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Short Communication

Effects of Various Nitrogen Sources on the Growth and Biochemical Composition of *Chlorella* sp.

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Abstract

Chlorella sp. is a potential microalgae species to be produced commercially for feed, growth accelerator, and immuno-modulator in fish and shrimp culture. This study aimed to evaluate the various nitrogen sources on the growth, biomass production, and biochemical composition of *Chlorella* sp. FNUB01. The nitrogen sources used in this study were urea $(NH_2)_2CO$, potassium nitrate (KNO_3), and ammonium nitrate (NH_4NO_3). Sodium nitrate ($NaNO_3$) was used as a control as it is a part of the commercial medium BG-11. Generally, the sources of nitrogen in the media affected the growth and chemical composition of *Chlorella* sp. FNUB01. This green microalga grew better in the urea-containing medium which accounted for 1.5 times the concentration of that cultured in BG-11 (40 x10^6 cells. mL^{-1}). Meanwhile, this microalgae species experienced the lowest growth when cultured in NH_4NO_3-containing medium. The biomass productivity of *Chlorella* sp. FNUB01 cultured in urea (0.93 $g.L^{-1}$) was comparable to those grown with $NaNO_3$ as the N source. A similar pattern was recorded for protein, chlorophyll, and carotenoid content as these biochemical contents were affected by N availability in the medium. Urea was an alternative low-cost N source for the culture of *Chlorella* sp. FNUB01. Replacement of $NaNO_3$ with urea could reduce the cost of the medium by 72.6%.

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1. Introduction

Microalgae are photosynthetic unicellular or multicellular microorganisms. These microorganisms can live in various ecosystems including fresh, brackish, and marine habitat. Microalgae can capture CO_2 ten times more efficiently than terrestrial plants due to energy savings for the formation of root, stem, and branch structures (Cheah *et al*., 2015). Several types of microalgae that have high efficiency in photosynthesis have been recorded, including *Chlorella* sp., *Tetraselmis suecica* and *Phaeodactylum tricornutum* (Singh and Singh, 2014).

Chlorella is eukaryotic, unicellular, free-living photosynthetic green microalgae with a diameter ranging from 1 to 20 µm. *Chlorella* sp. is an important feed source for aquatic organisms and has been commonly used in cosmetics and medicine. This species is a potential nutritional supplement as it produces significant pigments like chlorophyll and carotenoids (Fathi *et al*., 2013; Yaakob *et al*., 2014; Safafar *et al*., 2016). Furthermore, Ahmad *et al*. (2018) noted that *Chlorella* sp. biomass is used commercially for feed, growth enhancer, and immunostimulant in aquaculture.

Nitrogen (N) is the most crucial macronutrient for microalgal culture due to its necessity for the production of proteins, pigments, and nucleic acids (Ribeiro *et al*., 2020). The type, the amount, and the availability of nitrogen source would affect the microalgal growth including their biochemical content (Lin and Lin, 2011; Wang *et al*., 2013; Li *et al*., 2019). In mass microalgal culture, the frequently chosen N sources are among ammonium, urea, and nitrate. Ammonium and urea are often used due to their affordable prices (Matsudo *et al*., 2009; Bezerra *et al*., 2013). For physiological and practical reasons, nitrate is the most prevalent source. However, microalgae species respond differently to various kinds of N source. The ammonium was favored by *Spirulina platensis* (Li *et al*., 2019). Nitrate was largely used to cultivate *Arthrospira platensis* (Bezerra *et al*., 2013), while urea gave a high yield of *Mychonastes afer* and *Chlorella sorokiniana* culture (Podevin *et al*., 2015; Yuan *et al*., 2018).

Chlorella sp. FNUB01 was isolated and characterized morphologically. This microalga is a potential species to be developed as it has a high growth rate and high protein content. As each species of microalgae preferred a different supply of nitrogen (Lin and Lin, 2011), it is crucial to choose a suitable nitrogen source to improve biomass and biochemical compound production of *Chlorella* sp. FNUB01. In the present study, the effects of various nitrogen compounds on the growth and biochemical composition of batch *Chlorella* sp. FNUB01 cultures were investigated. A cost evaluation was also conducted to find an alternative low-cost medium for economically viable biorefinery.

