Influence of Salinity on The Growth and Fatty Acids Production of *Euglena* sp. Local Strain from Dieng Plateau, Indonesia

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Received: 29 August 2023

Accepted: 22 December 2023

Published: 31 December 2023

Academic Editor:
Dr. Endar Marraskuranto

 Accreditation Number:148/M/KPT/2020. ISSN: 2089-5690, e-ISSN: 2406-9272. https://doi.org/10.15578/squalen.812

Abstract

High salinity is a challenging environmental stressor for organisms to adapt to. In this work, the effects of added NaCl and KCl at various concentrations (0, 100 mM, and 200 mM) for 13 days in the growth medium were investigated in relation to the physiological, morphological, and proximate content of *Euglena* sp. Utilizing gas chromatography (GC), the amount of fatty acid methyl esters (FAMEs) was determined. *Euglena* sp. exhibited an obvious decline in growth rate and photosynthetic pigment with increasing salinity. Biomass, protein, carbohydrates had the highest quantities in KCl 100 mM medium, measuring 0.586 ± 0.096 μg/mL, 0.050 ± 0.00017 μg/mL, and 968.091 ± 81.197 mg/mL, respectively. The treatment with 200 mM NaCl had the highest lipid content, with a lipid concentration of 0.42 ± 0.060 mg/mL. After being cultivated in NaCl and KCl at a 200 mM concentration, respectively, the amount of polyunsaturated fatty acids (PUFAs) declined and the amount of saturated fatty acids (SFAs) increased in *Euglena* sp. The percentage of PUFAs, such as methyl linoleate and methyl linolenate, did not surpass the European B100 biodiesel standard limit of 12% (weight), despite the wide variety of PUFAs. It showed that the use of NaCl and KCl during salt stress significantly increases *Euglena* sp. biofuel production. For this reason, cultivating *Euglena* sp. at high salinity is suitable for producing biofuels.

Keywords: salinity, *Euglena* sp., growth rate, fatty acids, biofuel

Introduction

Microalgae were considered to be a good supply of fatty acids, primarily for aquaculture. Microalgae offers a possible way to address energy and fuel requirements and help achieve significant Sustainable Development Goals (SDGs). For instance, through photosynthesis, microalgae may transform CO_2, the main contributor to greenhouse gases and climate change, into energy (Olabi et al., 2023). A fascinating option for application-driven research and commercialization is *Euglena gracilis*, as one of the most promising species for use as microalgal feedstock for biofuels (Toyama et al., 2019). According to Gissibl et al. (2019), it is a prominent source of dietary protein, pro (vitamins), lipids, and the unique β-1,3 glucan paramylon discovered solely in euglenoids. There are more than 200 species in the genus *Euglena*, of which *E. gracilis* is a model organism with a comparatively high fatty acid content and a great potential source of biodiesel (Chen et al., 2022). However, due to expensive cultivation costs and poor productivity, *Euglena* sp. based biofuel production is still constrained. The conversion of biodiesel, microbial contamination, and expensive and energy-intensive culture are additional obstacles to the generation of microbial bioenergy (Pasha et al., 2021).

Environmental stress factors, such as high temperature, medium nutrient deficiency, light intensity, pH, nitrogen starvation, oxygen concentration, chemical modulator, and salinity, can increase the production of algal lipids. Two of these are the growth and biochemical composition of microalgae that maybe significantly impacted by variations in salinity (Haris et al., 2022). Research by Pandit et al. (2017), states that one of the major factors affecting microalgae development and biochemical composition is salt stress. Salinity stress may enhance the production of algal lipids, according to several studies (Kakarla et al., 2018; Yun et al., 2019; Mirizadeh et al., 2020; Fal et al., 2022). In the community of microalgae, salt stress may slow cell division, reduce size, stop motility, and cause palmelloid development, but some of these

changes are distinctly species-specific (Kholssi et al., 2023).

According to earlier research by Timotius et al. (2022), cultured *Euglena* sp. in 10 g/L NaCl can boost growth and metabolite production for biodiesel and medicinal applications, but studies that specifically use various salt types like NaCl and KCl to boost *Euglena*'s growth and lipid production have never been done before. According to a study by Srivastava et al. (2017), KCl and $MgCl_2$ give microalgae *Chlorella sorokiniana* CG12(KR905186) and *Desmodesmus* GS12(KR905187) structural integrity, which in turn promotes the accumulation of biomass. Contrarily, the addition of NaCl and $CaCl_2$ triggers a variety of metabolite synthesis mechanisms that can be chosen to produce neutral lipids and preserve cellular homeostasis. The current study evaluated the effects of certain salts, NaCl and KCl, on growth, proximate content, pigment content, and fatty acid synthesis under various salt types and concentrations. The *Euglena* used in this study was isolated from the Dieng Plateau, Central Java, Indonesia. This research also prepares local strains as renewable sources for the future from local resources that have the potential to be developed as a biofuel feedstock.

