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Effect of Different Sodium Nitrate Concentrations on the Growth, Biomass, and Biochemical Composition of *Tetraselmis chuii*

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Abstract

Nitrogen plays a significant role in the growth and metabolism of microalgae. The purpose of this study was to investigate the influences of different sodium nitrate concentrations on growth, biomass, and biochemical profile in *Tetraselmis chuii*. Four different nitrate concentrations, 0.5, 1.0, 1.5, and 2.0 g/L $NaNO_3$ were applied in *T. chuii* culture under a batch system. It was found that a low nitrate concentration of 0.5 g/L $NaNO_3$ produced the highest specific growth rate and biomass concentration of *T. chuii*. On the other hand, increasing nitrate concentration led to an increase in chlorophyll *a+b* and carotenoid in *T. chuii,* with the optimum nitrate concentration found at 1.5 g/L $NaNO_3$. Under the nitrogen limitation condition, protein content was significantly decreased, but lipid and carbohydrate content were highly accumulated in the cells. This study provides a unique phenomenon that low nitrogen concentrations not only produce higher biomass but also accumulate high lipid and carbohydrate content.

INTRODUCTION

Microalgae are unicellular or multicellular microorganisms that use carbon dioxide to synthesize biomass during photosynthesis and play an essential role as primary producers in aquatic environments (Fakhri *et al*., 2015; Pruvost *et al*., 2009). Microalgae have valuable biochemical content and are widely used as raw materials in various fields such as animal feed (Ansari *et al*., 2021), nutraceuticals (Zanella and Vianello, 2020), and functional food (Andrade *et al*., 2018). Microalgae also have an important role in fish and shrimp cultivation because of their protein, lipid, and carbohydrate content and cell size (Hemaiswarya *et al*., 2011). Additionally, they have been utilized in the generation of bioenergy (by the manufacture of biomethane, biodiesel, and biohydrogen) (Beer *et al*., 2009), cosmetics (because of their pigments and antioxidant characteristics), pharmaceuticals (as a source of bioactive compounds) (Morowvat and Ghasemi, 2016), and the environment (for wastewater treatment and CO_2 fixation) (Mohsenpour *et al*., 2021).

Numerous variables, including light intensity, temperature, pH, photoperiod, salinity, and nutrients, have a significant impact on the growth and biochemical

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profile of microalgae (Bartley *et al*., 2016; Kim *et al*., 2014a). Among these many components, nutrients, particularly nitrogen, are the most critical for growth and are found in all structural and functional proteins in algal cells, including peptides, enzymes, chlorophyll, energy transfer molecules, and genetic material (Cai *et al*., 2013; Hu *et al*., 2013). Importantly, the concentration of nitrogen in the culture medium has a significant impact on microalgae cell growth and biochemical composition (Wang *et al*., 2013; Yuniarti *et al*., 2023). Several investigations have demonstrated that when the culture medium's nitrogen level is restricted, microalgae slow down the cell growth rate, decrease protein synthesis, and increase the lipid (Ho *et al*., 2014) and carbohydrate content (Pancha *et al*., 2014). Nitrogen limitation has been shown in several studies to increase lipid accumulation but decrease biomass productivity (El-Kassas, 2013). The major challenge is not only to produce high-energy storage chemicals but also to obtain high biomass production (Li *et al*., 2008). Low concentrations of nitrogen alter the biochemical composition, including protein and carbohydrate content, pigments, and lipid content, as numerous studies have demonstrated (Pancha *et al*., 2014; Yaakob *et al*., 2021).

Tetraselmis sp. is a green microalga that serves as a vital live feed for aquaculture animals (Junior *et al*., 2007) due to its fast growth rate and ability to produce a variety of essential chemicals, including lipids, proteins (40–70% dry weight), and pigments (Singh and Gu, 2010). *T. chuii* was taken into consideration for this study because of its high nutritional value, which allows for its great potential as an aquaculture feed (Kim *et al*., 2016).