2. Materials and Methods

2.1. Microalgae Species and Standard Medium

The green microalgae *Chlorella* sp. FNUB001 used in this study is a collection from Laboratory of Aquaculture, Universitas Brawijaya, Indonesia. Standard cultivation of this *Chlorella* sp. was performed in Blue Green 11 (BG-11) medium (Table 1). *Chlorella* sp. was cultured in a 750 ml flat bottle with a volume of 500 ml. Preparation of *Chlorella* sp. inoculants was carried out for four days to reach the exponential phase. During the preparation of the inoculants, the room temperature was maintained at 28°C with a light intensity of 10,000 lux (24:0 of light: dark period).

2.2 Experimental Condition

The nitrogen sources used in this experiment were sodium nitrate ($NaN0_3$), urea ($(NH_2)_2CO$), potassium nitrate (KNO_3), and ammonium nitrate (NH_4NO_3). Determination of nitrogen amount from each source was conducted based on the N:P ratio (35:1) of $NaNO_3$ in BG-11 medium. If $NaNO_3$ (as a control) in BG-11 medium was 1.5 $g.l^{-1}$, the amount of urea, potassium nitrate, and ammonium nitrate used in this study were 0.53 $g.l^{-1}$, 1.85 $g.l^{-1}$, and 0.70 $g.l^{-1}$, respectively. The other components of the nutrients were used identically as the nutrient in BG-11 medium.

The treatment of various nitrogen sources was repeated four times to obtain independent biological replicates. All treatments were initially inoculated with a similar initial concentration of *Chlorella* sp. of 1×10^6 $cells.ml^{-1}$ and pH 7.7. All bottle cultures were exposed to the light intensity of 10,000 lux with white cool tubular lamps. Aeration was given continuously by air bubbling with 1 L min^{-1} airflow. During the experiment, the room temperature was maintained at 25°C.

2.3 Analysis of Growth and Biochemical Composition

To evaluate the *Chlorella* sp. growth, cell counting was conducted daily using a Neubauer hemocytometer (BOECO, Hamburg, Germany) (Fakhri *et al*., 2021). The specific growth rate of *Chlorella* sp. in each treatment was analyzed based on the equation as follows:

$$\mu\ (day^{-1}) = \frac{\ln T_b - \ln T_a}{t_b - t_a} \quad \text{.....Eq (1)}$$

Where:

μ = the growth rate per unit of biomass,

$T_{a,b}$ = the biomass at the certain time ($t_{a,b}$)

Doubling time (Td) was evaluated from the growth rate with the equation based on (Xu *et al*., 2016) as follows:

$$Td\ (day) = \frac{ln2}{\mu} \quad \text{.....Eq (2)}$$

Biomass, protein, and pigment analysis of *Chlorella* sp. was carried out at the end of exponential phase. The biomass analysis was carried out based on the method of Fakhri *et al.* (2021). A GF/C filter paper (Ø 47 mm) was dried inside an oven at 105°C for two hours until it reached a constant weight (A). The 25 ml of microalgae suspension was taken and filtered using previous GF/C filter paper, then washed with 25 ml of distilled water to remove salt contamination. The filter paper and microalgae were then oven-dried at 105°C for two hours until it reached a constant weight. After cooling, the filter paper was placed in a desiccator for 30 minutes (B). Biomass concentration (dry weight, $g.L^{-1}$) was quantified based on the following equation:

$$Biomass\ (dry\ weight, g.l^{-1}) = \frac{([B]-[A])\times 1.000}{volume\ sampel} \quad \text{.....Eq (3)}$$

Protein analysis was carried out based on the Lowry method (Fakhri *et al*., 2020). A total of 0.5 ml of 1 N NaOH was added to 0.5 ml of the *Chlorella* sp. suspension. The sample was placed in the ultrasonic cleaner for 15 minutes, heated at 100°C for 10 minutes in a water bath, and then cooled down. A 2.5 ml of reagent D (mixture of 50 ml of reagent A 5% Na_2CO_3 + 1 ml of reagent B 1% $CuSO_4$.5H2O + 1 ml of reagent C 2% $NaKC_4H_6O_6.4H_2O$) was added to each tube containing the *Chlorella* sp. suspension. It was homogenized until evenly distributed and allowed to stand for 10 minutes. The 0.5 ml of Folin - Ciocalteau reagent was added to the mixture, and it was homogenized until evenly distributed (vortex) and waited for 30 minutes for absorbance measurement with a spectrophotometer (750 nm). The slope used refers to the Bovine Serum Albumin (BSA) standard curve with the equation of y = 0,0011x + 0,1844, R^2 = 0,9769 (x = protein concentration, y = absorbance).

