CO_3^{2-}, respectively (Table 2).

3.3. Biochemical Composition

The effects of the three nutrients on the biochemical composition, including lipids, carbohydrates and proteins, were studied individually. *N. muscorum* and *A. variabilis* have different biochemical profiles (Figure 2). The highest carbohydrate yield was observed in *N. muscorum* with 285.33 mg L^{-1} over *A. variabilis* with 227.67 mg L^{-1} at −50% N-NO_3^-. Moreover, −50% N-NO_3^-enhanced the carbohydrate yield by 30% and 38% for *N. muscorum* and *A. variabilis*, respectively, and the complete depletion of phosphate to 0% improved the carbohydrate content over the control. These results are consistent with Deb et al. [49], who found that nitrate and phosphate depletion prompted the total cellular carbohydrate yield of both *A. variabilis* and *M. aeruginosa*. These findings may be attributed to the importance of nitrogen and phosphorus as two main nutrients, without which the cell's metabolic route is diverted, resulting in the accumulation of energy storage molecules [49,50]. While +50% C-CO_3^{2-} was the optimum condition for the maximum carbohydrate contents of 251.67 and 206 mg L^{-1} for *N. muscorum* and *A. variabilis*, respectively (Figure 3A), our finding was consistent with a previous study that observed improving the total carbohydrate yield and content of *A. variabilis* to 438.2 mg L^{-1} and 59.6% CDW, respectively, using 3X $NaHCO_3$ supplementation [5]. The reason behind such an increase in cellular carbohydrate content under high carbonate levels might be because such conditions induced the synthesis of carbonic anhydrase and bicarbonate transporters that increase the fixed level of CO_2, which helps to enhance their photosynthetic ability [51,52]. This finding possibly explains the enhanced biomass and total carbohydrate synthesis of the test cyanobacteria under a sufficient carbonate concentration.