Material and Methods

Microalgae Culture Condition and Growth

The freshwater microalgae strain employed in the study was *Euglena* sp., which was isolated from the Dieng Plateau in Central Java, Indonesia. The experiment was then run from February to April 2023, in a 500 mL bottle with 300 mL Cramer and Myers (CM) medium and 200 mL inoculant with pH 3.5. The initial culture concentration (OD_{680}) was 0.3. The microalgae were grown in varying salinities (0, 100, and 200 mM) of NaCl and KCl, respectively. There were three replicas in each culture group. The culture media was manually agitated twice daily, the temperature was 23 ± 2 °C, and the light intensity was 2100 Lux (MYVO LED Tube 15 watt, Indonesia) with a 24-hour light period. Every morning, the characteristics of *Euglena* sp. were assessed to measure the cell growth of *Euglena* sp. The optical density (OD) at 680 nm was measured using a UV/VIS spectrophotometer (Genesys 150) to measure *Euglena*'s development. The Inverted Biological Microscope (BDS400, China) and Opti Lab Viewer 4 (PT. Miconos, Indonesia) were used to observe the morphology of microalgal cells. The biomass was taken out when it reached its maximum growth (Day 12). The procedure began with the addition of a ten-millilitre sample to a Buchner Funnel vacuum (Amazon) that had been previously lined with Glass Micro Fiber GF/C Filter Paper 1822-047 (Whatman) and rinsed with distillate water. Following that, the harvested biomass was weighed using an analytical balance (AND GR-200, Japan) after drying for 24 hours at 40 °C in an oven (B-ONE OV-65, Indonesia).

Microalgae Growth Kinetic Modeling

The Logistic and Gompertz Models were used to model the growth patterns of *Euglena* sp. According to Phukoetphim et al. (2017) and Hanief et al. (2020), the logistics model was first developed using the following formula (equations 1 and 2), where X is cell density, X_o is initial cell density, X_{max} is maximum cell density, and μ_{max} is maximum specific growth rate.

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

$$X = \frac{X_o \exp(\mu max\ t)}{1 - \left[\frac{X}{X\ max}(1 - \exp(\mu max\ t)\right]} \quad (2)$$

Maximum cell production (r_m) and lag time (t_L) were the variables in the Gompertz model. SST is for sum square total, and SSR stands for sum square residual (Phukoetphim et al., 2017; Hanief et al., 2020). These formulas were used to determine the model.

$$X = Xo + [X_{max}.\exp[-\exp((\frac{rm.\exp(1)}{X_{max}})(t_L - t+1)]] \quad (3)$$

$$R2 = \left(1 - \frac{SSR}{SST}\right) \quad (4)$$

Measurement of Photosynthetic Pigment Content

At the height of growth, the photosynthetic pigments of *Euglena* sp. were extracted and quantified using the technique described by Pruvost et al. (2011). Two millilitres of microalgae culture were concentrated with a centrifuge (Thermo Fisher Scientific MicroCL 21, with dual row 18 x 2.0/0.5mL rotor (DISCONTINUED), Germany) at 12,000 rpm for five minutes, the supernatant was discarded, and the algal pellet was mixed with 99.9% methanol (Merck) before being incubated for 24 hours at 4 °C in the dark. Following incubation, the sample was evaluated using a UV-Vis spectrophotometer (Genesys 150) at wavelengths 480, 652, and 665 nm.

$$[\text{Chl-a}]\ \mu g/mL = -8{,}0962 x A_{652} + 16{,}5169 x A_{665} \quad (5)$$

$$[\text{Chl-b}]\ \mu g/mL = -27{,}4405 x A_{652} - 12{,}1688 x A_{665} \quad (6)$$

$$[\text{Carotenoids}]\ \mu g/mL = 4 x A_{480} \quad (7)$$

For this formula, A stands for absorbance. Similar to other research, the absorbencies at 480, 652, and 665 nm were adjusted by subtracting the absorbance at 750 nm in order to correct the turbidity of the growth media.

Analysis of Proximate Composition

The phenol sulfuric acid method from Dubois et al. (1956) was used to quantify the carbohydrate concentration in the sample. More specifically, 5 mL of sample was concentrated with a centrifuge (MPW-56 with angle rotor type, Berlin) at 4,000 rpm for 10 minutes. The remaining pellet was washed with one millilitre of distilled water after the supernatant had been removed. The material was then centrifuged once more after which the rinsing procedure was carried out twice. The pellet was then treated with 1.5 mL of sulfuric acid (H_2S0_4) (Merck) and 0.5 mL of 5% phenol (Merck). The material was then vortexed and incubated for 30 minutes. Following that, measurements of absorbance at a wavelength of 490 nm were made using a UV-Vis spectrophotometer (Genesys 150). As a standard, glucose was used.