Chlorophyll and carotenoid analysis were carried out using a methanol extraction based on the method recommended by Fakhri *et al.* (2021). The 10 ml of Chlorella suspension was centrifuged at 6,000 rpm for 10 minutes. Cell pellet was disrupted using freezing (-20°C) and thawing (25°C) method for three cycles. The pellet was mixed with 10 mL methanol absolute and incubated at 70°C in water bath for 30 minutes. The mixture was centrifuged at 6000 rpm for 10 minutes. The supernatant was incubated in a refrigerator in the dark condition for 30 minutes. The concentration of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid was calculated using the equations:

$$Chlorophyl\_a\ (\mu g.mL^{-1}) = (16.72 \times Ab665) - (9.16 \times Ab652) \quad \text{Eq (4)}$$

$$Chlorophyl\_b\ (\mu g.mL^{-1}) = (34.09 \times Ab652) - (15.28 \times Ab665) \quad \text{Eq (5)}$$

$$Carotenoid\ (\mu g.mL^{-1}) = 4 \times Ab480 \quad \text{Eq (6)}$$

2.4 Statistical Analysis

A significant difference in growth rate, doubling time, biomass, and pigment content among the treatments was analyzed using Analysis of Variance with SPSS v.20.

3. Results and Discussion

There was an increase in the population of *Chlorella* sp. during the first three days of culture in all treatments (Figure 1). On the fourth day, there was a decrease in the population of *Chlorella* sp. in all media except those grown in the media with $(NH_2)_2CO$ as a source of nitrogen. On the fourth day, *Chlorella* sp. in the media with $(NH_2)_2CO$ reached the highest concentration. On the fifth day, all *Chlorella* sp. experienced a slowly growth and reached the lowest concentration of culture. In general, *Chlorella* sp. grown in media with $(NH_2)_2CO$ as a source of nitrogen had the highest growth curve compared to other treatments. On the other hand, *Chlorella* sp. grown in NH_4NO_3-containing media had the lowest growth curve. During this study, the lag period of *Chlorella* sp. culture in all media could not be identified. It is suggested that *Chlorella* sp. could adapt and utilize all nitrogen sources directly in the media even though they were previously cultured in BG-11 media with $NaNO_3$ as nitrogen source.

In this study, the different sources of nitrogen in the growth media affected the maximum growth

rate, doubling time, and maximum cell concentration of *Chlorella* sp. significantly ($p<0.05$) (Table 1). The highest growth rate (1.76 day^{-1}) of *Chlorella* sp. was observed in the media with $NaNO_3$ as nitrogen source with double time of 9.49 hours. On the contrary, the lowest growth rate (1.54 day^{-1}) of *Chlorella* sp. was found in the media with KNO_3. The highest maximum growth rate was not necessarily followed by the maximum cell concentration. It was possible that the growth rate of the following period was not constantly high.

Table 1. Composition of BG-11 medium

Component	Amount (g.l^{-1})
$NaNO_3$	1.5
$CaCl_2.2H_2O$	0.036
$EDTA.Na_2.2H_2O$	0.001
$C_6H_8O_7.xFe.xH_3N$	0.012
K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
Na_2CO_3	0.02
Trace mineral mix*	1 (ml.l^{-1})

* H_2BO_3 (2.89 g.l^{-1}); $MnCl_2.4H2O$ (1.81 g.l^{-1}), $ZnSO_4.7H_2O$ (0.222 g.l^{-1}), $Na_2MoO_4.2H_2O$ (0.39 g.l^{-1}; $CuSO_4.5H_2O$ (0.079 g.l^{-1}), $Co(NO_3)_2.6H_2O$ (0.049 g.l^{-1})

