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Research Article

Characterization of *Euglena* sp. Amino Acids and Fatty Acid Methyl Ester (FAME) in Correlation to Ammonium Sulfate $(NH_4)_2SO_4$ Variation: Large-Scale Cultivation

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Abstract: Ammonium sulfate $(NH_4)_2SO_4$ as a source of N compounds in the growth medium of *Euglena* sp. has a major role in growth, vegetative cell formation, protein formation, lipid, and other organic compounds. *Euglena* sp. are microalgae that can grow in environments with acidic pH and high ammonia levels. In this study, variation of ammonium sulfate treatments at different concentrations includes low nitrogen level/F1 (0.5 g L^{-1}), control (1 g L^{-1}), and high nitrogen level/F2 (2 g L^{-1}). This study aimed to define and quantify the amount of FAME, amino acids, growth, and biomass of *Euglena* sp. cultured on a large scale over a period of 16 days. FAME components were tested using Gas Chromatography Flame-Ionization Detector (GC-FID) and amino acids were characterized using Ultra Performance Liquid Chromatography (UPLC). The highest lipid content in F1 (0.209±0.0133 mg mL^{-1}), while the highest protein content (23.1792±2.2607µg mL^{-1}), and the highest biomass content (0.1444±0.0406 mg mL^{-1}) found in F2. The SFA components in *Euglena* sp. elevated with low nitrogen treatment, with methyl arachidate (C20:0), methyl heneicosanoate (C21:0), and methyl palmitoleate (C16:1) with values of 18.76%; 13.54%; and 10.18%, respectively. The amino acid characterization generated 18 compounds, with the highest amino acid concentration of L-Arginine in the control group (15233.09 mg kg^{-1}), and typical amino acids obtained were L-Alanine, L-Tyrosine, L-Histidine, L-Tryptophan, L-Aspartic acid, and L-Serine (VIP score>1).

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1. Introduction

Microalgae have been widely used in numerous areas due to their capacity to swiftly and easily create biomass that is mass-produced. Moreover, microalgae represent a viable supply of diverse chemicals, several of which may be subject to further development (Haris et al., 2022). The nutrient composition of microalgal species varies widely, each of the main components are primarily composed of protein (25–40%), fat (10–30%), and carbs (5–30%) (Ahmed et al., 2023). Various studies and isolation of new strains have been widely carried out as one of the methods of microalgae cultivation to produce various macromolecular materials and products that have high quality (Irhamni et al., 2014).

To increase metabolite production and growth rate, it is necessary to pay attention to the right procedures in the cultivation process, including environmental conditions and the media used (Widiyanto et al., 2014). Production of large quantities of microalgae can be done with various cultivation techniques. One of them is by using an open pond system with a non-aseptic environment (Di Caprio, 2020). This cultivation technique is considered more efficient for producing high amounts of biomass than laboratory-scale cultivation but requires a larger volume of culture media. In addition, the influence of environmental parameters such as pH, temperature, light intensity, weather, and biotic elements originating from pollutants such as bacteria, fungi, etc. cannot be separated from large-scale cultivation (Molina et al., 2019).

Euglena sp. is one type of microalgae that is currently being cultivated on a large scale. This species has several advantages over other types of microalgae and its ability to produce various high-value products. *Euglena* sp. is a unicellular organism with a fast growth rate and can synthesize various bioactive chemicals through very complex metabolic pathways (Kottuparambil et al., 2019). This species produces important metabolites such as fatty acids, carbohydrates, proteins, pigments, and 59 essential nutrients needed by the body (Vieira et al., 2021).

Lipids and fatty acids in microalgae have important roles as storage products, membrane components, metabolites, as well as energy sources. Algal oil is now widely used in various applications due to its similar characteristics to vegetable oil and fish oil. Its application as an alternative energy source as a substitute for fossil oil products is starting to be widely applied. The fatty acid composition of microalgae varies depending on the species. *Euglena* sp. has a fatty acid composition dominated by C16 (42%) and C18 (50%) methyl esters. This composition has similarities with the composition of vegetable oils (Kottuparambil et al., 2019). In addition, *Euglena* also has significant palmitic, linolenic, and linoleic acid components, which are 46%, 23%, and 22%, respectively. This fatty acid composition in microalgae is related to its function as a biodiesel fuel (Amelia et al., 2023). The overall protein content in microalgal biomass is contingent upon the specific microalgal species and may attain up to 70% of the dry weight (Andreeva et al., 2021). According to Villegas et al. (2015), the protein found in microalgae can be used as fuel, chemicals for industry, human food, and animal feed. Essential amino acids such glutamic acid, asparagine, arginine, leucine, and lysine are found in *Euglena* sp. (Ahmed et al., 2023).

Biomass production, especially lipid and protein content will be affected by the composition and concentration of nutrients in the growth medium. One method that can be applied to increase growth as well as the quantity and quality of metabolites in microalgae is through regulating the level of nitrogen (Yaakob et al., 2021). Nitrogen is one of the macronutrients that has a major effect on microalgae cell growth. Jung et al. (2021) stated that large amounts of biomass will be produced under ideal growth conditions. This theory has been tested previously through experiments by Tossavainen et al. (2019), where the availability of high N content in *Euglena gracilis* culture will produce high protein, chlorophyll, and carotenoid content. Meanwhile, limited nitrogen in the culture leads to the production of lipids in the microalgae. Under low nitrogen conditions in the culture, microalgae will accumulate lipids produced in the cells. This can occur because, under stressful conditions, cell division is inhibited, which does not affect the production of microalgal lipids (Ulya et al., 2018).

Microalgae can utilize nitrogen sources in the form of nitrate, ammonia or organic nitrogen such as urea. Nitrogen sources in the form of ammonia are the most preferred by microalgae and plants in general (Afifah et al., 2021). This is because the energy used during the metabolic process to reduce ammonium to organic matter is lower than the energy for the reduction of other forms of nitrogen (Mandal et al., 2018). The use of ammonium will avoid energy consumption due to nitrate/nitrite reduction and energy from the production of nitrate reductase (NR) and nitrite reductase (NiR) enzymes

(Lachman et al., 2018). Apart from N-ammonia, microalgae also consume nitrogen in the form of N-nitrite and N-nitrate. In algal cells, N-nitrate and N-nitrite will undergo shrinkage and $N\text{-}NO_2^-$ will turn into $N\text{-}NH_4^+$. Therefore, microalgae more easily accumulates nitrogen sources in the form of ammonia.

Different medium compositions will affect variations in nutrient amounts that can significantly alter the biochemical composition of microalgae. Therefore, it is important to perform media optimization to achieve higher growth yields and value-added products from microalgae. This research will examine the effect of nitrogen variation on lipid and protein content, and determine the composition of fatty acid and amino acid content of *Euglena* sp. in large-scale cultivation, as well as to obtain the most optimal microalgae growth conditions.