According to Bligh and Dyer (1959), lipid analysis was performed using methanol (Merck) /chloroform (Merck) mixture (2:1 v/v) and then supplemented with chloroform (Merck) /distilled water (1:1 v/v) mixture. After concentrating (MPW-56 with angle rotor type, Berlin), the mixture was incubated until three layers emerged, the bottom layer was taken out and kept at 30 °C for a full day in an oven (B-ONE OV-65, Indonesia). The lipid was then put on an analytical balance (AND GR-200, Japan) and weighed.

$$Total\ lipids(mg/mL) = \frac{Final\ sample weight - Initial\ sample weight}{5\ mL} \quad ...(8)$$

A formula from Bradford (1976) was used to determine the protein content of microalgae cells. The microalgae pellet was first centrifuged with a centrifuge (Thermo Fisher Scientific MicroCL 21, with dual row 18 x 2.0/0.5mL rotor (DISCONTINUED), Germany) at 12,000 rpm for five minutes. A 200 µL of 10% sodium dodecyl-sulfate (SDS) (Chemix, Indonesia) was then added, and the mixture was incubated in a water bath for 30 minutes at 95 °C. Following that, the incubation was maintained at 4 °C for a further five minutes. The sample was then added to 200 µL of Bradford solution (Chemix, Indonesia). Elisa Reader Biotech (Biotek Instruments SN 236114, USA) with a 595 nm wavelength was used to read the absorbance. As a reference, bovine serum albumin (BSA) (Chemix, Indonesia) was employed.

Fatty Acids Methyl Ester (FAME) Analysis

Through the direct transesterification method and Gas Chromatography Flame-Ionization Detector (GCFID-Agilent Technologies 7890B), the fatty acid methyl ester (FAME) concentration of the microalgal biomass was determined following method from Breuer et al. (2013) with minor modification. In a litre bottle, the sample with the highest amount of lipids was cultivated. At the end of the experiment, a sample from each test was taken, and the algae and bulk water were separated by centrifuging the sample with a centrifuge (Hettich Zentrifugen Universal 320 R, Swing-out rotor, Germany) for five minutes at 4000 rpm. The particle was then washed with 50 mL of distilled water after the supernatant had been removed. The second centrifugation of the material followed by three rinses was performed. Following that, the sample was hydrolyzed using 10 mL of concentrated HCl. then heated for three hours at 80 °C, and then cooled to ambient temperature (25 °C). Next, 25 mL of diethyl ether and petroleum ether (1:1 v/v) were used to extract the sample. The top layer was removed after being determined to be oil. The sample is then evaporated using N_2 gas in a water bath. The sample was then methylated by putting 0.5 mL of oil, and 1.5 mL of methanolic sodium solution, heated at 60 °C for 5–10 minutes through shaking, and chilled at room temperature (25 °C) in a small, closed test tube. After that, 2 mL of boron trifluoride methanoate was added and incubated at room temperature (25 °C) after heating for 5–10 minutes at 60 °C. Finally, 1 mL of heptane and 1 mL of saturated NaCl were used to extract the material. The top layer was put in the GC vial and 1 µL of the sample was injected into the GC (7890B Agilent Technologies, Column HP-88).

Statistic Evaluation

Data were analyzed using Microsoft Excel and presented as mean SE. One-way ANOVA is used to examine the significant levels at a 0.05 significance level. Tukey's and Duncan's post-hoc tests were used to compare the means numerous times in order to identify trends and the pattern of data distribution.

Results and Discussion

The growth rate of *Euglena* sp. was measured using optical density ($OD_{680\ nm}$). The highest growth rate was obtained in the control treatment, followed by KCl 100 mM, NaCl 100 mM, KCl 200 mM, and NaCl 200 mM with sequential values of 0.73 ± 0.12, 0.69 ± 0.059, 0.58 ± 0.015, 0.45 ± 0.077, and 0.43 ± 0.024 throughout the 13 days cultivation (Figure 1). Up until

day 12, the number of cells increased. On day 13, however, it declined. *Euglena* sp. shows a decreased algal growth with increasing salinity for both NaCl and KCl in its growth curve at various NaCl and KCl concentrations. All of the cultures reached the stationary phase on the fifth day until the eighth day, although there were slower growth in the salt-treated cultures.