The highest cell concentration (40.07 x10^6 cells. mL^{-1}) of *Chlorella* sp. was found in the media with $(NH_2)_2CO$. This concentration was calculated as 1.5 times the concentration of *Chlorella* sp. grown in the commercial media (BG-11) with $NaNO_3$ as nitrogen source (26.02 x10^6 cells. mL^{-1}). The comparable concentration of *Chlorella* sp. was observed in the media with $NaNO_3$ and KNO_3. Meanwhile, the lowest cell population of *Chlorella* sp. (12.07 x10^6 cells. mL^{-1}) was detected in the media with NH_4NO_3 which accounted for 0.5 times of those cultured in the commercial media. Algae can utilize a variety of nitrogen sources combination such as NO_3^-, NH_4^+, NO_2^- and urea. Raven and Giordano (2016) found that most algae can use NO_3- and NO_2-, and all of them can use NH_4+, urea, and amino acids N. Each species preferred a different supply of nitrogen (Lin and Lin, 2011). For example, the green alga *Dunaliella salina* preferred NO_3, while NH_4NO_3 caused cell death (Borowitzka and Borowitzka, 1988). With ammonium as the nitrogen source, *Scenedesmus* sp. LX1 grew the fastest (Xin *et al*., 2010).

With different sources of nitrogen, ammonium (NH_4+) is first absorbed by *Chlorella* sp. through Amt (Ammonia channel transporter) (Ribeiro *et al*., 2020). Ammonium is able to inhibit both nitrate transport and reduction (Scherholz and Curtis, 2015). Furthermore, upon ammonium reduction, suppression of the nitrate assimilatory pathway was reduced then transportation nitrate to the cell could occur. The assimilation of nitrate

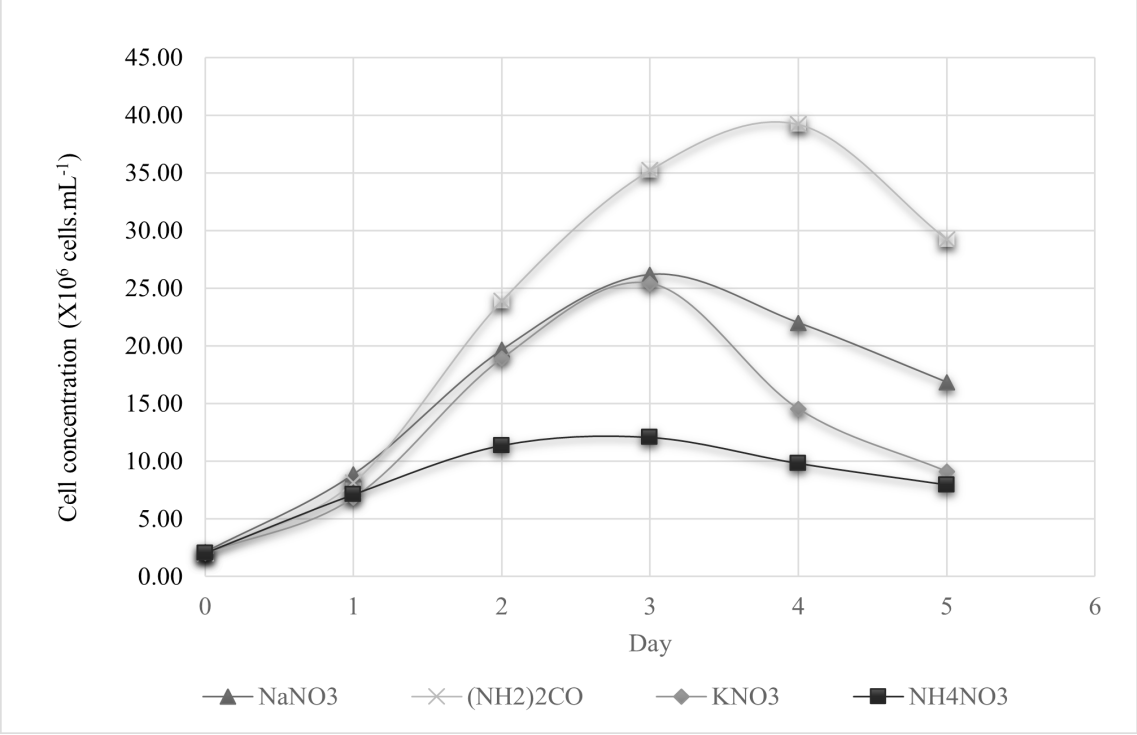

Figure 1. Growth curve of *Chlorella* sp. FNUB001 under different sources of nitrogen.