2. Material and Methods

2.1. Materials

The research was done from December 2023 to February 2024. *Euglena* sp. was cultivated at Biodiversity Research Station II, Karanggayam. The culture of *Euglena* sp. was obtained from the IDN33 culture collection at the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada. Cultivation was carried out by growing *Euglena* sp. in Cramer & Myers (CM) modification medium for large-scale 500 L volume with composition of MKP Pak Tani PT. Saprotan Utama (P_2O_5 52% and K_2O 34%), ZA Fertilizer PT. Petrokimia Gresik [$(NH_4)_2SO_4$], $MgSO_4$, KCl, $FeSO_4.7H_2O$, $MnCl_2.4H_2O$, $CoSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_20$, $Na_2MoO_4.2H_2O$, vitamin B1, and vitamin B12.

2.2. *Euglena* sp. cultivation

The preparation of inoculant stock was carried out on laboratory scale with initial culture concentration (OD 680_{nm}) was 0.3. The starter culture was left for three weeks to equalize the age of *Euglena* sp. Then, stock culture was scaled up from laboratory scale to large-scale. The cultivation of mass-scale composition consisted of 100 L inoculant microalgae with pH 3.5 and 400 L of Cramer & Myers (CM) medium modification with a total volume of 500 L. The microalgae were grown in varying nitrogen of 1 g L^{-1} (control), 0.5 g L^{-1} (F1), and 2 g L^{-1} (F2). Nitrogen source that was used in the culture medium was ammonium sulfate. There were five repetitions in each culture group (n=5).

2.3. Measurement of the growth

The growth rate of the sample was determined daily during the research by measuring optical density (OD) and the number of cells. The optical density was measured using a Thermo Scientific Genesys 150s UV/Vis spectrophotometer to determine the absorbance at wavelengths of 680 nm. The number of cells were determined using a neubauer hemocytometer under a OLYMPUS CX22 microscope. The sample volume utilized for cell density computation was 900 µL, with the addition of 100 µL of 70% alcohol to the microtubes. The amount of cells number was then analyzed using the following formulas (Equation 1).

$$cell/mL = \frac{Counted\ cell\ quantity}{4} \text{ x } 10^4 \quad (1)$$

2.4. Growth kinetic modeling

The Logistic and Gompertz Models were employed to characterize the growth kinetics of *Euglena* sp. The logistic model has various parameters, with X denoting cell densities, X_0 indicating beginning cell densities, X_{max} signifying maximum cell density, and μ_{max} representing the specific growth rate per day during the exponential and stationary stages of microalgal growth. This model did not consider the mortality of microalgae following the stationary phase (Phukoetphim et al., 2017; Maghfiroh et al., 2023). The logistic model could be calculated using the following formulas (Equations 2 and 3).

$$\frac{dx}{dt} = \mu max \left(1 - \frac{X}{\mu max}\right) x \quad (2)$$

$$X = \frac{Xo.exp(\mu max\ t)}{1 - \left[\left(\frac{Xo}{xmax}\right)(1 - exp\ (\mu max\ t))\right]} \quad (3)$$

The Gompertz model was relatively more complex when compared to the Logistic model. This model has three parameters that could describe several characteristics of microalgae. The maximum cell production rate (rm) and time lag (tL) are variables in the Gompertz model (Phukoetphim et al., 2017; Maghfiroh et al., 2023). The Gompertz model was determined using the following formula (Equation 4).

$$x = Xo + [\ Xmax.exp\ [-exp\ ((\left(\frac{rm.exp\ (1)}{Xmax}\right)\ (tl - t) + 1] \quad (4)$$

The Logistic model and Gompertz model were determined using the residual sum of squares between the cell density and the calculated results, where SSR was the residual sum of squares and SST was the total sum of squares (Equation 5).

$$R^2\ = (1 - \frac{SSR}{SST}) \quad (5)$$

2.5. Biomass measurement

A sample of *Euglena* sp. mass culture, taken up to 50 mL in a conical tube and filtered using 47 mm Whatman GF/C filter paper with 1.5 µm. Buchner funnel was used to measure biomass or dry weight. The filter paper with the biomass was dried for approximately an hour at 100 °C in an oven incubator. The biomass was weighed using an AND GR-200 analytical balance after it had dried (Erfianti et al., 2023).

$$Dry\ weight\ (mg/mL) = \frac{Filter\ paper\ final\ weight\ -\ filter\ paper\ initial\ weight}{Sample\ volume} \quad (6)$$

2.6. Measurement of Lipid Content

The total lipid content of *Euglena* sp. was determined using the Bligh and Dyer (1959) method. This method was one of the standard procedures performed for the extraction and isolation of total fat fraction from biological matrices based on polar and non-polar solvent systems. Fifty milliliters of *Euglena* sp. culture was centrifuged in a Hettich Zentrifugen Universal 320R centrifuge, and the resultant biomass was combined with solvents including chloroform, methanol, and distilled water with a constant ratio of 2:2:1 (v/v/v). The solution was centrifuged at 4000 rpm until three layers formed. The yellow bottom layer was taken using a micropipette and transferred to a petri dish that had previously been weighed. The petri dish was put into the oven at 50 °C for 24 hours. Calculation of lipid content using the following formula (Equation 7):

$$Lipid\ content\ (mg/mL) = \frac{Petri\ dish\ final\ weight\ -\ petri\ dish\ initial\ weight}{Sample\ volume} \quad (7)$$

2.7. Protein extraction and quantification

Protein extraction of *Euglena* sp. used buffer lysis which contains 50 mM tris buffer pH 8,3; SDS 10% solution, protease inhibitor using PMSF (Fenilmetilsulfonil fluorida), glycerol, MilliQ (Anjos et al., 2022). Analysis of protein content began with protein extraction from *Euglena* sp. cells. 50 mL of sample from each treatment was centrifuged with a Hettich Zentrifugen Rotofix 32A centrifuge for 10 minutes at 3500 rpm. The pellet that settled on the tube was weighed and transferred into a 1.5 mL microtube. A total of 10 µl of buffer was added per 1 mg sample weight and then homogenized for 1 minute. The sample was extracted in a sonicator at a cold temperature for 2×10 min with a 1 min pause in between, then incubated in a dry block thermostat (bioSan Bio TDB-100) for 10 min at 95 °C. Next,

the sample was centrifuged in Hettich Zentrifugen Universal 320R at 13000 rpm for 5 minutes at 4 °C, and the supernatant formed can be transferred to a new microtube (Knoshaug et al., 2020). Protein quantification was determined by Bradford assay and standard solution of Bovine Serum Albumin (BSA) analyzed by BioTek ELX800 Microplate reader at 595 nm. The 96-well microplate was filled with 200 µl Bradford and added 8 µl of both standard solution and isolated samples, calculation of protein content by substituting the absorbance value into a linear regression equation so that the formula y = ax + b is obtained with y is the absorbance value (Roche, 2024).