Euglena sp. is a species of the *Euglena* genus that was successfully isolated in difficult conditions, according to Erfianti et al. (2023). A significant environmental issue limiting plant growth and productivity is salt stress (Yang & Guo, 2018). Treatment with salinity has an impact on *Euglena* sp. growth. As salinity rose, *Euglena* sp. grew less quickly. When the culture of *Euglena* sp. treated with 5 g/L of NaCl has the highest specific growth rate, with values of 0.678 ± 0.479/day. The environment culture with a salt treatment of 5 g/L NaCl is tolerated and may be adjusted well. The cultures exposed to salinities of 10 and 20 g/L NaCl showed the lowest specific growth rate (Indahsari et al., 2022). However, the harsh cultivation conditions cause algae to undergo programmed cell death (PCD), which hinders their ability to grow and produce as much (Elloumi et al., 2020). When there is an imbalance of ions, it disrupts the dynamic equilibrium between reactive oxygen species (ROS) generation and consumption, cell death occurs (Ji et al., 2018).

It was crucial to comprehend microalgal growth and development. As a result, growth kinetic models are needed. Figure 2 illustrates the usage of Gompertz and logistic models in this investigation. In the Gompertz model, the control treatment had the highest cell production rate (rm) of 0.04 cells/ mL. The lag time values (tL) sequentially for control, NaCl 100 mM, NaCl 200 mM, KCl 200 mM, and KCl 200 mM were 0.41/day, 0.09/day, 0.20/day, 0.78/day, and -1.10/day, respectively. Furthermore, the highest R^2 error value for the control was 0.88. Using logistic modelling, it was shown that the maximal specific growth rates (μmax) for the control, NaCl 100 mM, NaCl 200 mM, KCl 100 mM, and KCl 200 mM were 0.29/day, 0.31/ day, 0.31/day, 0.23/day, and 0.33/day, respectively.

The highest R^2 error value was present on the control treatment with values of 0.91. Figure 2 demonstrates that the Logistic model, when applied to microalgae under salt stress, more closely approximates the microalgae growth curves than the Gompertz model. In the study, the goodness of fit of the Logistic model

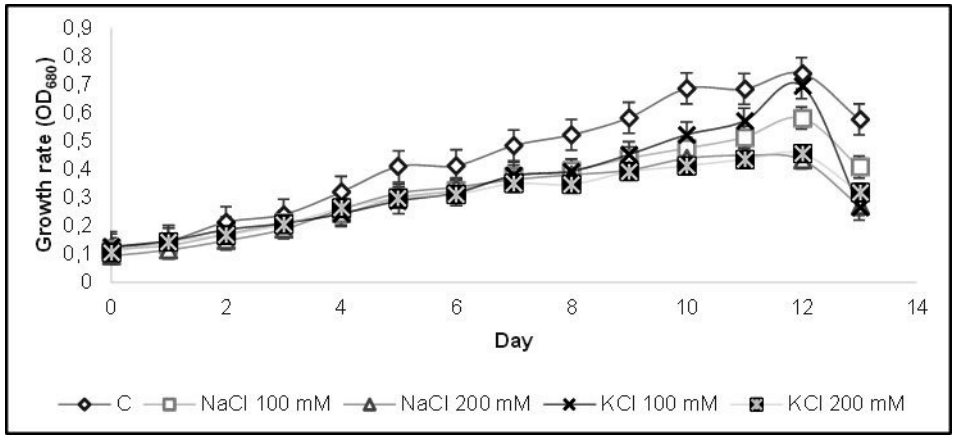


Figure 1. Salinity's impact on *Euglena* sp. growth rate.

over the Gompertz model was determined by the coefficient of determination R^2 values.

Since these models were previously used for species with fast growth in populations, the logistic and Gompertz nonlinear models were selected for this investigation (Soni et al., 2017). Previous research by Nur et al. (2023) shows that the Gompertz model for three species of microalgae (monoculture Glagah, mixed culture Glagah, and *Euglena* sp., and monoculture *Euglena* sp.) matches the growth curves more accurately than the Logistic model. The Gompertz model was found to fit better than the logistic model based on the values of the coefficient of determination (R^2). This suggests that depending on the species and treatment used, different organisms have different optimal growth models.

Compared to the microscopic representation of algal cells following salt treatment (Figure 3). At the end of the observation, microscopic photographs were taken with a 40-times magnification. Details of algal morphology were revealed in the minuscule images magnified 40 times. The shape of the control algal sample was spindle. In addition, a spindle format was also observed after treatment with KCl 100 mM and