follows a simple pathway involving: (1) uptake of nitrate by NRT2.1/NAR2, (2) reduction of cytosolic nitrate by Eukaryotic Nitrate reductase (NR), (3) transportation of nitrite into chloroplast by NAR1.1, (4) reduction of nitrite to ammonium by NIR (nitrite-reductase), and (5) incorporation of the ammonium into carbon skeleton by GS/GOGAT cycle which synthesizes glutamate (Cabello *et al*., 2019). Urea ($(NH_2)_2CO$) is absorbed by DUR3 (the active urea transporter) system into the cell (Pinton *et al*., 2016). The urea was then hydrolyzed by allophanate and carboxylase which resulted in two molecules of NH_4^+ and one molecule of carbon dioxide (CO_2) (Caspi *et al*., 2016). This hydrolysis process requires ATP, K^+, and Mg^{2+}. The main route of nitrogen assimilation is GS-GOGAT, and its main product is glutamine, which becomes a component for amino acid and protein synthesis (Ribeiro *et al*., 2020).

The use of urea $(NH_2)_2CO$ as an alternative nitrogen source was studied in several studies and provided positive results. Makarevičienė *et al.* (2011) noted that *Scenedesmus* sp. and *Chlorella* sp. had the highest biomass when cultured in BG-11 modified with urea. Study by Lin and Lin (2011) on *Scenedesmus rubescens* like alga observed that mixture of urea-N and $NaNO_3$-N had the highest productivity. Another research with *Mychonastes afer* found that using $NaNO_3$ and urea gave higher biomass yield than NH_4Cl and NH_4NO_3 (Yuan *et al*., 2018). However, the substitution with urea as nitrogen source in the culture media was not always satisfying. Xu *et al.* (2001) demonstrated that the use of ammonium-N (NH4Cl) in culture medium produced higher biomass and lipid content for *Ellipsoidion* sp. compared to those with nitrate (NaNO3) and urea. Another study noted that several microalgae (*Chlorella minutissima*, *Dunalilella tertiolecta*, *Skeletonema costatum*, *Synechococcus subsalsus*, *Tetraselmis gracilis* and *Nannochloropsis oculate*) grew better in medium with Ammonium-N instead of those in urea (Lourenço *et al*., 2002).

pH is an essential factor for regulation of cell metabolism and biomass development. Variations of pH in the media with $NaNO_3$, $(NH_2)_2CO$, and KNO_3 exhibited a quite similar pattern with slight changes between pH 7 and 9 (Figure 2). On the contrary, a significant change of pH occurred in the media with NH_4NO_3, from 7 to 2.8 over the five-day period of culture. As the pH in the media dropped beyond the optimal pH of *Chlorella* (pH 6-10), the growth and biomass formation were disrupted. Therefore, the cell concentration of *Chlorella* sp. grown in the media with NH_4NO_3 was found to be the lowest among others. Tolerance level of microalgal species to culture medium pH varied, which then affected the growth rate (Chowdury *et al*., 2020). Furthermore, the most common optimal pH value of microalgae culture varied from 6 to 8. Methods for controlling pH in algae cultivation included CO_2 injection and buffer addition (Qiu *et al*., 2017).