For the first time, a higher concentration of nitrogen was used to determine the growth and biochemical composition of *T. chuii*, which will aid in optimizing the nitrogen concentration for higher growth of *T. chuii*. Most previous studies had used low concentrations of nitrogen. The main interest was to enhance the lipid and carbohydrate productivity of *T. chuii*. In this present study, we applied sodium nitrate ($NaNO_3$) as a source of nitrogen. In general, synthetic media such as BG-11, Walne, and Zarrouk use nitrate as a source of nitrogen, especially sodium nitrate ($NaNO_3$) (Grobbelaar, 2004). We investigated the effect of various nitrate concentrations on growth, biomass, and biochemical composition, especially the lipid and carbohydrate of *T. chuii.*

METHODOLOGY

Ethical Approval

Not applicable because none of the writers of this paper have ever worked with humans or animals in this study.

Place and Time

This study was carried out at Indoalgae Aquaculture, Sukorejo District, Pasuruan Regency, East Java, Indonesia. This research was conducted from 2 September to 15 December 2023.

Research Materials

For cell counting, a Neubauer hemocytometer (BOECO, Hamburg, Germany) was applied and the cells were observed using an inverted microscope (Olympus IX53). A spectrophotometer (GENESYSTM 10S UV-Vis, Thermo Scientific, USA) was used to determine the pigment, carbohydrate, and protein content of *T. chuii*. Biomass and cell harvesting were conducted by centrifugation (Kokusan, model H-11n, Tokyo, Japan).

In this study, BG-11 (all compounds from Merck, Darmstadt, Germany) was used as a growth medium. Methanol (Merck, Darmstadt, Germany) was utilized for pigment analysis. Bovine serum albumin (Sigma-Aldrich, USA), Na_2CO_3, $CuSO_4.5H_2O$ (Supelco, Inc), $NaKC_4H_6O_6.4H_2O$ (Supelco, Inc), and Folin-Ciocalteau reagent were applied for protein analysis. For lipid analysis, we used methanol (Merck, Darmstadt, Germany) and chloroform (Merck, Darmstadt, Germany), while phenol (Supelco, Inc) and H_2SO_4 (Supelco, Inc)

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were utilized for carbohydrate determination.

Research Design

The experimental approach employed in this study included four treatments (0.5, 1.0, 1.5, and 2.0 g/L $NaNO_3$) with three replications. Cell counting was analyzed daily, while biomass, pigment, carbohydrate, protein, and lipid content were determined at the end of the cultivation period.

Work Procedure

Strain and experimental cultivation conditions

Tetraselmis chuii was obtained from the Center for Brackish Water Aquaculture, Jepara, Central Java, Indonesia. A logarithmic phase of *T. chuii* was applied as an inoculum. *T. chuii* was cultured in 500 mL sterilized seawater enriched with BG-11 medium. The composition of BG-11 is shown in Table 1. All compounds in the BG-11 medium were added in the same amount in all treatments except for the sodium nitrate. Four different sodium nitrate concentrations (0.5, 1.0, 1.5, and 2.0 g/L $NaNO_3$) were applied to the medium. The strain was cultured at a temperature of 28±1°C with a light intensity of 150 μmol $m^{-2}s^{-1}$ and a photoperiod of 24:0 light/dark cycle under a batch system (Fakhri *et al*., 2015). *T. chuii* was grown at a salinity of 30 ppt and given continuous aeration with sterilized air. An initial cell concentration of 5 x 10^5 cells/mL was applied for all treatments. All treatments were conducted in triplicate.

Table 1. Composition of BG-11 medium used in this study.

Compound	Amount (g/L)
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
$Na_2EDTA.2H_2O$	0.001
Na_2CO_3	0.02
Ferric ammonium citrate	0.012
Trace metal solution	1 mL/L

[a]H_3BO_3, 2.86 g/L; $MnCl_2.4H_2O$, 1.81 g/L; $ZnSO_4.7H_2O$, 0.222 g/L; $Na_2MoO_4.2H_2O$, 0.39 g/L; $CuSO_2.5H_2O$, 0.079 g/L; $Co(NO_3)_2.6H_2O$, 0.049 g/L.