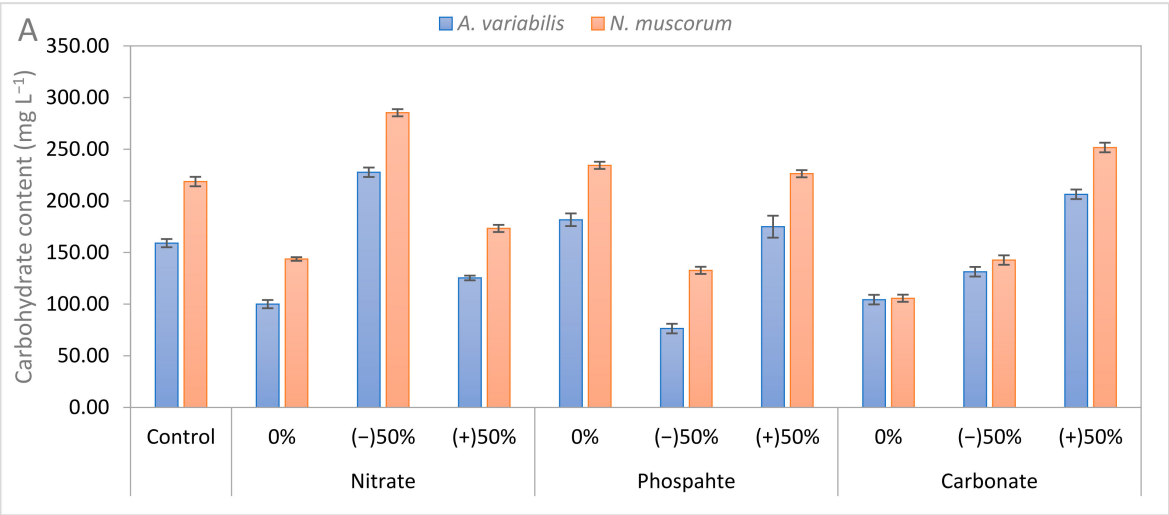

Figure 2. *Cont.*

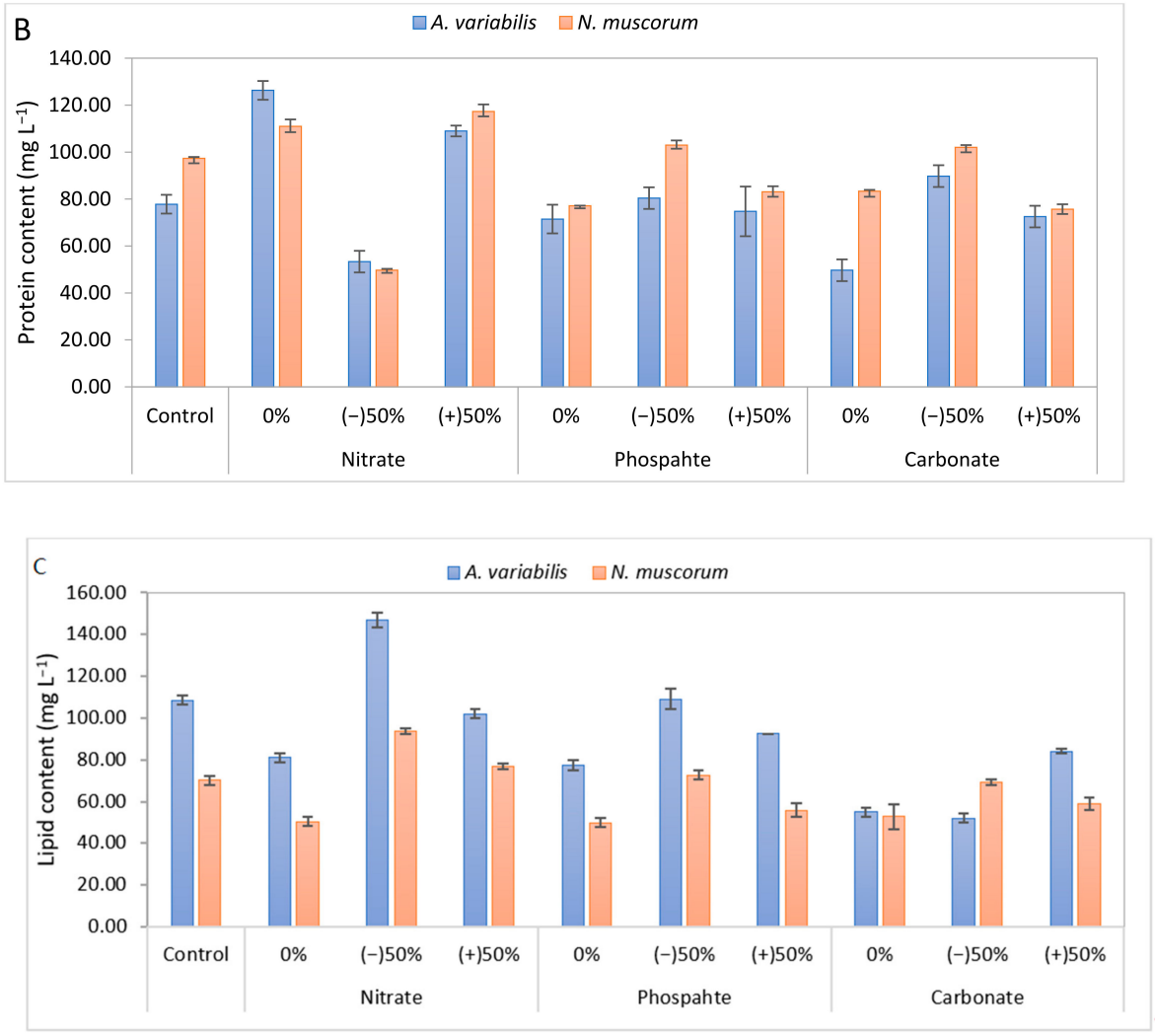

Figure 2. Carbohydrate (**A**), protein (**B**) and lipid contents (**C**) of *A. variabilis* and *N. muscorum* under different nutrient concentrations.