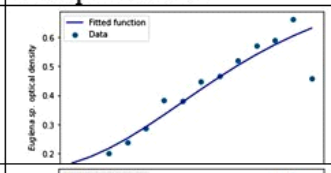
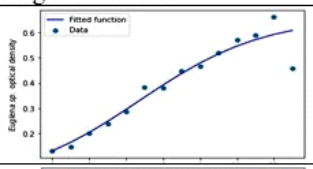
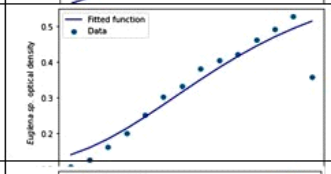
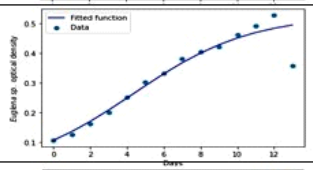
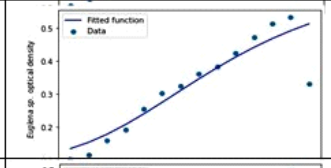
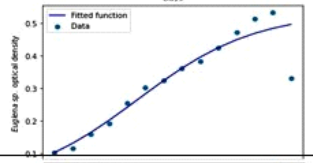
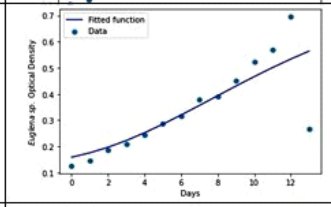
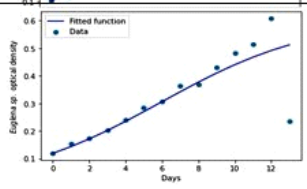
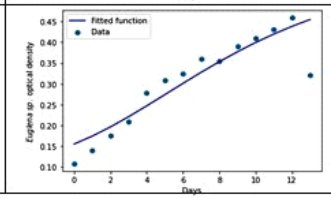
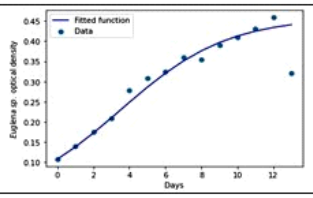
Salt type cons.	Gompertz Model	Parameter	Logistic Model	Parameter
Control		rm: 0.04 tL: 0.41 R^2: 0.88		μmax: 0.29 R^2: 0.91
NaCl 100 mM		rm: 0.03 tL: 0.09 R^2: 0.87		μmax: 0.31 R^2: 0.90
NaCl 200 mM		rm: 0.03 tL: 0.20 R^2: 0.83		μmax: 0.31 R^2: 0.87
KCl 100 mM		rm: 0.03 tL: 0.78 R^2: 0.66		μmax: 0.23 R^2: 0.66
KCl 200 mM		rm: 0.02 tL: -1.10 R^2: 0.83		μmax: 0.33 R^2: 0.89

Figure 2. Fitting of Gompertz and Logistic Model (Cell Density) on the growth of *Euglena* sp. after salinity treatment.

NaCl 100 mM. An increased salinity causes cells to shorten, condense, and take a spherical shape (NaCl 200 mM and KCl 200 mM).

Microalgae's reactions to different salinities can also be a reflection of the cellular stress brought on by the cells' short-term salinity stress, which can cause a variety of morphological and biochemical changes (Borowitzka, 2018). The Na+/K+ pump's activity, which also contributes to the formation of osmotic potential, affects turgor pressure and cell volume (Erdmann & Hagemann, 2001). Increased salt (either NaCl or KCl) induced a considerable color change and cell expansion. Under KCl and NaCl stress conditions, algal cell diameters increased by around five times (Janchot et al., 2019). In response to stress conditions, the *E. gracilis* cells displayed morphological changes, and commonly rounded cells were seen (Azizullah et al., 2012; Peng et al., 2015). This could be an adaptation to harsh conditions. The microalgal cell wall is particularly important in these types of environments because it is the first barrier in constantly interacting with the surrounding, ever-changing environment (Gonzales-Haurcade et al., 2023).

Figure 4 demonstrates KCl was successful in raising the dry cell weight of microalgae *Euglena* sp. The concentration of dry cell weight that was determined to be greatest was 0.586 ± 0.096 mg/mL in KCl 100 mM, which was 1.2 times higher than the control (0.466 ± 0.125 mg/mL). The growth of the microalgae *Euglena* sp. was suppressed by the presence of NaCl 200 mM and KCl 200 mM, and as a result, the dry cell weight was drastically decreased.

According to Fal et al. (2022), one of the most important environmental factors limiting the production of microalgal biomass is salinity. According to other research, *Scenedesmus almeriensis* survived in low salinities of between 0 and 5 g/L of NaCl concentrations. At a salinity of 5 g/L NaCl, it was able to produce the highest levels of lutein content and biomass production (Romanenko et al., 2017). Despite the limitation in growth rate, microalgae also demonstrated notable biomass accumulation. Under salinity stress, compared to the control, the cell and its organelles (such as the chloroplast, mitochondria, and vacuole) are larger and heavier (Sinetova et al., 2021). This may occur as a defense mechanism and aid in maintaining cellular functions under salinity stress.