Gonad Maturation Rate (Ovarian Growth analysis

A Neubauer hemocytometer (BOECO, Hamburg, Germany) was applied to count the cell concentration of *T. chuii*. A specific growth rate (μ) was calculated as stated by (Feng *et al*., 2012):

$$\mu\ (/\text{day}) = \frac{\text{Ln(x2)}-\text{Ln(x1)}}{\text{t2}-\text{t1}}$$

where, x1 and x2 are cell concentration of *T. chuii* at the initial (t1) and final times (t2).

The doubling time (td) of the cell represents the mean of the generation time of biomass. The doubling time (day) of the growth rate was calculated according to the equation:

$$\text{dt (day)} = \frac{Ln2}{\mu}$$

Biomass determination

Microalga biomass was determined by using the gravimetric method (Fakhri *et al*., 2021a). A Twenty-five mL algal sample was centrifuged at 4,000 rpm for 15 min. Then, 25 to 50 mL distilled water was added to the pellet to dissolve the remaining salt in the sample before it was centrifuged at 4,000 rpm for 15 min. After two hours of drying at 105°C, the sample was weighed and allowed to cool in desiccators. Biomass was determined as dry weight, g/L.

Chlorophyll *a*+*b* and carotenoid measurement

Chlorophyll (chl a and b) and carotenoid of *T. chuii* were extracted by 90% methanol according to Ritchie (2006) with

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some modifications. Five ml algal cultures were centrifuged at 4,000 rpm for 10 min. Then, 5 ml methanol was added to the extract and vortexed for 10 sec. Furthermore, the mixture was sonicated for 10 mins. After 10 min of incubation at 70 °C in the water bath, the mixture was centrifuged for 10 mins at 4,000 rpm. A supernatant was measured by spectrophotometer (GENESYSTM 10S UV-Vis, Thermo Scientific, USA) to quantify chlorophyll and carotenoid content. While carotenoid quantification was carried out at a wavelength of 480 nm (Kim *et al*., 2014b), the absorbance of chl a and b was measured at 665 and 652 nm and evaluated according to Ritchie's (2006) method.

Chl *a* (μg/mL) = 16.5169 x A665 – 8.0962 x A652

Chl *b* (μg/mL) = 27.4405 x A652 – 12.1688 x A665

Car (μg/mL) = 4 x A480

Total lipid measurement

Total lipids were extracted following the protocol of Bligh and Dyer (1959) and Feng *et al*. (2012) with slight modifications. The cells (5 ml) were collected by centrifuging at 4,000 rpm for 10 min. Then, the supernatant was removed, and 1 mL chloroform, 2 mL methanol, and 1 mL distilled water were added to the pellet and vortexed for 10 sec. The extracts (supernatant) were collected into a pre-weighed glass tube (X1 g) after centrifuging at 4,000 rpm for 10 min. For phase separation, 1 mL of distilled water and 1 ml of chloroform were then added to the extracts. After removing the top layer, N2 was used to dry the bottom layer. The residue and glass tube were then dried until the weight was constant (X2 g) at 105°C. Lipid content was estimated by subtracting X1 from X2 and represented as a percentage of dry weight.

Determination of total carbohydrate

Total carbohydrate was determined according to the method from Dubois *et al*. (1956). One mL microalgal sample was collected by centrifugation at 4,000 rpm for 10 min. Then, 0.5 mL of 5% phenol (v/v) was added to the pellet. Immediately, 2.5 mL of concentrated H_2SO_4 was added to the solution, then the mixture was incubated for 30 min. Finally, the sample was read at an absorbance of 490 nm.