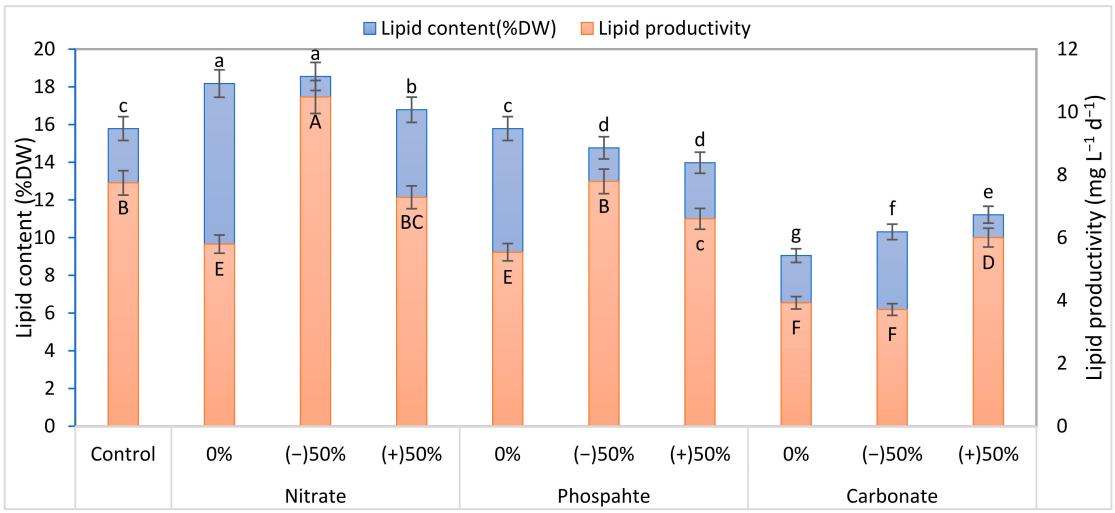

Figure 3. Comparison of the individual factor studies showing the lipid content (%DW) and lipid productivity (mg L^{-1} d^{-1}) in *A. variabilis* under different nutrient concentrations. Different letters in each plotted series indicate significant differences at $p < 0.05$ using Duncan's multiple range test.

A. variabilis was superior in protein content over *N. muscorum*; −50% nitrate reduced the protein yield to the lowest value in both cyanobacteria, while zero N-NO_3^- was the most inducible treatment for *N. muscorum* of 117 mg L^{-1}. On the other hand, −50% P-PO_4^{3-} and −50% C-CO_3^{2-} were the optimum conditions for protein content yield for both cyanobacteria, with no significant differences at $p < 0.05$ (Figure 2).

Nitrate starvation to −50% showed the most lipid induction (146.67 mg L^{-1}) for *A. variabilis* and 93.67 mg L^{-1} for *N. muscorum*, an increase of 35% and 32%, respectively, over the control treatment. Meanwhile, the maximum lipid yield under phosphate treatment was observed at −50% P-PO_4^{3-} (109.07 mg L^{-1}) for *A. variabilis*, with no significant difference ($p < 0.05$) compared to the control treatment (108.4 mg L^{-1}). The same induction was observed in *N. muscorum* but showed a lower yield (Figure 2). On the other hand, all C-CO_3^{2-}treatments showed low lipid yields compared to the control treatment for both species. This finding demonstrates the effectiveness of nitrate level in lipid induction compared to phosphate or carbonate. In this context, nitrogen, as a vital element in the algal cell and a component of high-value biological macromolecules, plays a key role in microalgal metabolism. Variations in nitrogen concentration can significantly affect the growth rate and the synthesis of carbohydrates, lipids and proteins in microalgae [53,54].

In general, *A. variabilis* was the leading species for lipid production over *N. muscorum*. Numerous microalgal species preferentially synthesize lipids under nitrogen-deprived conditions [19,21,55]. The current study was well agreed with the previous reports where both lipid yield and lipid productivity recorded the maximum values at the −50% N-NO_3^- concentration for both cyanobacteria. These results were supported by a previous study on *Chlorella pyrenoidosa* and *A. circinalis* that recorded >20% and >30% lipid yields at nitrogen depletion [41]. The maximum lipid productivity derived from *Synechococcus* sp. HS01, which was 2.8^{2-}times higher than the control, was achieved in a medium containing only 0.1 g L^{-1} $NaNO_3$ compared to a control of 1.5 g L^{-1} [9]. This increase under nitrogen deficiency can be explained by the fact that algal cells often accumulate an excess of carbon metabolites as lipids under nitrogen-starving conditions [56,57]. It was also found that microalgae adapt to the nitrogen deprivation state by breaking nitrogenous biomolecules to stockpile carbon reserve components, such as polysaccharides and lipids [58,59]. In respect to the previous data, *A. variabilis* is the most recommended species for biodiesel production according to its high lipid content. On the other hand, according to low lipid content and high carbohydrate content in *N. muscorum*, it may be recommended for bioethanol production instead of biodiesel production.

3.4. Comparative Summary of the Individual Factors Analyses versus Control

Selection of the promising microalgae for biodiesel production depends mainly on lipid content (%DW) and lipid productivity, which are influenced by biomass yield [21,22,60,61].

