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 (NH₂CO), potassium nitrate (KNO₃), and ammonium nitrate (NH₄NO₃). Sodium nitrate (NaNO₃) 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 x10⁶ cells. mL⁻¹). Meanwhile, this microalgae species experienced the lowest growth when cultured in NH₄NO₃-containing medium. The biomass productivity of Chlorella sp. FNUB01 cultured in urea (0.93 g.L⁻¹) was comparable to those grown with NaNO₃ 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 NaNO₃ with urea could reduce the cost of the medium by 72.6%.

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 CO₂ 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 microalga 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 microagal 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 microagal 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 Arthropsira 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 microalgal 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 (NaNO₃), urea (NH₄)₂CO), potassium nitrate (KNO₃), and ammonium nitrate (NH₄NO₃). Determination of nitrogen amount from each source was conducted based on the N:P ratio (35:1) of NaNO₃ in BG-11 medium. If NaNO₃ (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 × 10⁶ 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:
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:

\[
\text{Chlorophyll}_a (\mu g. ml^{-1}) = (16.72 \times \text{Abs}665) - (9.16 \times \text{Abs652}) \quad \text{Eq (4)}
\]

\[
\text{Chlorophyll}_b (\mu g. ml^{-1}) = (34.09 \times \text{Abs665}) - (15.38 \times \text{Abs665}) \quad \text{Eq (5)}
\]

\[
\text{Carotenoid (\mu g. ml^{-1})} = 4 \times \text{Abs480} \quad \text{Eq (6)}
\]

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 \((\text{NH}_2)_2\text{CO}\) as a source of nitrogen. On the fourth day, Chlorella sp. in the media with \((\text{NH}_2)_2\text{CO}\) 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 \((\text{NH}_2)_2\text{CO}\) as a source of nitrogen had the highest growth curve compared to other treatments. On the other hand, Chlorella sp. grown in \(\text{NH}_4\text{NO}_3\)-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 \(\text{NaNO}_3\) 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 NaNO₃ 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 KNO₃. 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**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g.l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>1.5</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.036</td>
</tr>
<tr>
<td>EDTA.Na₂.2H₂O</td>
<td>0.001</td>
</tr>
<tr>
<td>C₆H₈O₇.xFe.xH₃N</td>
<td>0.012</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.04</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.075</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.02</td>
</tr>
<tr>
<td>Trace mineral mix*</td>
<td>1 (ml.l⁻¹)</td>
</tr>
</tbody>
</table>

*H₂BO₃ (2.89 g.l⁻¹); MnCl₂.4H₂O (1.81 g.l⁻¹); ZnSO₄.7H₂O (0.222 g.l⁻¹); Na₂MoO₄.2H₂O (0.39 g.l⁻¹); CuSO₄.5H₂O (0.079 g.l⁻¹); Co(NO₃)₂.6H₂O (0.049 g.l⁻¹)

The highest cell concentration (40.07 x10⁶ cells. mL⁻¹) of *Chlorella* sp. was found in the media with (NH₂)₂CO. This concentration was calculated as 1.5 times the concentration of *Chlorella* sp. grown in the commercial media (BG-11) with NaNO₃ as nitrogen source (26.02 x10⁶ cells. mL⁻¹). The comparable concentration of *Chlorella* sp. was observed in the media with NaNO₃ and KNO₃. Meanwhile, the lowest cell population of *Chlorella* sp. (12.07 x10⁶ cells. mL⁻¹) was detected in the media with NH₄NO₃ which accounted for 0.5 times of those cultured in the commercial media. Algae can utilize a variety of nitrogen sources combination such as NO₃⁻, NH₄⁺, NO₂⁻ and urea. Raven and Giordano (2016) found that most algae can use NO₃⁻ and NO₂⁻, and all of them can use NH₄⁺, 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 NO₃⁻, while NH₄NO₃ 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 (NH₄⁺) 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...
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$_2$)$_2$CO) 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$_2$)$_2$CO 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$_4$Cl and NH$_4$NO$_3$ (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, Dunaliella tertiolecta, Skeletonema costatum, Synechococcus subsalsus, Tetraselmis gracilis and Nannochloropsis oculata) 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$_2$)$_2$CO, 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$_4$NO$_3$, 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$_4$NO$_3$ 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 microalgal 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.
The use of ammonium-N, either NH$_4$NO$_3$ or NH$_4$Cl, 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 CO$_2$ (Rodrigues et al., 2011), NaOH (Yuan et al., 2018), and feed batch addition of nitrogen (Scherholz and Curtis, 2015).