2.8. Fatty Acids Methyl Ester (FAME) analysis

Fatty Acids Methyl Ester (FAME) concentration was analyzed through the direct transesterification method and Gas Chromatography Flame-Ionization Detector (GC FID-Agilent Technologies 7890B). Transesterification reaction is a reaction that occurs between oil (triglyceride) and alcohol (Amelia et al., 2023). The commonly used alcohol in this reaction was methanol because it is one of the stable short-chain alcohols. Biomass from *Euglena* sp. microalgae was harvested by centrifugation technique at 4000 rpm for 5 minutes. Samples from each treatment were taken on the last day of cultivation (day 16^{th}). The samples were then centrifuged for 5 minutes at 4000 rpm to separate the pellet and supernatant. The pellet was then washed using 50 mL of distilled water after the supernatant was discarded. After that, a second centrifugation was carried out followed by three rinses using distilled water. The sample was hydrolyzed using 10 mL of concentrated HCl, then heated for 3 hours at 80 °C, and then cooled at room temperature (25 °C). Next, the sample was extracted using 25 mL of diethyl ether and petroleum ether (1:1 v/v). The top layer of the sample was discarded, then the sample was evaporated with N_2 gas in a water bath. The sample was methylated by adding 0.5 mL of oil and 1.5 mL of sodium methanol solution, then heated at 60 °C for 10-15 minutes with stirring and cooled at room temperature (25 °C) in a small-closed test tube. After that, 2 mL of boron trifluoride methanol was added and incubated at room temperature (25 °C) after heating for 5-10 minutes at 60 °C. 1 mL of heptane and 1 mL of saturated NaCl were used for sample extraction. The top layer was put into a GC vial and 1 µL of sample was injected into the GC.

2.9. Amino acids characterization

The hydrochloric acid (HCl) hydrolysis method was used to characterize the amino acids from frozen biomass using 0.1–1 g of sample with standard solution (SIGMA-ALDRICH, AAS18–10X1ML, Lot# SLCL 2556). The hydrolysis products were mixed with distilled water in a 50 mL volumetric flask, filtered using a syringe filter, and then introduced into an Ultra Performance Liquid Chromatography (UPLC) apparatus. The C18 column, mobile phase (eluent accQ, ultra tag, aquabidest), column temperature (49 °C), and detector (PDA Ch1 260 nm) were the chromatographic conditions for this test (Liw et al., 2019).

2.10. Statistical analysis

The results were analyzed using Microsoft Excel and IBM Statistical Product and Service Solution software (Version 26, IBM Corporation, USA). A one-way ANOVA test with a data significance threshold determined by a *p*-value <0.05 at the 5% level and post-hoc analysis using the Duncan Multiple Range Test (DMRT) is used to examine the mean differences in each variable. The kinetics of growth models using the Anaconda Navigator application with the Logistics model and the Gompertz model. Data processing graph visualization was displayed using the Origin-Pro application. The results of fatty acids methyl ester and amino acid profiles are analyzed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/).

3. Results

3.1. Cell growth characteristic of *Euglena* sp.

The augmentation of cell size and cell quantity is a determinant of the growth rate of microalgae in culture (Martinez-Ruiz et al., 2022). Growth properties of *Euglena* sp. can be determined through specific growth rate and doubling time. The specific growth rate (SGR) quantifies the rate of microalgal

cell proliferation over time, influencing nutrient production and the cell division mechanism. Doubling time refers to the duration necessary for microalgae cells to increase their population twofold (Nurafifah et al., 2023). The number of cells was measured using a hemocytometer.

Table 1. Specific growth rate (SGR) and doubling time (Td) of *Euglena* sp.

Treatment	SGR	*Doubling Time* (T_d)
Control (1 g L^{-1})	0.108 ± 0.008^{a}	6.416 ± 0.186^{c}
F1 (0.5 g L^{-1})	0.125 ± 0.007^{b}	5.541 ± 0.265^{a}
F2 (2 g L^{-1})	0.115 ± 0.004^{ab}	6.026 ± 0.089^{b}

Notes: Mean values followed by different letters indicate significant differences at the α = 5% test statistical level.

Based on table 1, it is known that the correlation between specific growth rate and division time is inversely proportional, where when the specific growth rate is high, the cell division time will be low and vice versa. In the table, F1 treatment has the highest specific growth rate value compared to the other two treatments which is 0.125 ± 0.007 with the lowest division time of 5.541 ± 0.265. While the lowest specific growth rate was obtained in the control culture with the highest division time. Through statistical analysis, it is known that the specific growth rate in each treatment shows a significant difference ($p<0.05$). While at doubling time, there was no significant difference in F1 and F2 treatments ($p>0.05$) but the control treatment was significantly different from the F1 treatment.

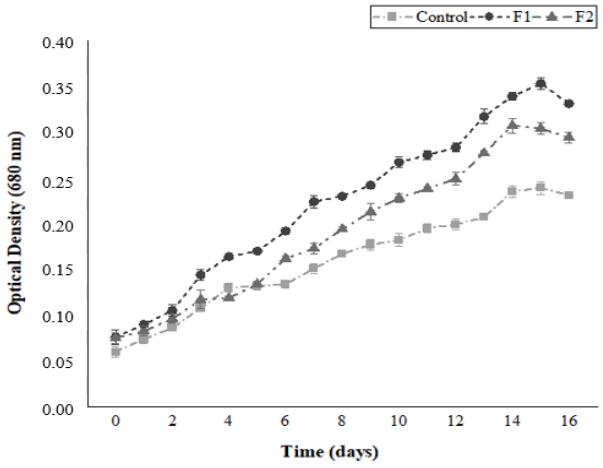

Figure 1. Cell density of *Euglena* sp. on various ammonium sulfate concentration treatments.

According to Figure 1, it can be seen that the cell density in *Euglena* sp. both in the control, F1, and F2 treatment cultures continued to increase until the end of the cultivation period. The highest cell density was obtained in the F1 treatment, while the culture with the lowest cell density was shown in the control. The highest cell density was found on the 14th day of cultivation in each treatment. The cell density pattern obtained is positively correlated with the cell growth rate, where the absorbance of cell density will increase if the number of *Euglena* sp. cells also increase. From day 11 to day 14, the growth of the control group reached a stationary phase, which was absent in treatments F1 and F2, as illustrated in Figure 1. The stationary phase commences when the high density of *Euglena* cells experiences diminished growth due to intercellular competition for limited food resources essential for metabolism. The wavelength of 680 nm was used to determine the cell density of *Euglena* sp., because this wavelength is commonly applied for measuring cell density in microalgae (Toyama et al., 2019).

3.2. Growth kinetic modeling

Based on Figure 2, it was found that through the Gompertz model, the high concentration ammonium sulfate treatment showed a maximum cell growth rate (rm) of 6.33 cells mL^{-1}. The lag time values (tL) sequentially for control, F1, and F2 were 0.333/day; -0.541day; 0.365/day. Using the Logistic model, the maximum specific growth rate (μ_{max}) in each treatment was 0.366/day (control);

0.3365/day (F1); and 0.338/day (F2). The highest R^2 error values for control, F1, and F2 were 0.955; 0.988; and 0.995.