Total lipid content was also impacted maximally with nitrate variation, where −50% N-NO_3^- showed the highest content of 18.55% for *A. variabilis* followed by 0% nitrate. The variation in phosphate and carbonate showed lower content compared to control (Figure 3). Nitrate depletion by −50% induced lipid productivity up to ~1.3 fold higher (up to 35%) than the control for *A. variables*. These results concluded that the optimum conditions for the highest lipid content and lipid productivity were obtained under the starving condition of N-NO_3^- by 50% and keep P-PO_4^{3-} and C-CO_3^{2-} at the control level (Figure 3).

Despite the low lipid content (18.55%) presented in *A. variabilis* in the current study compared to green microalgae that may be reached to 44.5% in *Micractinium reisseri* [21] or 37.71% in *Nannochloropsis oceanica* [37], *A. variables* showed a comparable lipid yield, content and productivity (146.67 mg L^{-1}, 18.55% and 10.48 mg L^{-1}·d^{-1}, respectively) with the recommended cyanobacteria for biodiesel production, i.e., *Anabaena variabilis* MBDU 013, *Nostoc calcicola* MBDU 602, *N. entophytum* MBDU 679, *Nostoc* sp. MBDU 013 and *Desmonostoc muscorum* MBDU 105 showed lipid content ranged from 9.8–18.921% and lipid productivity ranged from 1.649–3.2 mg L^{-1} d^{-1} [62]. As a result, *A. variabilis* showed

comparative biomass and lipid productivities, making it an economically viable biodiesel feedstock, more so than *N. muscorum*, and will be selected for the rest of the study.

3.5. Fatty Acid Profiles Properties

Although higher lipid content and lipid productivity are key factors in determining the best conditions for biofuel production, lipid quality or the fatty acids (FAs) composition determines whether a lipid is suitable for biodiesel production. Several studies have studied this phenomenon [21,37,60,61]. As a result, the current research included a thorough analysis of fatty acid composition. Figure 4 shows the FAs content at different nutrient conditions. Saturated fatty acid (SFA) showed the highest proportion, which varied from 36.02% to 59.91%, followed by monounsaturated fatty acids (MUFAs) (5.58% to 46.57%) and polyunsaturated fatty acids (PUFAs) (13.44% to 34.57%). Under the −50% N-NO_3^- condition, the biomass produced a highest percentage of SFA (59.81%), corresponding to more than 23% of those produced by the biomass grown in the control.

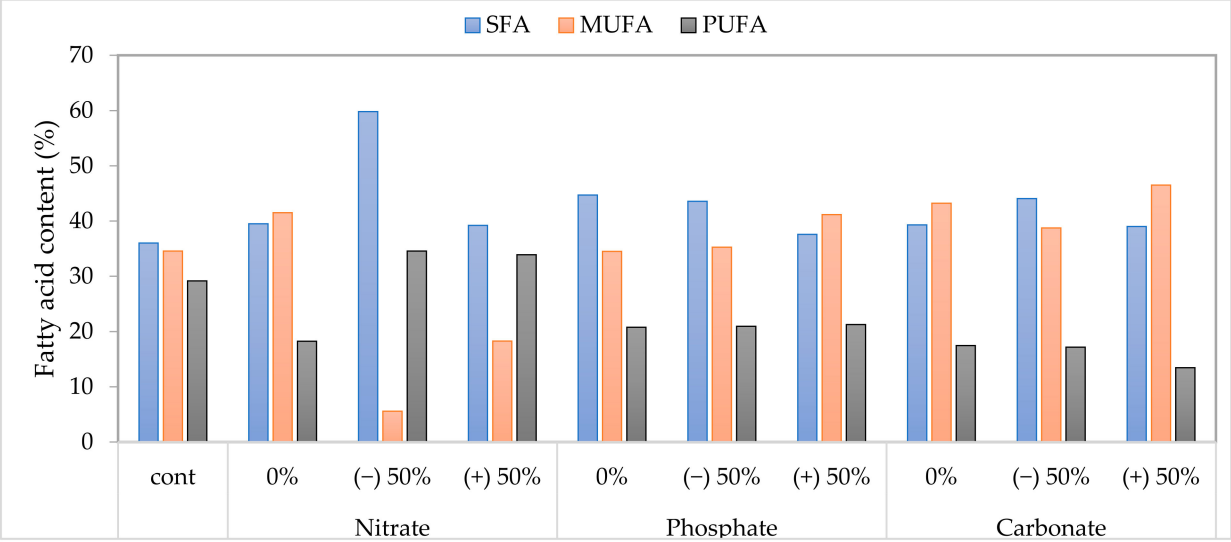

Figure 4. Fatty acid contents (%) under different treatment conditions. (SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; Cont, control).