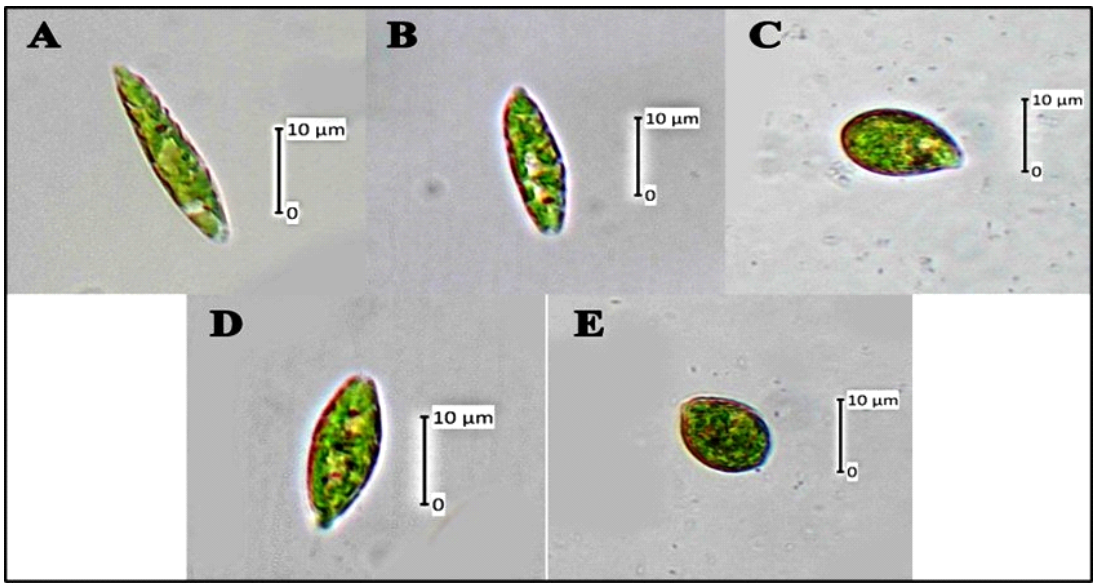


Figure 3. Morphology of *Euglena* sp. after salinity treatment. (A) Control (B) NaCl 100 mM (C) NaCl 200 mM (D) KCl 100 mM (E) KCl 200 mM.

Table 1 displays the variance of pigment content in *Euglena* sp. species that were cultivated at various starting NaCl and KCl concentrations. With NaCl 200 mM and KCl 200 mM, *Euglena* sp.'s levels of chlorophyll-a, chlorophyll-b, chlorophyll a+b, and carotenoid content were significantly decreased. This was also evidently apparent from the fact that the color of the culture solution turned yellow. The control, NaCl 100 mM, and KCl 100 mM, turn dark green in contrast. The highest content of chlorophyll-a, chlorophyll-b, chlorophyll a+b, and carotenoid was found in the control treatment with values of 9.727 µg/mL, 1.160 µg/mL, 10.888 µg/mL, and 1.589 µg/mL, respectively. The KCl treatment had more chlorophyll-a, chlorophyll-b, chlorophyll a+b, and carotenoids than the NaCl treatment did. In cultures containing NaCl 100 mM or more and KCl 100 mM or more, the ratio of chlorophyll a/b or carotenoid/total chlorophyll considerably

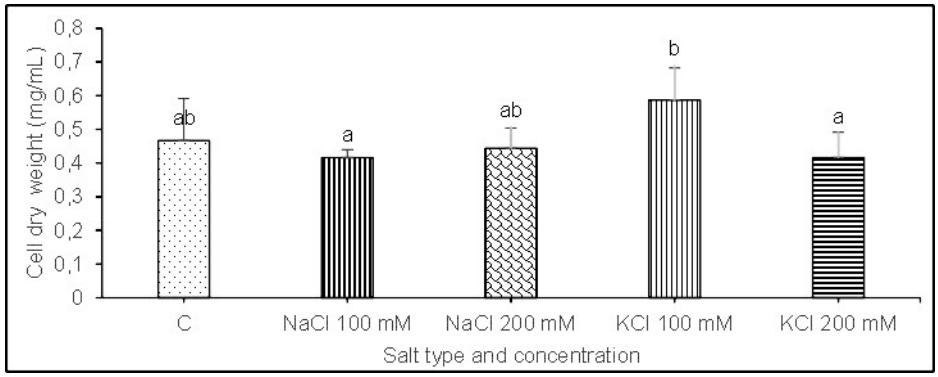


Figure 4. Cell dry weight of *Euglena* sp. after salinity treatment. (Different lowercase letters with every bar indicate significant differences according to Duncan's multiple range test at $p < 0.05$ between treatment concentrations.)

increased, which may serve as a defensive mechanism to stop photooxidative damage under stressful circumstances. Chlorophyll a/b ratio contents increased, but there was no difference between treatments that was significant.