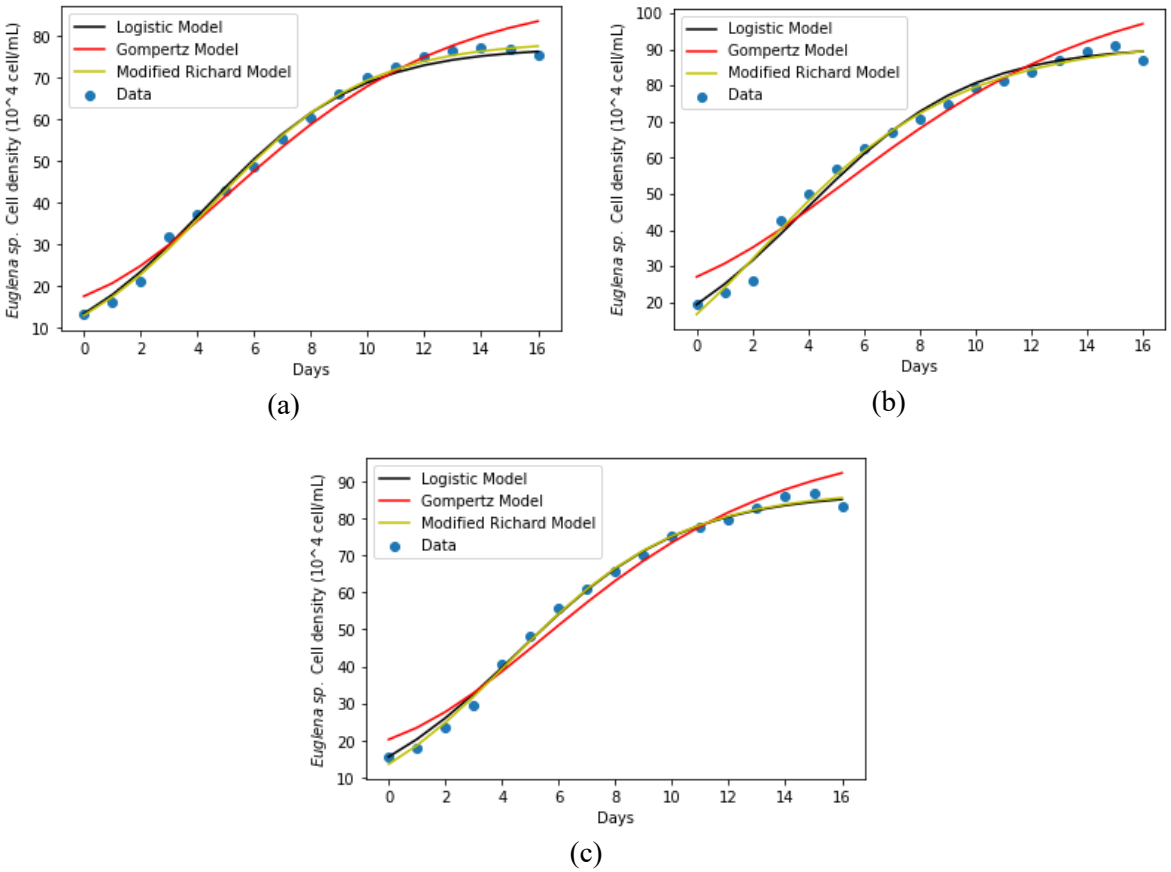

Figure 2. Fitting of Gompertz and Logistic Model (cell density) on the growth of *Euglena* sp. (a) control; (b) low ammonium sulfate concentration, (c) high ammonium sulfate.

3.3. The effect of variation nitrogen on biomass

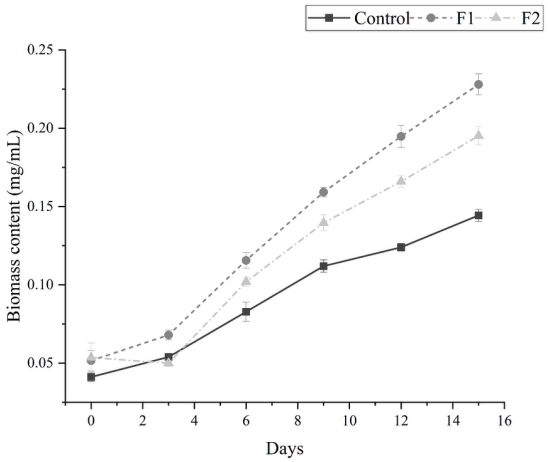

Figure 3. The content of *Euglena* sp. biomass under $(NH_4)_2SO_4$ variation treatment.

Based on the figure above, the daily biomass production rate of *Euglena* sp. aligns with the growth rate curve depicted in figure 5, based on optical density measurements. On the fifteenth day, the F1 group exhibited the highest average biomass value of 0.228±0.007 mg mL^{-1}, while the F2 group

recorded a biomass of 0.195±0.006 mg mL^{-1}, and the control group yielded a biomass of 0.144±0.004 mg mL^{-1}. In this experiment, the biomass production growth observed in each treatment group did not correspond with established theory and prior research, such as that by Erfianti et al. (2023), which indicated that medium variations with a nitrogen concentration of 1.5 g L^{-1} yielded greater biomass of *Euglena* sp. than media with nitrogen concentrations of 0.75 g L^{-1} and 0.375 g L^{-1}. The nitrogen source in this study, specifically ammonium sulfate, can degrade into ammonia.

3.4. Lipid content and fatty acids methyl ester of *Euglena* sp.

Figure 4 shows the lipid content produced by *Euglena* sp. during 16 days of cultivation and the productivity of lipids produced per day. The highest lipid content of *Euglena* sp. was produced by treatment F1 (low nitrogen content) at 0.209 mg mL^{-1}, followed by treatment F2 at 0.151 mg mL^{-1} and the lowest lipid content in the control treatment at 0.138 mg mL^{-1}. The same trend was shown in the productivity results, where the highest productivity was produced in the F1 treatment, followed by the F2 treatment and the lowest in the control, with values of 0.0165 mg mL^{-1}/day; 0.0123 mg mL^{-1}/day; and 0.0104 mg mL^{-1}/day, respectively. Through these results, it can be known that F1 treatment is more effective in increasing lipid content and productivity in *Euglena* sp. The above results also show that *Euglena* sp. lipid productivity is directly proportional to biomass productivity (Figure 4). When biomass productivity is high, lipid productivity will follow (Sobari et al., 2013). Based on the statistical test, it is known that each treatment has a significant difference ($p<0.05$).

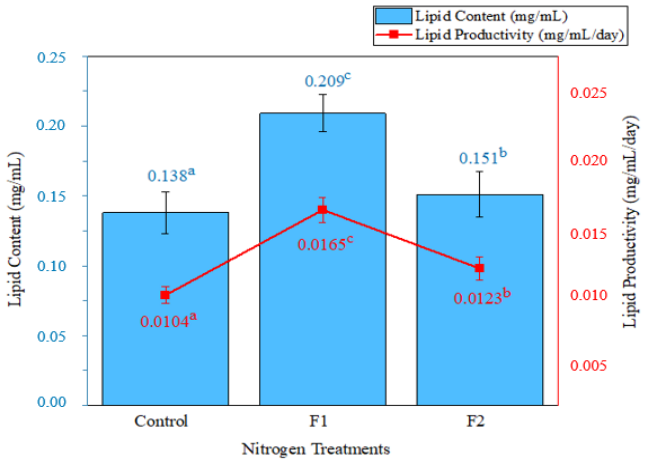

Figure 4. The content and productivity of *Euglena* sp. lipid under $(NH_4)_2SO_4$ variation treatment during mass-cultivation of. Data means ± SD (n=5), one-way ANOVA was used to compute significant differences between treatments, and the Duncan Multiple Range Test (DMRT) was used to confirm the results ($p<0.05$).