PUFAs content was the maximum at −50% N-NO_3^- condition, while MUFAs content was the highest content at +50% C-CO_3^{2-} compared to other treatments, and the lowest content was observed at +50% N-NO_3^- condition. The percentage of SFA, MUFA and PUFA in the FAs' compositions showed a significant variation under different conditions at $p < 0.05$ (Figure 4). This may be due to variations in the lipid production and the fatty acid composition in response to the various stressors [21,63,64]. The high content of SFA in lipids displays the high ignition properties of the fuel. Nevertheless, the fuel viscosity increases at low temperatures, resulting in decreased flow properties [65]. As a result, the presence of UFA in a mixture of SFAs improves fuel efficiency at low temperatures. Nevertheless, high UFA is considered unfavorable for biodiesel production because of higher polymerizing tendency of unsaturated components [66]. Of note, high SFA and PUFA and low MUFA in the N-NO_3^{-1} (−50%) condition would be a favorable feature for biodiesel quality [61].

Compared to traditional biodiesel feedstocks such as soybean and canola oils, algal lipids had a higher proportion of fully saturated FAs. Regarding biodiesel's traditional soybean and canola oil feedstock, algal lipids had higher SFA levels [67]. Higher quantities of PUFA and SFA both play a key role in terms of fuel properties.

Table 2 shows the fatty acid profiles under different nutrient conditions. Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) fatty acid species have

previously been found in vegetable oils [68] and other microalgae species [4,69]. The fatty acid profiles of *A. variabilis* revealed the presence of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), Linoleic acid (C18:2) and γ-linolenic acid (C18:3). Palmitic, linoleic and oleic acids were among the abundant fatty acids detected in *A. variabilis*, comprising 15.77–30.80%, 18.10–33.50% and 5.58–45.04% of the total fatty acids, respectively (Table 3).

Table 3. Fatty acid profile of *A. variabilis* (%) grown under the different nutrient concentrations.

Fatty Acids	Types	Ctrl	Nitrate			Phosphate			Carbonate		
			0%	(−)50%	(+)50%	0%	(−)50%	(+)50%	0%	(−)50%	(+)50%
Lauric acid	C12:0	-	-	-	-	-	6.2	-	-	-	-
Tridecanoic acid	C13:0	-	-	-	4.08	5.64	-	-	-	-	-
Myristic acid	C14:0	0.92	8.55	14.48	-	-	3.5	-	-	-	-
Pentadecanoic acid	C15:0		4.42	5.8	-	-	-	7.5	-	4.23	-
Palmitic acid	C16:0	30.4	15.77	24.64	28.53	30.8	22.5	20.14	26.13	12.66	21.73
Stearic acid	C18:0	4.3	3.93	4.55	4.11	5.53	2.88	8.15	12.32	14.57	11.6
Archidic acid	C20:0	0.4	6.85	10.34	2.5	2.73	2.5	7.8	0.85	12.6	5.7
Palmitoleic acid	C16:1	1.3	2	-	1.78	6.4	4.75	8.86	1.78	9.01	1.45
Heptadecanoic acid	C17:1	5.21	8.06	-	-	-	2.08	-	-	-	-
Oleic acid	C18:1	27.8	28.22	5.58	13.37	27.7	32.3	22.55	40.84	14.5	45.047
Erucic acid	C22:1	0.25	3.23	-	3.13	0.4	2.03	3.85	0.61	15.25	-
Linoleic acid	C18:2	27.7	13.1	33.5	28.54	16.3	18.1	15.5	16.02	17.18	12.53
Lenolenic acid	C18:3	1.48	5.15	1.07	5.36	4.46	3.16	5.44	1.44	-	0.91

SFA consisting of myristic acid (C18:0) and palmitic acid (C16:0) presented at a major percentage in the studied strain varied under different conditions. Our findings support a previous study that found that tested cyanobacterial strains had higher levels of palmitic acid, stearic acid and myristic acid than other algal species [62,70–72]. The current study observed that FA profile was altered under different nutrient levels, resulting in variation in the fatty acid content. These findings concurred with Srinuanpan et al. [22], who observed that nutrient manipulation resulted in a characteristic shift in the fatty acid profile that was considered favorable for biodiesel quality. According to the previous results, *A. variabilis* under −50% N-NO_3^- was the optimum condition for lipid productivity and FA profile of the highest SFA content, so it was selected to characterize biodiesel properties.