The primary photosynthesizers on earth are microalgae, which also offer potential supplies of natural colorants such as lutein, astaxanthin, and β-carotene, as well as chlorophylls and phycobiliproteins. Carotenoids generated from microalgae are produced industrially (Garcýa-Vaquero et al., 2021). Due to their strong antioxidant and photoprotective abilities, carotenoids are crucial for all photosynthetic organisms (Yao et al., 2022). In photosynthetic species, chlorophyll-a and b are naturally present. Green algae and terrestrial plants both use chlorophyll-a and

Table 1. The effect of different salt types and concentrations on pigment content of *Euglena* sp. (Different superscript letters in the column indicate significant differences according to Duncan's multiple range test at $p < 0.05$)

Salt type con. (mM)	Chlorophyll-a (µg/mL)	Chlorophyll-b (µg/mL)	Chlorophyll a + b (µg/mL)	Carotenoid (µg/mL)	Chlorophyll a/b	Carotenoid/Chlorophyll a + b
0	9.727 ± 1.763 ^{b}	1.160 ± 0.274 ^{b}	10.888 ± 2.035 ^{b}	1.589 ± 0.222 ^{b}	8.459 ± 0.496 ^{a}	0.147 ± 0.008 ^{ab}
NaCl 100 mM	6.185 ± 3.647 ^{ab}	0.795 ± 0.297 ^{ab}	6.980 ± 3.943 ^{ab}	1.199 ± 0.498 ^{ab}	7.174 ± 2.449 ^{a}	0.193 ± 0.055 ^{b}
NaCl 200 mM	4.424 ± 0.541 ^{a}	0.502 ± 0.052 ^{a}	4.926 ± 0.580 ^{a}	0.729 ± 0.097 ^{a}	8.819 ± 0.723 ^{a}	0.149 ± 0.018 ^{ab}
KCl 100 mM	9.024 ± 1.574 ^{b}	1.056 ± 0.227 ^{b}	10.080 ± 1.800 ^{b}	1.203 ± 0.215 ^{ab}	8.608 ± 0.443 ^{a}	0.119 ± 0.002 ^{a}
KCl 200 mM	4.650 ± 0.890 ^{a}	0.510 ± 0.060 ^{a}	5.160 ± 0.945 ^{a}	0.705 ± 0.097 ^{a}	9.078 ± 0.926 ^{a}	0.138 ± 0.011 ^{a}

chlorophyll-b as significant components of their photosynthetic systems. For their photosynthesis, chlorophyll-b is not required, but chlorophyll-a is necessary for photochemistry. The primary light-harvesting chlorophyll-binding proteins, however, require chlorophyll-b to be stabilized (Tanaka & Tanaka, 2011).

Salt stress adversely impacts *Chlamydomonas reinhardtii*'s carbon fixation and carbon concentrating processes, which are necessary for Rubisco to have access to CO_2 (Hounslow et al., 2021). Additionally, cells collect more ROS when exposed to salinity-induced oxidative stress; growth is inhibited and the amount of photosynthetic pigments is decreased when NaCl concentrations are raised to 0.4 M. (Romanenko et al., 2017). Other research from Kanna et al. (2021) showed that the highest salt stress had a negative impact on the cell growth of *E. gracilis* and resulted in a drop in the amount of chlorophyll. The lower concentration of chlorophyll may be an indication of changes in the photosynthetic machinery under salt stress. In our study, the addition of KCl resulted in better pigment content compared to NaCl, it might be because the ion Na^+ interacts with the cell membrane more strongly than the ion K^+, then produces an extracellular hypertonic condition. Due to this circumstance, water molecules leak out of the cell, causing it to contract and eventually harming both the cell and its constituent parts (Suyono et al., 2015). This causes growth inhibition in NaCl treatment than in KCl treatment, so the photosynthetic activity was also hampered. This relates to the growth curve of *Euglena* sp. in Figure 1.

The proximate composition of *Euglena* sp. following treatment with various salt types and concentrations is displayed in Table 2. The KCl 100 mM treatment had the highest protein content, followed by the NaCl 200 mM, control, KCl 200 mM, and NaCl 100 mM treatments. The maximum protein content in the NaCl salt treatment was detected at the highest concentration of 200 mM, whereas the highest protein content in the KCl salt treatment was detected at the concentration of 100 mM. However, the protein content generally tends to rise with increasing salt.

In general, the effects of salinity level on growth and biochemical composition depend on the species and the type of salt, respectively. It might occur as a result of the systems for acquiring nutrients being separated from those for fixing carbon (Frank & Dubinsky, 1999). Under hypo- or hypersaline circumstances, photosynthetic organisms net rates of photosynthesis and carbon fixation typically decrease (Kirst, 1989; Reed et al., 1980), although nitrogen uptake rates mostly stay unaltered (Lartigue et al., 2003) or even rise. As a result, there is a higher concentration of proteins in the biomass as compared to other components (Roy et al., 2014). From other research, known that *Tetraselmis chuii* had considerably higher protein content after grown at 30 ppt and 40 ppt, with values of 34.42 ± 4.51 and 40.47 ± 0.51 % of dry weight, respectively. With rising salinity at 10 ppt, 20 ppt, 24 ppt, 30 ppt, and 40 ppt, the total protein content shows a modest tendency to rise (Haris et al., 2022).