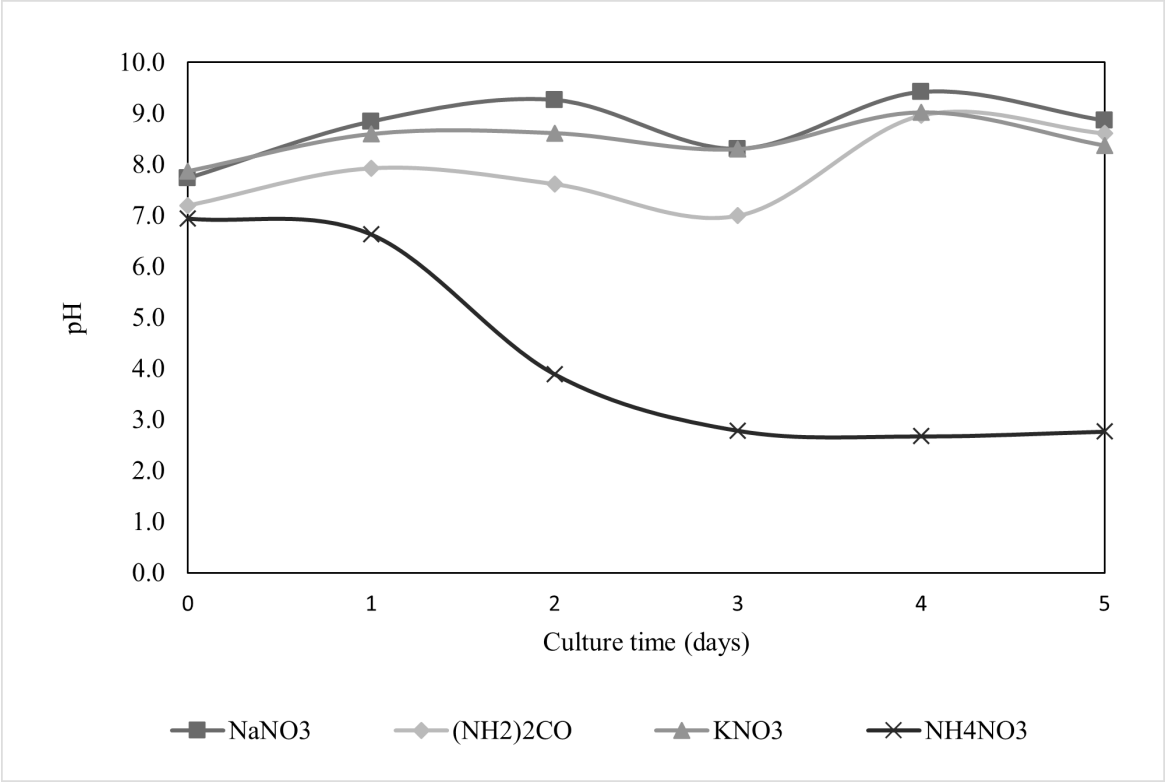

Figure 2. pH values of *Chlorella* sp. cultured in the media with different nitrogen sources.

Table 2. Specific growth rates, doubling time, and maximum cell concentrations of *Chlorella* sp. under different nitrogen sources.

Source of Nitrogen	Max. Specific Growth Rate (Day^{-1})	Doubling Time (hours)	Max. Cell Concentration (10^6 cells. mL^{-1})
$NaNO_3$	1.76 ± 0.16^{b}	9.49 ± 0.43^{b}	26.20 ± 1.07^{b}
$(NH_2)_2CO$	1.75 ± 0.09^{b}	9.54 ± 0.53^{b}	40.07 ± 2.50^{c}
KNO_3	1.54 ± 0.13^{a}	10.84 ± 0.89^{ab}	25.41 ± 0.95^{b}
NH_4NO_3	1.61 ± 0.07^{ab}	10.37 ± 0.43^{a}	12.07 ± 0.82^{a}

Table 3. Biomass, protein, chlorophyll, and carotenoids accumulated in *Chlorella* sp. under different nitrogen sources in the culture media.

Parameter	$NaNO_3$	$(NH_2)_2CO$	KNO_3	NH_4NO_3
Biomass concentration (g DW.L^{-1})	0.91 ± 0.03^{b}	0.93 ± 0.03^{b}	0.77 ± 0.05^{a}	0.67 ± 0.01^{a}
Protein (%)	42.59 ± 1.25^{b}	42.41 ± 1.37^{b}	35.10 ± 1.05^{a}	34.48 ± 1.94^{a}
Chlorophyll a (µg. mL^{-1})	12.89 ± 0.55^{c}	14.94 ± 0.59^{d}	9.72 ± 0.75^{b}	3.05 ± 0.52^{a}
Chlorophyll b (µg. mL^{-1})	9.13 ± 0.41^{c}	13.52 ± 1.68^{d}	6.45 ± 0.60^{b}	1.46 ± 0.88^{a}
Carotenoids (µg. mL^{-1})	3.80 ± 0.20^{c}	4.96 ± 0.31^{d}	2.81 ± 0.30^{b}	0.76 ± 0.14^{a}