Table 2, shows the fatty acid methyl ester (FAME) components found in *Euglena* sp. with different nitrogen variation treatments. Based on the results obtained, it can be seen that the most compounds found in the three treatments are methyl heptadecanoate (C17:0), methyl arachidate (C20:0), methyl heneicosanoate (C21:0), and methyl palmitoleate (C16:1). In general, *Euglena* sp. in this study produced more saturated fatty acids and less unsaturated fatty acids. The F1 treatment with low N content produced the highest concentration of saturated fatty acids (43.24%), followed by the F2 treatment (42.44%) and the control (41.38%). The highest monounsaturated fatty acid was produced in treatment F1 (28.36%), followed by treatment F2 (26.44%), and the lowest in the control (25.18%). Meanwhile, polyunsaturated fatty acids (PUFA) were produced more in the control treatment (24.65%) compared to F2 (23.72%) and F1 (20.81%). Based on the above results, ammonium sulfate with low concentration can produce more saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in *Euglena* sp.

Table 2. Fatty acids methyl ester (FAME) on *Euglena* sp. grown in various nitrogen treatment

Parameters	C.Number	Control	Treatment 1/F1	Treatment 2/F2
Methyl laurate	C12:0	0.40	0.36	0.40
Methyl tridecanoate	C13:0	1.11	1.03	1.47
Methyl myristate	C14:0	6.33	5.77	6.15
Methyl palmitate	C16:0	1.77	1.90	1.84
Methyl heptadecanoate	C17:0	7.51	7.82	7.42
Methyl arachidate	C20:0	7.37	10.18	9.04
Methyl heneicosanoate	C21:0	13.51	13.04	13.04
Methyl lignocerate	C24:0	3.38	3.14	3.08
Methyl cis-10-pentadecenoate	C15:1	1.56	1.93	1.82
Methyl palmitoleate	C16:1	16.98	18.76	17.83
Methyl cis-10-heptadecanoate	C17:1	5.05	6.98	6.19
Methyl linolelaidate	C18:2	5.40	3.23	6.70
Methyl cis-8,11,14-eicosatrienoate	C20:3	1.37	1.36	1.33
Methyl erucate	C22:1	0.59	0.69	0.60
Methyl cis-5,8,11,14-eicosatetraenoate	C20:4	4.75	4.05	3.42
Methyl cis-5,8,11,14,17-eicosapentaenoate	C20:5	7.02	6.18	6.38
Methyl cis-4,7,10,13,16,19-docosahexaenoate	C22:6	6.11	5.99	5.89
SFA		41.38%	43.24%	42.44%
MUFA		25.18%	28.36%	26.44%
PUFA		24.65%	20.81%	23.72%
%Relative Area			100.00	

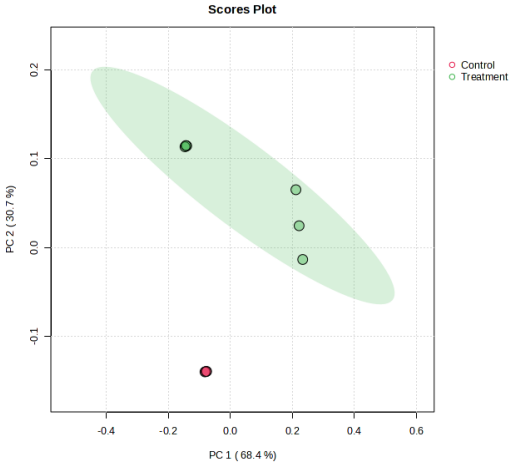

Figure 5. Principal component analysis (PCA) of the fatty acid methyl ester (FAME) *Euglena* sp. under nitrogen variation treatment.

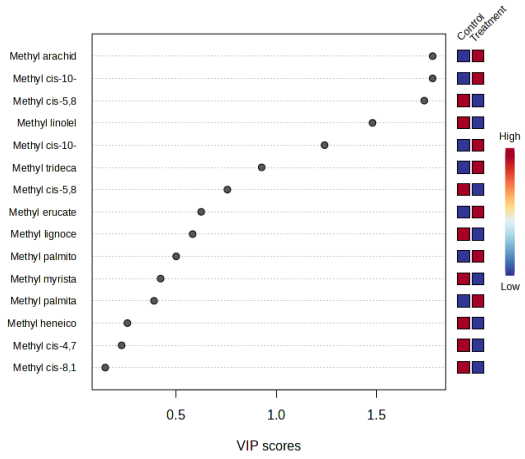

Figure 6. Importance features of the fatty acid methyl ester (FAME) *Euglena* sp. under nitrogen variation treatment.

Figure 5 shows the results of PCA analysis on fatty acids of *Euglena* sp. obtained from UPLC analysis. PC 1 explains 68.4% of the total variance while PC 2 accounts for 30.7% of the total variance. The PCA model in these results can be said to be good because the cumulative Q2 value obtained is the total of the sum of PC1 and PC2 >50%. The F1 and F2 treatments are in the same cluster (cluster 2), while the control is in a different cluster (cluster 1). Based on Figure 6, methyl arachid, methyl cis-10-heptadecenoate, and methyl cis-5,8,11,14,17-eicosapentaenoate are known to have VIP values greater than (VIP>1.5). Based on the results of significance features, methyl arachid is the most common and significant compound of *Euglena* sp. in nitrogen variation treatment.

3.5. Protein content and amino acids of *Euglena* sp.

Figure 7 shows the fluctuation in *Euglena* sp.'s protein content and productivity values treated with ammonium sulfate. The examination revealed that the F2 treatment group had a significantly higher protein content of *Euglena* sp. (23.1792±2.2607 µg mL^{-1}) than the control group. Furthermore, treatment group F2 produced the highest protein productivity of all treatments in this investigation, with a value of (0.4183±0.0975 µg mL^{-1}/day), as shown in Figure 7, which also displays the value of protein productivity daily.

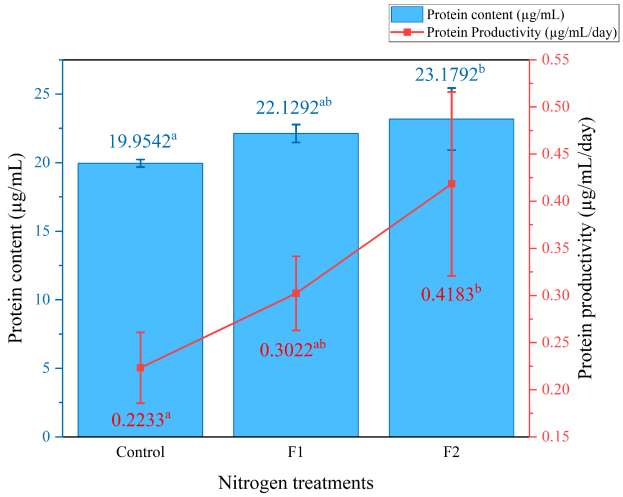

Figure 7. The content and productivity of *Euglena* sp. protein under $(NH_4)_2SO_4$ variation treatment during large-scale cultivation. Data means ± SD (n=5), one-way ANOVA was used to compute significant differences between treatments, and the Duncan Multiple Range Test (DMRT) was used to confirm the results ($p<0.05$).