Determination of protein

The Lowry method was performed for protein analysis (Lowry *et al*., 1951), and bovine serum albumin (BSA) was used for calibration with a concentration range of 0–2,000 μg/mL (R^2 = 0.98). A total of 0.5 mL microalgal suspension was mixed with 0.5 mL 1 N NaOH. The sample was sonicated for 10 min using an ultrasonic cleaner, then heated in a water bath at 100°C for 10 min before cooling. 2.5 mL of reagent D (mixture of 50 ml of reagent A 5% Na_2CO_3 + 1 mL of reagent B 1% $CuSO_4.5H_2O$ + 1 mL of reagent C 2% $NaKC_4H_6O_6.4H_2O$) was added to each tube containing the microalgal suspension. It was homogenized, evenly distributed, and allowed to stand for 10 minutes. The 0.5 ml of Folin-Ciocalteau reagent was added to the mixture and vortexed for 10 sec. The mixture was incubated for 30 min before it was determined using a spectrophotometer at an absorbance of 750 nm.

Data Analysis

The IBM SPSS statistic version 27 was utilized for conducting statistical studies. Data of biomass, pigment, lipid, and carbohydrate were examined using one-way ANOVA followed by Tukey's test with a 95% confidence level (α = 0.05). The data was presented as mean ± standard deviation, with n = 3.

RESULTS AND DISCUSSIONS

Growth and Biomass of *Tetraselmis chuii* under different sodium nitrate concentrations

The growth performance of *T. chuii* at various sodium nitrate concentrations is depicted in Fig. 1A. The cell density of *T. chuii* was increased with cultivation time for all nitrate concentrations. The growth of cells decreased with increasing nitrate

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concentration, with the maximum cell density of 26.10 x 10^4 cells/mL produced under 0.5 g/L $NaNO_3$. Supplementation of 0.5 g/L sodium nitrate resulted in 1.21 times higher growth compared to 2 g/L $NaNO_3$ at day 5 of the cultivation period. We also observed the highest specific growth rate (0.33 /day) under 0.5 g/L $NaNO_3$ (Table 2). This result indicated that the cells grew faster under low nitrate concentration with a doubling time of 2.1 days. In a similar study, Sánchez-García *et al*. (2013) reported that the low initial concentration of nitrate (0.2 g/L) produced the highest growth rate of *T. suecica*.

The biomass of *T. chuii* sp. grown under different concentrations of sodium nitrate is shown in Fig. 1B. There was a significant difference ($p<0.05$) in the dry weight of *T. chuii* among the treatments. Interestingly, the results showed that enhancing nitrate concentration led to a decrease in the biomass of *T. chuii*. The highest biomass (0.92±0.01 g/L DW) was obtained under 0.5 g/L $NaNO_3$, which was 1.08, 1.16, and 1.25-fold higher than that of 1.0 g/L, 1.5 g/L, and 2.0 g/L $NaNO_3$, respectively. These results indicate that the nitrate requirement for higher biomass of *T. chuii* was relatively low (0.5 g/L $NaNO_3$). Similarly, Sánchez-García *et al*. (2013) reported that low concentrations of sodium nitrate (0.2 g/L) produced the highest biomass of *T. suecica*. This study indicates that our strain required a low amount of nitrate. In contrast to our study, Kim *et al*. (2016) indicated that nitrate repletion (1.5 g/L) in an f/2 medium obtained the maximum dry weight in *Tetraselmis* sp. KCTC 12236BP. These contrary results suggest that the utilization of nitrate in the *Tetraselmis* strain was species-specific.

Table 2. Specific growth rate and doubling time of *Tetraselmis chuii* under different sodium nitrate concentrations.

Sodium nitrate concentration (g/L)	Specific growth rate (/day)	Doubling time (days)
0.5	0.33	2.10
1.0	0.31	2.26
1.5	0.31	2.26
2.0	0.25	2.82

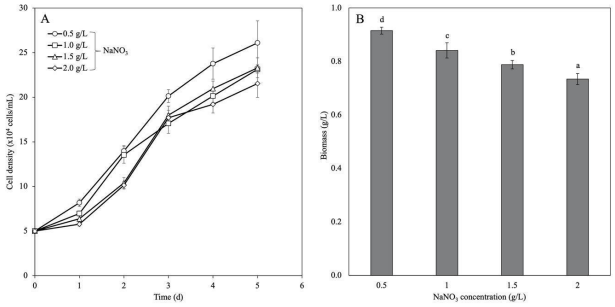

Figure 1. Growth of *Tetraselmis chuii*. A. Cell concentration of *T. chuii*; B. Biomass of *T. chuii* under various sodium nitrate concentrations. Standard deviations from the triple cultures (n = 3) are displayed by error bars. The same letters demonstrate that, at a 95% confidence level, there was no significant difference between the treatments.