The biomass, protein, chlorophyll, and carotenoid 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-11 with 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 microalgae biomass and lipid increased significantly (Ramanna et al., 2014). On the other hand, culture with NH$_4$NO$_3$ proved the least effective nitrogen source only producing 0.67 g. L$^{-1}$ of dry weight bio-

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

<table>
<thead>
<tr>
<th>Source of Nitrogen</th>
<th>Max. Specific Growth Rate (Day$^{-1}$)</th>
<th>Doubling Time (hours)</th>
<th>Max. Cell Concentration (10$^6$ cells. mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO$_3$</td>
<td>1.76±0.16$^b$</td>
<td>9.49±0.43$^b$</td>
<td>26.20±1.07$^b$</td>
</tr>
<tr>
<td>(NH$_2$)$_2$CO</td>
<td>1.75±0.09$^b$</td>
<td>9.54±0.53$^b$</td>
<td>40.07±2.50$^c$</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>1.54±0.13$^a$</td>
<td>10.84±0.89$^{ab}$</td>
<td>25.41±0.95$^b$</td>
</tr>
<tr>
<td>NH$_2$NO$_3$</td>
<td>1.61±0.07$^{ab}$</td>
<td>10.37±0.43$^a$</td>
<td>12.07±0.82$^a$</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NaNO$_3$</th>
<th>(NH$_2$)$_2$CO</th>
<th>KNO$_3$</th>
<th>NH$_4$NO$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass concentration (g DW.L$^{-1}$)</td>
<td>0.91±0.03$^b$</td>
<td>0.93±0.03$^b$</td>
<td>0.77±0.05$^a$</td>
<td>0.67±0.01$^a$</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>42.59±1.25$^b$</td>
<td>42.41±1.37$^b$</td>
<td>35.10±1.05$^a$</td>
<td>34.48±1.94$^a$</td>
</tr>
<tr>
<td>Chlorophyll a (µg. mL$^{-1}$)</td>
<td>12.89±0.55$^c$</td>
<td>14.94±0.59$^d$</td>
<td>9.72±0.75$^b$</td>
<td>3.05±0.52$^a$</td>
</tr>
<tr>
<td>Chlorophyll b (µg. mL$^{-1}$)</td>
<td>9.13±0.41$^c$</td>
<td>13.52±1.68$^d$</td>
<td>6.45±0.60$^b$</td>
<td>1.46±0.88$^a$</td>
</tr>
<tr>
<td>Carotenoids (µg. mL$^{-1}$)</td>
<td>3.80±0.20$^c$</td>
<td>4.96±0.31$^d$</td>
<td>2.81±0.30$^b$</td>
<td>0.76±0.14$^a$</td>
</tr>
</tbody>
</table>

### Table 4. Biomass productivity and the cost of tested media

<table>
<thead>
<tr>
<th>Estimation</th>
<th>NaNO$_3$</th>
<th>(NH$_2$)$_2$CO</th>
<th>KNO$_3$</th>
<th>NH$_4$NO$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass productivity (mg.L$^{-1}$day$^{-1}$)</td>
<td>300</td>
<td>310</td>
<td>260</td>
<td>220</td>
</tr>
<tr>
<td>Cost for 1 L medium (IDR)</td>
<td>2,686</td>
<td>737</td>
<td>810</td>
<td>2,248</td>
</tr>
<tr>
<td>Cost of medium to produce 1 g Chlorella sp. (IDR)</td>
<td>2,952</td>
<td>810</td>
<td>1,052</td>
<td>3,35</td>
</tr>
</tbody>
</table>
mass. Ramanna et al. (2014) found that high amounts of \( \text{NH}_3\text{NO}_3 \) 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 \( \text{NaNO}_3 \)-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 \( \text{NH}_3\text{NO}_3 \) 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 \( \text{NaNO}_3 \) as nitrogen sources. Nitrogen supplies from \( \text{KNO}_3 \) and \( \text{NH}_3\text{NO}_3 \) 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 \( \text{CO}_2 \) 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 \( \text{NH}_3\text{NO}_3 \). It was due to a nitrogen deficiency for supporting algal growth. With \( \text{NH}_3\text{NO}_3 \) 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 Čoban, 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 \( \text{NH}_3\text{NO}_3 \) 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 (Coullombier 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 \( \text{NaNO}_3 \) with urea and \( \text{KNO}_3 \) 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 \( \text{NH}_3\text{NO}_3 \), the cost of producing 1 g *Chlorella* sp. FNUB01 was even bigger (13.7%) than those with \( \text{NaNO}_3 \). 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$_2$)$_2$CO 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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