Table 3. Amino acid on *Euglena* sp. grown in various nitrogen treatment

Name	Abbreviations	Amino acid content (mg kg^{-1})			Molecular formula
		Control	F1	F2	
L-Alanine	Ala	9474.67	7114.54	5908.90	$C_3H_7NO_2$
L-Arginine	Arg	15233.09	9919.30	13581.21	$C_6H_{14}N_4O_2$
L-Aspartic Acid	Asp	9837.21	7517.98	5939.41	$C_4H_7NO_4$
Glycine	Gly	6019.12	4384.87	4370.28	$C_2H_5NO_2$
L-Glutamic Acid	Glu	10655.58	8315.72	5394.30	$C_5H_9NO_4$
L-Histidine	His	1303.25	1144.76	1023.57	$C_6H_9N_3O_2$
L-Isoleucine	Ile	4263.35	2959.31	1405.57	$C_6H_{13}NO_2$
L-Cystine	Cys	7398.56	5362.21	6711.27	$C_6H_{12}N_2O_4S_2$
L-Leucine	Leu	8866.46	6623.70	4473.19	$C_6H_{13}NO_2$
L-Lysine	Lys	6369.82	5044.25	2886.28	$C_6H_{14}N_2O_2$
L-Methionine	Met	1446.25	1196.90	1412.52	$C_5H_{11}NO_2S$
L-Tryptophan	Trp	1494.03	563.08	1188.96	$C_{11}H_{12}N_2O_2$
L-Valine	Val	7044.66	5093.27	2736.61	$C_5H_{11}NO_2$
L-Phenylalanine	Phe	4043.01	3071.35	2395.63	$C_9H_{11}NO_2$
L-Proline	Pro	7979.34	5484.11	3545.70	$C_5H_9NO_2$
L-Serine	Ser	3379.44	2532.86	2163.26	$C_3H_7NO_3$
L-Threonine	The	3778.28	2885.57	2046.48	$C_4H_9NO_3$
L-Tyrosine	Tyr	2827.23	2874.68	2526.04	$C_9H_{11}NO_3$

The results of the amino acid characterization of *Euglena* sp. impacted by ammonium sulfate $(NH_4)_2SO_4$ are displayed in Table 3. It is evident that *Euglena* sp. under the control, F1, and F2 treatments, contains eighteen different amino acid molecules. The largest known amino acid is L-arginine ($C_6H_{14}N_4O_2$), which is found in the control (15233.09 mg kg^{-1}), F1 as much as (9919.30 mg kg^{-}

^{1}), and F2 (13581.21 mg kg^{-1}). In the F1 group, L-tryptophan ($C_{11}H_{12}N_2O_2$) was the amino acid with the lowest levels, whereas L-histidine ($C_6H_9N_3O_2$) was discovered at the lowest quantities in the control and F2 treatments.

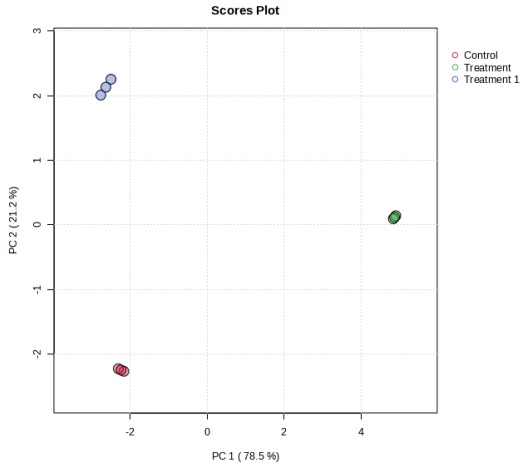

Figure 7. Principal component analysis (PCA) of *Euglena* sp. amino acid under nitrogen variation treatment.

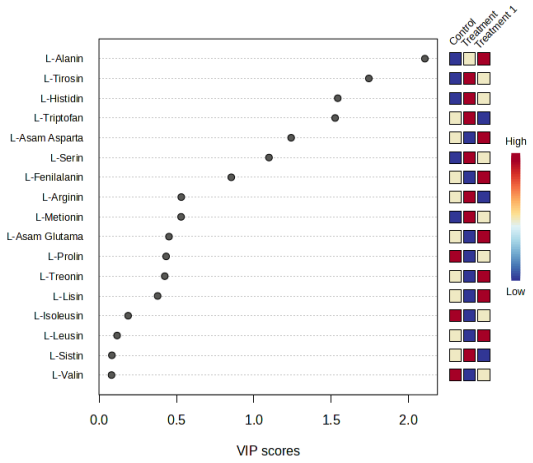

Figure 8. Importance features identified by PLS-DA of the *Euglena* sp. amino acid under nitrogen variation treatment.

The amino acid PCA analysis data for *Euglena* sp., obtained from UPLC analysis, is presented in Figure 7. Three distinct clusters, each comprising 18 compounds, have emerged without intersection, signifying that the chemicals in PC1 and PC2 are very well grouped, with their combined contribution exceeding 50%. L-Alanine, L-Tyrosine, L-Histidine, L-Tryptophan, L-Aspartic acid, and L-Serine are the six molecules depicted in Figure 8 that possess VIP scores over 1, VIP scores greater than 1 signify that certain amino acid molecules are distinctive or significant in *Euglena* sp. (Stoessel et al., 2018). The major feature results indicate that L-Alanine is the predominant and most significant chemical from *Euglena* sp. in addressing nitrogen variation.

4. Discussion

The success rate of a cultivation method can be characterized in the early phase of microalgae growth. This can be seen from the short lag phase or from the exponential phase where the ratio of the increase in the number of microalgae populations per unit time is very high due to rapid cell division (Hudaidah et al., 2013). In this study, the lag phase of *Euglena* sp. occurred very briefly, which is less than 24 hours from observation day 0 to day 1. This can be seen from the lag time value in each treatment based on the Gompertz growth model kinetics, where each treatment has a lag phase of 0.333/day (control), -0.541/day (F1), and 0.365/day (F2). On day 2, *Euglena* sp. cells in each treatment began to enter the exponential phase characterized by increased cell growth. The peak exponential phase occurred on day 14 in the control and day 15 in the F1 and F2 treatments. After that, cell growth began to stagnate and experienced a decrease in growth rate after the peak phase. Nutrients become a factor that limits cell growth in the growth decline phase, which results in decreased cell division (Muchammad et al., 2013). The results obtained show that the increase in growth patterns in each culture is directly proportional to the increase in cell density, whereas as cell density increases, the number of cells also increases.

Specific growth rate and doubling time are one of the growth characteristics of *Euglena* sp. Specific growth rate can be defined as the speed of microalgae cells to grow per unit of time and to determine the capacity of nutrients produced as well as the cell division process. While doubling time is the length of time for microalgae to double their number (Nurafifah et al., 2023). Based on table 1, the low ammonium sulfate concentration treatment (F1) produced the highest specific growth rate of 0.125 ± 0.007 with the shortest division time of 5.541 ± 0.265. The specific growth rate has an inversely proportional relationship to the division time, where when the division time is low, the specific growth rate will be high and vice versa. Microalgae cells will double when entering the logarithmic phase where

microalgae cells will actively divide at a rapid and continuous rate (Amelia et al., 2023). Microalgae species with short cell division times and high specific growth rates will be more efficient because the length of cultivation until harvest can be achieved with a shorter duration (Maghfiroh et al., 2023).