3.6. Biodiesel Properties

There are several criteria to determine the suitability of FA for biodiesel, which depend mainly on the chain length and degree of saturation. The values of KV, CN, IV and ρ estimated in this study (Table 4) were found to be in accordance with those recommended by the international standards (ASTM D6751 and EN 14214). The ADU of *A. variabilis* FAMEs was 0.76, which shows a relatively high saturation degree. The CN is a metric of biodiesel ignition efficiency that rises as the saturation level of fatty acids rises [73]. CN should be ≥47.0 and ≥51.0, respectively, according to the international standards of ASTM D6751 and EN14214. The biodiesel made from *A. variabilis* had a relatively high CN of 57.82 compared to the control of 56.58, which allows the engine to start quickly and quietly, indicating good ignition efficiency and low NO_x emissions [74,75], and showed better oxidative stability.

The biodiesel characteristics are comparable with those of *A. variabilis* reported by Anahas and Muralitharan [62]. The maximum iodine value (IV) allowed by EN 14214 standards was 120 g I_2/100 g oil, which excluded several lipid-rich biomasses such as soybean and sunflower from being used as biodiesel feedstocks [76]. However, in this study, *A. variabilis* showed a low iodine value compared to the control. It should be noted that the lowest value of IV indicates the most saturated level and have high stability against oxidation [22]. Our results were consistent with different biodiesels derived from different

green microalgae [21,37] or with cyanobacteria [61,62], as shown in Table 4. Specific gravity (ρ) and kinematic viscosity (KV) were also matched with ASTM D6751 of 0.85–0.90 kg^{-1} and 1.9–6.0 mm^{-2} s^{-1}, respectively. The cloud point (CP) (9.87 °C) in this study improves biodiesel suitability at low temperatures.

The amount of heat generated by the complete combustion of a unit quantity of fuel is referred to as HHV (higher heating value). The HHV value (39.87 MJ kg^{-1}) observed in this study was acceptable according to the previous studies [77–79], and it agrees with previous findings for *Anabaena variabilis* MBDU 013 and *Anabaena anomala* MBDU 629 (41.44 and 40.26 MJ kg^{-1}, respectively) [62]. Overall, *A. variabilis* under −50% N-NO_3^- produced qualified biodiesel that met international requirements and could compete with fossil diesel.

Table 4. The estimated properties of biodiesel derived from *A. variabilis* in comparison with the international standards and those reported by other studies.

Biodiesel Parameters	ADU	KV (mm^2 s^{-1})	CN	IV (g $I_2$100 g^{-1} Oil)	Cp (°C)	ρ (g m^{-3})	HHV (MJ kg^{-1})	Reference
Control	0.94	4.61	56.58	82.92	7.39	0.88	40.20	This study
+50% NO_3	0.76	4.73	57.82	69.08	9.87	0.88	39.87	This study
ASTM D6751	-	1.9–6.0	≥47	-	-	0.85–0.90	-	[80]
EN 14214	-	3.5–5	≥51	≤120	>4	0.86–0.90	-	[81]
A. variabilis MBDU 013		1.56	69.09	30.93	9.15	0.87		[62]
Arthrospira platensis NIOF17/003		19.3	52.9	85.5	-		40.71	[61]
Micractinium reisseri	0.33	5	60.65	37.55	15.53	0.87	39.19	[21]

4. Conclusions

The present investigation emphasizes the effect of nutrient manipulation of NO_3^-, PO_4^{3-} and CO_3^{2-} on growth and biochemical constituents using two cyanobacterial strains. The overall the large biomass of both cyanobacteria was due to the high nitrogen level. However, the depletion of nitrate by 50% induced lipid and carbohydrate contents and reduced protein content. *A. variabilis* showed the highest lipid content, while *N. muscorum* showed the highest carbohydrate content. As a result, *A. variabilis* was chosen for biodiesel production. Compared to control, adding −50 N-NO_3^- to *A. variabilis* resulted in 1.28- and 1.3-fold higher biomass and lipid productivity (up to 29 and 35%, respectively), which directly impacts the cost of cultivation by decreasing nutrient consumption. Additionally, the FAMEs profile and biodiesel properties of *A. variabilis* were improved, demonstrating the potential of cyanobacteria biomass as a biodiesel feedstock. However, further scale-up and confirmatory tests for fuel qualities are required. From these findings, it can provide a useful small-scale application of *A. variabilis* biomass for biodiesel production in terms of fuel quality parameters under low nitrate level.

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