Table 4. Biomass productivity and the cost of tested media

Estimation	$NaNO_3$	$(NH_2)_2CO$	KNO_3	NH_4NO_3
Biomass productivity (mg.L^{-1}day^{-1})	300	310	260	220
Cost for 1 L medium (IDR)	2,686	737	810	2,248
Cost of medium to produce 1 g Chlorella sp. (IDR)	2,952	810	1,052	3,35

The use of ammonium-N, either NH_4NO_3 or NH_4Cl, caused a big drop of pH in several species of microalgal culture such as *Haematococcus pluvialis* (Göksan *et al*., 2011), *Chlorella* sp. M2 (Amin *et al*., 2013), and *Mychonastes afer* HSO-3-1 (Yuan *et al*., 2018). Assimilation of ammonium resulted in H+ release, which led to acidification of culture medium (Ribeiro *et al*., 2020). The same pattern was observed as soil acidification also happened when plants absorbed ammonium. The concentration of ammonium N will affect the acidifying strength (Göksan *et al*., 2011). The higher the concentration of ammonium N, the higher the fluctuation of pH in the media will be. Several efforts on pH control when using ammonium N were conducted with the addition of CO2 (Rodrigues *et al*., 2011), NaOH (Yuan *et al*., 2018), and feed batch addition of nitrogen (Scherholz and Curtis, 2015).

The biomass, protein, chlorophyll, and carot enoid biomass productivity of *Chlorella* sp. FNUB01 was significantly affected by the source of nitrogen ($p<0.05$) (Table 3.). Media supplemented with urea gave the highest biomass production (dry weight) of 0.93 g. L^{-1}. However, the result was statistically similar to the control BG-11with $NaNO_3$. Urea dissociates in solution to produce CO_2 and ammonium through pathway of urea amido hydrolase (Kim *et al*., 2013). Later, this ammonium enters the cell directly and builds up to produce amino acids that are helpful in the production of chlorophylls, which is necessary for the photosynthetic process. Moreover, with the addition of CO_2, the production of microalga biomass and lipid increased significantly (Ramanna *et al*., 2014). On the other hand, culture with NH_4NO_3 proved the least effective nitrogen source only producing 0.67 g. L-1 of dry weight bio-

mass. Ramanna *et al.* (2014) found that high amounts of NH_4NO_3 in solution dissociated into ammonium and nitrate which then inhibited biomass production. Moreover, they explained about the inability of microalgal cells to control passive diffusion of ammonia, which is in equilibrium with ammonium ions, across the plasma membrane.

When considering microalgae as fish and shrimp feed, protein is a crucial component to be analyzed. The protein content of *Chlorella* sp. FNUB01 varied when cultured in the media with different sources of nitrogen. The $NaNO_3$-containing media supported the accumulation of the highest protein of *Chlorella* sp. FNUB01 with the value of 42.59%. Meanwhile, the lowest protein content was observed in the medium with NH_4NO_3 as nitrogen source. On the basis of dry mass, the majority of microalgae species have crude protein contents that are over 40% (Chacón-Lee and González-Mariño, 2010). *Chlorella* sp. FNUB1 has a standard protein content with urea and $NaNO_3$ as nitrogen sources. Nitrogen supplies from KNO_3 and NH_4NO_3 provided not only a slower growth rate, but also a lower protein content. Decrease in protein content was a typical physiological response of microalgae during nitrogen deficiency (Lin and Lin, 2011). The microalgal growth, as well as their biochemical composition, would be influenced by the type, quantity, and availability of nitrogen sources (Lin and Lin, 2011; Wang *et al*., 2013; Li *et al*., 2019). Moreover, protein synthesis was strongly affected by the concentration of macronutrient nitrogen. Chen *et al.* (2015) noted that the high protein content of *C. vulgaris* FSP-E would be achieved if the concentration of urea as the nitrogen source in basal medium was 12.4 mM.