Pigment content under different sodium nitrate concentrations

Chlorophyll is essential to green algae's photosynthesis because it absorbs CO_2 and light energy, which creates the metabolic flux required for cell growth and the accumulation of some biochemical products (Lv *et al.*, 2010). Chl *a* and *b* are the most prevalent pigments found in green microalgae (Chen *et al*., 2015).

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In this study, the influence of sodium nitrate in different concentrations on chl *a*+*b* and carotenoid was investigated. An enhancing concentration of sodium nitrate in the medium led to a remarkable increase ($p<0.05$) in the chl *a*+*b*, and carotenoid of *T. chuii*, with the highest chl *a*+*b* and carotenoid of 7.32±0.07 μg/mL and 1.68±0.04 μg/mL was obtained at 1.5 g/L $NaNO_3$, respectively (Fig. 2). Zarrinmehr *et al*. (2020) indicated that nitrogen limitation led to a decrease in all photosynthetic pigments of *Isochrysis galbana*. Surprisingly, we observed a slight decrease in the chlorophyll and carotenoid content of *T. chuii* when nitrogen was increased from 1.5 to 2 g/L. We suggest that higher nitrogen concentrations affect the synthesis and metabolism of photosynthetic pigment in *T. chuii*. Oxborough (2004) indicated that chlorophyll content is influenced by the type and concentration of nitrogen sources.

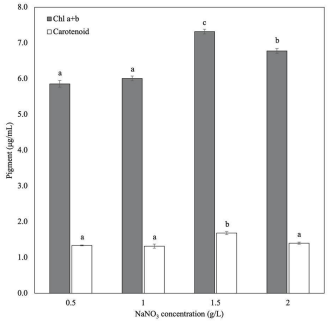

Figure 2. Chl *a*+*b* and carotenoid content of *Tetraselmis chuii* under various sodium nitrate concentrations. Standard deviations from the triple cultures (n = 3) are displayed by error bars. The same letters demonstrate that, at a 95% confidence level, there was no significant difference between the treatments.

Protein synthesis under different sodium nitrate concentrations

We investigated the influence of different sodium nitrate concentrations on the protein production of *T. chuii* (Fig. 3). The synthesis of protein increased remarkably as a response to sodium nitrate concentrations. The highest protein content of 43.15±0.01% was obtained at 2.0 g/L (Fig. 3), which was 1.1 times higher than 0.5 g/L $NaNO_3$ ($p<0.05$). This finding was consistent with Delgado *et al*. (2021), who reported that the increasing nitrate supply significantly increased the protein content of microalga *Picocystis salinarum*. Nutrient availability significantly influences the biochemical composition of microalgal cells (Griffiths and Harrison, 2009). In addition, according to some studies, the amount of nitrogen present in the medium significantly influences the amount of proteins that accumulate in microalgae (Fakhri *et al*., 2021b; Markou, 2015).

Under high nitrogen concentrations, the net nitrogen consumption increases, and microalgae accumulate protein. On the other hand, under low concentrations of nitrogen, the production of nitrogen-containing compounds (Morales-Sánchez *et al*., 2013; Wen and Chen, 2003), including chloroplastic proteins and certain photosystem (PSs) proteins, was decreased (Liefer *et al*., 2018; Young and Beardall, 2003). Furthermore, one explanation for the decline in protein content could be that the cells broke down the nitrogenous molecules to preserve the amount of nitrogen inside the cell necessary for regular metabolic processes. Nitrogen is a significant component of $NADP^+$ (Kiran *et al*., 2016) and an essential element that affects the production of proteins (Araujo *et al*., 2020). This study indicated that *T. chuii* requires a high amount of nitrogen in the medium for protein synthesis.