Based on the growth kinetics model in Figure 2, the Logistic model when applied to *Euglena* sp. with nitrogen variation treatment is closer to the microalgae growth curve than the Gompertz model. This suitability is determined based on the coefficient of determination R^2 in each model, where the Logistic model shows the highest R^2 value. Based on previous research conducted by Maghfiroh et al. (2023), the Logistic model and the Gompertz model are two non-linear models that are suitable for the growth of organism populations, especially microalgae. In addition, these two models are considered the simplest models and can be used to describe the growth rate of microalgae in general.

The treatment of low ammonium sulfate levels was able to produce the highest cell growth of *Euglena* sp. compared to the treatment of additional ammonium sulfate concentration. Research conducted by Khanra et al. (2020) states that many microalgae species prefer nitrogen in the form of ammonium and high ammonium concentrations in the growth medium can increase the ability of microalgae to absorb NH_4^+ ions. However, ammonium concentrations that are too high can inhibit the conversion of ammonium into amino acids rather than its entry into cells, which can reduce growth under conditions of high nitrogen concentration. This is also supported by the research of Bakku et al. (2023) that excessive NH_4^+ flux can inhibit ATP formation and photosynthesis regulation, causing ammonium poisoning.

Previous research by Rios et al. (2014) showed that the growth curve in *Desmodesmus* sp. with different nitrogen concentrations in each treatment produced similar growth behavior and there was an increase in the number of cells at 0% nitrogen until the 4th day of cultivation. Another study revealed that the growth rate of *C. vulgaris* was higher in nitrogen-limited media (Agirman and Cetin, 2017). In addition, N additional conditions in the mass cultivation of *E. gracilis* resulted in lower cell density. The N addition and N reduction treatments became similar as the cultivation period progressed and the biomass formed in the low nitrogen treatment became higher. This suggests that *E. gracilis* is able to adjust to the cyclic process of N acquisition under N^- conditions resulting in higher growth rates compared to N^+ conditions (Bakku et al., 2023).

The growth of *Euglena* sp. as shown in Figure 1 has a close connection with dry biomass formation. The biomass of *Euglena* sp. in the F1 treatment group was the highest when compared to other treatments. This treatment contains 0.5 g L^{-1} of ammonium sulfate, compared to 1 g L^{-1} in the control group. The high biomass of *Euglena* sp. in medium with low nitrogen levels is linked to its growth rate. According to Nigam et al. (2011), growth rate is directly related to microalgae biomass because healthy microalgae are more active in converting CO_2 into biomass, resulting in high biomass productivity. At the beginning of the culture phase, the amount of biomass is still low because microalgae are still in the adaptation stage with their environment. After that, the biomass will increase exponentially over time. An exponential increase in biomass implies that microalgae are in a growing phase. During the logarithmic phase, the number of cell divisions increases, resulting in higher cell counts and biomass content (Krishnan et al., 2015).

Previous research states that biomass production is influenced by the concentration of nitrogen given to the culture medium (Menegol et al., 2017). Figure 3 shows that the F1 treatment produced the highest amount of biomass compared to the F2 treatment and the control, so that the F1 treatment was effective in increasing the biomass of *Euglena* sp. According to Hudaidah et al. (2013), intensive environmental pressure, especially in media containing low nitrogen elements, can affect microalgae biomass. The results obtained in this study are supported by the research of Wang et al. (2018), that the algae species *Chlorella* sp. proved to accumulate starch granules and oil after three days in N-deficient conditions, and resulted in an increase in total biomass and energy yield. Biomass results in this study were positively correlated with density (cells/ml), meaning that reducing the concentration of ammonium sulfate has a positive effect on cell density which affects biomass production.

In this study, the lipids produced were influenced by the content of biomass produced, which is in line with research by Griffiths et al. (2011). Lipid productivity is also directly proportional to biomass productivity, where when there is an increase in biomass content, lipid content will also increase. This is evidenced by the results obtained in this study that treatment F1 with low ammonium sulfate concentration produced the highest total biomass so that the resulting lipid content in treatment F1 was also the highest compared to the other two treatments. Meanwhile, lipid productivity or the mass of

lipids produced per unit volume of microalgae per day is also influenced by the growth rate of microalgae (Chisti, 2007).

Lipid content can increase even under nitrogen deprivation conditions because nitrogen depletion causes inhibition of cell division without a gradual decrease in lipid production which results in the accumulation of lipids in cells. This suggests that microalgae are able to adapt metabolic pathways to store large amounts of lipids under nitrogen deprivation conditions (Li et al., 2015). *Euglena* sp. will store the end result of photosynthesis from the Calvin cycle in the form of carbohydrates (paramylon) in the cell as the main additional energy source of the cell which also functions in cell defense against environmental osmotic pressure, can also be stored in the form of lipids when under stress conditions (Pareek et al., 2017).

Microalgae will modify lipid biosynthetic pathways to produce and accumulate neutral lipids, especially in the form of triacylglycerol (TAG) when under stress conditions. The increased content of neutral lipids, especially TAG, is caused by a shift in lipid metabolism from membrane lipid synthesis to neutral lipids or storage lipids (Hu et al., 2008), in other words, the carbon skeleton needed for protein and amino acid synthesis is diverted into carbon and energy sources for TAG biosynthesis (He et al., 2017). In addition, the increased lipid production under stress conditions is also due to the role of lipids as cell protectors because they are compounds that make up the cell membrane (Wardana et al., 2023).

The composition of fatty acids in microalgae is highly dependent on environmental conditions, including the availability of nutrients in the growth medium. According to Chiu et al. (2009), the factors that affect the fatty acid composition in microalgae consist of temperature, CO_2 gas, the addition of stressors such as heavy metals and the composition of the growth medium. Under nitrogen-limited conditions, the proportion of total lipids consisting of TAG increases. Stephenson et al. (2010) mentioned that *C. vulgaris* cultured after 12 days with nitrogen limitation accumulated more than 50% of total lipids. The fatty acid composition of lipids was also reported to change with nitrogen restriction. Previous research by Griffiths et al. (2014) explained that the metabolism of *C. vulgaris* shifted from the production of polyunsaturated fatty acids (C18:2 and C18:3) to the production of saturated or monounsaturated fatty acids (C18:0 and C18:1) under nitrogen deprivation conditions.

Table 2 shows that the composition of fatty acids in *Euglena* sp. contains saturated fatty acids (SFA) which are higher than monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). This is in accordance with the research of Zarrinmehr et al. (2020) that the amount of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) increases when under low nitrogen conditions, while the range of polyunsaturated fatty acids (PUFA) increases under high nitrogen concentration conditions. The fatty acid components in *Euglena* sp. were dominated by arachidic acid (C20:0), heneicosanoic acid (C21:0), and palmitoleic acid (C16:1). The dominating fatty acid components are classified into saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Based on the research of Kottuparambil et al. (2019), lipids in *Euglena* sp. have a composition dominated by C16 methyl esters (42%). In addition, *Chlorella* sp. showed high amounts of saturated fatty acids and monounsaturated fatty acids, consisting of 36.45% stearic acid; 16.78% arachidic acid, 7.02% heneicosanoic acid, and 19.91% linoleic acid (Praveenkumar et al., 2012). Zienkiewicz et al. (2020) using different microalgae also showed that fatty acids belonging to the C16:1 group produced >40% of fatty acids under a concentration of 0 g L^{-1} nitrogen. The differences in fatty acid components in microalgae indicate that differences in treatment of the composition and concentration of nutrients in the culture greatly affect the composition of fatty acids.