All photosynthetic organisms have chlorophyll. An integral part of green algae's photosynthesis, chlorophyll is responsible for absorbing CO_2 and sunlight energy to provide the metabolic flux necessary for both cell development and lipid build-up (Lv *et al*., 2010). Chlorophyll a and b are the most abundant pigments in all photosynthetic organisms, including green algae (Chen *et al*., 2015). In this study, nitrogen source influenced the chlorophyll a and b concentration of *Chlorella* sp. FNUB1 ($p<0.05$). In urea-containing medium, both chlorophyll concentrations achieved the highest level compared to that with other nitrogen sources. On the other hand, the lowest concentration of chlorophyll was detected in the *Chlorella* sp. FNUB1 culture with NH_4NO_3. It was due to a nitrogen deficiency for supporting algal growth. With NH_4NO_3 as nitrogen source in the media, the availability of macronutrient N for growth was limited. A study with *C. vulgaris* showed that lowering the availability of nitrogen in the growth medium resulted in decreased growth rate and chlorophyll content (Lv *et al*., 2010). Lai *et al.* (2019) also found that when nitrogen was deficient in *Dunaliella viridis*, the chlorophyll concentration immediately decreased, and once it had considerably decreased, the new algal cell development ended.

Carotenoids are prominent pigments found in microalgae in addition to chlorophyll. In Chlorophyceae, distribution of both β-carotene and α-carotene is high in almost all species (Takaichi, 2011). While β-carotene is responsible for giving fish their orange and red color, α-carotene is accountable for yellow (Keleştemur and Çoban, 2016). In this study, the total carotenoids in *Chlorella* sp. FNUB01 was significantly influenced by the source of nitrogen ($p<0.05$, Table 3). *Chlorella* sp. FNUB01 grown in urea-containing medium had the highest total carotenoids, while those with NH_4NO_3 had the lowest. Total carotenoids in this study followed a similar pattern with growth, total chlorophyll a, and b. When nutrient N was available, microalgae would continue to grow and reach the highest concentration. Once the nitrogen source was reduced, the concentration of chlorophyll and carotenoid dropped quickly. This finding was in line with study in *Nephroselmis* sp. which evaluated the concentration of pigments including β-carotene with various concentrations of N (Coulombier *et al*., 2020). Nitrogen supply will influence the photosynthesis system. Protein synthesis was reduced as a result of N-limitation, which eliminated chloroplastic proteins and certain photosystem (PSs) proteins (Young and Beardall, 2003; Liefer *et al*., 2018). Then, chlorophyll and carotenoids related to PSs decreased too.

3.1 Evaluation of biomass productivity and cost of medium

Biomass productivity of *Chlorella* sp. FNUB01 in urea-containing media was comparable with that of the control (Table 4). This indicates that urea is a potential alternative nitrogen source for *Chlorella* sp. culture. Prediction of medium cost was conducted to ensure their economic potential. For producing 1 g of *Chlorella* sp. FNUB01, the replacement of $NaNO_3$ with urea and KNO_3 reduced the cost of the medium by 72.6% and 64.4%, respectively. However, even though the cost of 1 L medium reduced with the use of NH_4NO_3, the cost of producing 1 g *Chlorella* sp. FNUB01 was even bigger (13.7%) than those with $NaNO_3$. The new application for *Chlorella* biomass as a source of high-value feed additive in aquaculture industry is being found and developed together with efforts to optimize biomass production. This then will create more economically viable possibilities for future algal biorefineries.

4. Conclusion

The nitrogen sources affected the growth, biomass, protein, chlorophyll, and carotenoid of *Chlorella* sp. FNUB01. Urea $(NH_2)_2CO$ was shown to be the best nitrogen source for *Chlorella* sp. FNUB01. Urea was also found to be the low-cost medium for future *Chlorella* sp. FNUB01 biorefineries.

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Authors' Contributions

The contribution of each author is as follows, MF and NBA; worked on preparing experimental alga culture. AMH; analyzed the data and proofread the article manuscript. ATY; designed the research and wrote the article manuscript. All authors have discussed and contributed to the final manuscript.

Conflict of Interest

This research has no potential conflict of interest.

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