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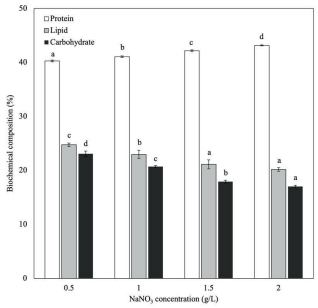

Figure 3. Protein, lipid, and carbohydrate of *Tetraselmis chuii* under various sodium nitrate concentrations. Standard deviations from the triple cultures (n = 3) are displayed by error bars. The same letters demonstrate that, at a 95% confidence level, there was no significant difference between the treatments.

Lipid and carbohydrate accumulation under sodium nitrate limitation

We studied the effect of sodium nitrate on the synthesis of lipids and carbohydrates of *T. chuii* (Fig. 3). Our investigation revealed that energy storage compounds including lipids and carbohydrates were highly accumulated under low nitrate concentration. The highest lipid content of 24.76±0.31% were observed under 0.5 g/L $NaNO_3$ which was 1.08, 1.17, 1.23-fold higher than those of 1.0 g/L, 1.5 g/L, and 2 g/L $NaNO_3$, respectively ($p<0.05$). Furthermore, at the lowest nitrate concentration (0.5 g/L), the largest carbohydrate content (23.07±0.51%) was likewise obtained, which was 1.11, 1.29, and 1.36 times greater than those of 1.0 g/L, 1.5 g/L, and 2.0 g/L $NaNO_3$, respectively ($p<0.05$). Some studies reported that nitrogen deprivation promotes the lipid and carbohydrate content in microalgae. For example, the production of neutral lipids was accumulated up to 75% in *Acutodesmus dimorphus*, and a 93% increase in lipid content was observed in *Chlamydomonas reinhardtii* (Chokshi *et al*., 2017; Yang *et al*., 2018). In terms of carbohydrates, Pancha *et al*. (2014) found that carbohydrate production of *Scenedesmus* sp. CCNM 1077 increased with decreasing nitrate concentration in the medium. A similar pattern of carbohydrate accumulation under nitrogen limitation was also seen in *Isochrysis galbana* (Mishra *et al.*, 2019). These findings suggest a favorable correlation between the amount of carbohydrates and lipids in cells and a reduction in the nitrate concentration in the growth medium.

When there is a shortage of nutrients, photosynthetic carbon flow in microalgae adapts to direct metabolic energy into different energy-rich compounds like lipids and carbohydrates (Siaut *et al*., 2011). This suggests that when nitrogen levels are low, the cells divert and store lipids instead of synthesizing new membrane components, proteins, nucleic acids, and other nitrogen-containing molecules. Similarly, a decrease in the glycolipid and phospholipid composition of the thylakoid membrane, the activation of acyl hydrolase, and an increase in phospholipid hydrolysis are the three alterations that nitrogen constraint tends to cause (Wettern, 1980; Takagi *et al*., 2000; Li *et al*., 2010). The amount of fatty acid acyl-CoA inside cells may rise as a result of these modifications. Acyl-CoAtotriglyceride (TAG) is converted by diacylglycerol acyltransferase, which may be activated by nitrogen constraint (Sánchez-García *et al*., 2013). As a result, microalgal cells under nitrogen limitation may have higher TAG and lipid contents (Courchesne *et al*., 2009).

CONCLUSION

This present study suggested that different nitrate concentrations significantly affected the growth and biochemical composition of *T. chuii*. Increasing nitrate

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concentration increased the pigment and protein of *T. chuii* cells. Interestingly, low nitrate concentration produced the highest storage compounds, such as lipids and carbohydrates, and the highest biomass of *T. chuii*. This study provides an optimal nitrogen concentration of 0.5 g/L $NaNO_3$ for the further mass scale of green microalgae *T. chuii*.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

MF: Methodology, Formal analysis, Visualization, Validation, Writing – original draft, review & editing. AAA: Investigation, Methodology. FES: Methodology, Validation, Writing – review & editing. AY: Resources, Writing – review & editing. NBA: Conceptualization, Formal analysis, Project administration, Investigation, Methodology, Validation, Writing – review & editing.

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