Based on Figure 6, methyl arachid, methyl cis-10-heptadecenoate, and methyl cis-5,8,11,14,17-eicosapentaenoate are known to have VIP values greater than (VIP>1.5). Based on the results of significance features, methyl arachid is the most common and significant compound of *Euglena* sp. in nitrogen variation treatment. These saturated fatty acids have very important biochemical roles, including being direct precursors of bioactive lipid mediators such as prostaglandins and leukotrienes. In addition, arachidonic acid also keeps fatty acid liquids pure, even at sub-zero temperatures, and helps provide proper fluidity cell membranes at physiological temperatures (Martin et al., 2016).

The fatty acid composition of *Euglena* sp. will be very influential in the potential of *Euglena* sp. as a source of lipids that can be utilized for biofuel production. According to Demirbas (2010), high-quality biodiesel generally contains saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Saturated fatty acids (SFAs) with chain lengths from C10 to C18 have properties such as low viscosity, higher cetane number, and lower pollutant emissions that make them superior for biodiesel

production. Monounsaturated fatty acids (MUFAs) in the range of e.g. C18:1, also exhibit characteristics suitable for biodiesel production (Suyono et al., 2015).

PUFAs exhibit different characteristics than SFAs and MUFAs, namely high viscosity and low cetane number, which can reduce the quality of biodiesel. These characteristics cause saturated fatty acids and monounsaturated fatty acids to be preferred for use in biodiesel (Knothe, 2013). Through the results obtained, it can also be recognized that cultivation of *Euglena* sp. under nitrogen deficit resulted in lower PUFA and higher SFA. Previous research by Ho et al. (2012) stated that under nitrogen deficiency conditions, the content of C16 or C18 fatty acid groups will increase and dominate the total fatty acids which are indicators of biodiesel quality. Based on Table 2, it is also known that *Euglena* sp. has less EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) content where the highest content of the two fatty acids is 4.75% and 6.11%. Through fatty acid characterization tests in this study, it was found that low ammonium sulfate concentrations produced highly saturated fatty acids suitable for biodiesel production.

In addition to lipid and FAME levels, nitrogen variation treatment can also impact protein content. In Figure 7, the F2 treatment group with ammonium sulfate levels of 2 g L^{-1} has the highest protein content and productivity, which is significant to the control group ($p<0.05$). However, the protein content in F2 is not significant to the F1 group (0.5 g L^{-1}) ($p>0.05$). According to recent research by Tossavainen et al. (2019), a medium with high nitrogen levels can boost the content and productivity of *Euglena* sp. protein, making it an ideal alternative for the production of protein-rich biomass. A common reaction to reduced cellular N content and increased C:N ratio in biomass is a decrease in both protein and non-protein N molecules (Xie et al., 2023).

The study's analysis of the amino acids that comprise the protein in *Euglena* sp. revealed that each treatment had 18 amino acid compounds, with L-Arginine having the greatest amino acid content. Arginine is a semi-essential amino acid obtained from food intake for protein biosynthesis; however, arginine can also be synthesized by the body via the urea cycle mechanism using L-citrulline as a substrate; the synthesis of arginine compounds can aid in wound healing, cell division, and ammonia removal. (Al-Koussa et al., 2020). The PLS-DA analysis of *Euglena* sp. amino acids revealed that eight compounds had a VIP score value more than 1, indicating that these amino acids are the most influential or typical amino acids of *Euglena* sp. (IDN33). The amino acids are L-alanine, L-histidine, L-tyrosine, L-tryptophan, L-aspartic acid, and L-serine. Alanine was the most influential in the F1 treatment group, whereas histidine, tyrosine, and tryptophan were the most influential in the F2 treatment group.

L-alanine, L-histidine, L-tyrosine, and L-tryptophan are amino acids that are needed for a variety of biological functions, including food metabolism and nutrition. L-alanine is a non-essential amino acid that acts as a precursor to the synthesis of other amino acids, including glutamate and aspartate. L-histidine is an important amino acid that aids in the production of histamine. L-tryptophan is an important amino acid that acts as a precursor for the synthesis of serotonin, which is involved in the production of melatonin, a hormone that regulates sleep-wake cycles. L-tyrosine is an important amino acid that acts as a precursor to the synthesis of neurotransmitters such as dopamine, norepinephrine, and epinephrine (Pokorný et al., 2021). L-Alanine, identified as a notable amino acid in this study, is a non-essential amino acid that can be produced from pyruvate, a transient result of glycolysis (Li et al., 2024). Alanine synthesis is facilitated by the enzyme Alanine Aminotransferase (ALT), which transfers the amino group from glutamate to pyruvate, resulting in the formation of alanine (Ingrisano et al., 2023). Alanine production transpires during the vigorous development phase when microalgae must equilibrate energy and nitrogen metabolism.

Conclusion

The variation of ammonium sulfate [$(NH_4)_2SO_4$] to *Euglena* sp. in large scale cultivation (500 L) with variation of 0.5 g L^{-1}, 1 g L^{-1}, and 2 g L^{-1} cultivated over 16 days. The modifications tested had a significant impact on growth, biomass, lipid content, and protein content. *Euglena* sp. grew the best in the F1 treatment group, which was also the group with the most significant biomass. The F1 treatment group exhibited a notable reduction in lipid levels due to fluctuations in ammonium sulfate concentration, whereas the F2 treatment demonstrated a considerable augmentation in protein content. The Fatty Acid Methyl Ester (FAME) content detected was 17 fatty acids, of which methyl heptadecanoate (C17:0) and methyl palmitoleate (C16:1) were the highest concentrations of fatty acids

characterized and belonged to saturated fatty acid (SFA) and monounsaturated fatty acids (MUFA). Nitrogen levels substantially influence the protein content of *Euglena* sp. during large cultivation, with treatment group F2 yielding 11.87% protein, which is 1.16 times greater than that of the control group. The study identified 18 amino acids from *Euglena* sp., with L-Arginine having the highest concentration in the control group and identifying distinctive amino acids like L-Alanine, L-Tyrosine, L-Histidine, L-Tryptophan, L-Aspartic acid, and L-Serine.

Ethical Statement

This study does not require ethical approval as it does not include human or animal subjects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Author Contribution

E.A.S conceptualization , A.N.D. and I.W. collected the data, performed data processing and analysis, and composed the preliminary draft of the manuscript., S.N.Z. and T.A.P. preparation and collected data, D.K., T.E., R.A., and K.Q.M. assisted in formulating research strategies and overseeing the execution of research, R.A.E.P assisted in developing and amending the text in accordance with reviewer suggestions, and contributed to the data analysis. All authors examined, revised, and validated the final paper.

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