Along with the rise in salinity, the amount of carbohydrates also increased. Cells grown with KCl 100 mM had the highest concentration of carbohydrates, with values of 968.091± 87.197 mg/mL. However, there were no significant differences ($p<0.05$) in other treatments. In the current investigation, a relationship between growth rate, dry cell weight, and biochemical composition (protein and carbohydrate) was discovered. The effects of salt stress on the productivity of the biofuel-producing *Euglena* sp. were examined using lipid analysis at the end of the growth peak (day 12). The highest concentration of lipids produced in CM medium was 0.420 ± 0.060 mg/mL in NaCl 200 mM, followed by 0.393 ± 0.117 mg/mL in KCl 200 mM. In *Euglena* sp., a rise in salinity results in an increase in lipid content.

High salinity will cause osmotic stress. To endure significant osmotic shock, cells produce a variety of low molecular weight organic osmolytes under salinity stress, such as proline and glycine betaine. Additionally, in conditions of high salinity, cell respiration activity rises, which leads to the breakdown of energy-dense storage materials like lipids and starch in microalgae

Table 2. Proximate composition of *Euglena* sp. grown on different salinity treatments. (Different superscript letters in the column indicate significant differences according to Duncan's multiple range test at $p < 0.05$).

Salt type and concentration	Protein (µg/mL)	Carbohydrate (mg/mL)	Lipid (mg/mL)
Control	0.049 ± 0.026^{a}	777.846± 77.353^{a}	0.260± 0.034^{a}
NaCl 100 mM	0.049 ± 0.014^{a}	729.562± 38.081^{a}	0.286± 0.046^{ab}
NaCl 200 mM	0.049 ± 0.032^{ab}	635.131± 82.341^{a}	0.420± 0.060^{c}
KCl 100 mM	0.050 ± 0.017^{b}	968.091± 87.197^{b}	0.366± 0.041^{abc}
KCl 200 mM	0.049 ± 0.028^{b}	756.629± 45.701^{a}	0.393± 0.117^{bc}

(Yao et al., 2013). To maintain homeostasis and ensure osmotic adjustment under salt stress, microalgae utilize carbohydrates as osmoprotectants (Wang et al., 2018; Tietel et al., 2019). According to a study by Fal et al. (2022), in 200 mM NaCl circumstances, *C. reinhardtii* also showed an increase in carbohydrate and lipid accumulation as cell storage molecules to ensure microalgal survival.

For microalgae to survive in salt-stress conditions, lipid content in microalgae may increase under stressful conditions. Microalgae will alter their fatty acid metabolism in response to nutrition stress in order to produce and accumulate triacylglycerols, which can make up as much as 80% of a cell's total lipid content (Ratomski & Hawrot-Paw, 2021). The impact of nitrogen (N) deficiency on lipid content is amplified by salt. If N is depleted in the presence of salt, the salinity stress may impede the growth of cellular components and increase lipid synthesis (Shen et al., 2015). Because the ion Na^{+} interacts with the cell membrane more powerfully than the ion K^{+} in the current investigation, NaCl 200 mM was determined to have the highest concentration of lipids. To maintain cell homeostasis, it causes cells to create more lipids.

Fatty acids from *Euglena* sp. were esterified at the end of each test, and the main fatty acid compositions were identified using GCFID (Table 3). The most prevalent compounds in both treatment were methyl myristate (C14:0), methyl palmitate (C:16), methyl cis-10-heptadecenoate (C17:1), and methyl linoleate (C18:2). The treatment KCl 200 mM resulted in the highest concentration of saturated fatty acids (36.82%), followed by the treatment NaCl 200 mM (36.53%) and the control (32.02%). The amount of polyunsaturated fatty acids (PUFAs) and mono-unsaturated fatty acids (MUFAs) decreased at treatment of NaCl 200 mM and KCl 200 mM. According to fatty acids methyl ester (FAME) profile results, *Euglena* sp. cultivated in high salinity produced more saturated fatty acids and fewer unsaturated fatty acids (UFA), which led to higher-quality lipids for biodiesel generation than *Euglena* sp. grown in low salinity.

In response to salt stress, neutral lipid accumulation plays a function in maintaining membrane integrity by lowering osmotic pressure and fluidity in cell membranes (Ji et al., 2018). Particularly in the membranes of organelles, polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) function as membrane monomers. Then, multiple research revealed how essential unsaturated fatty acids are for salt stress adaptation and tolerance by defending the plasma membrane and the photosynthetic apparatus (Rismani & Shariati, 2017). According to the results of the current study, the range of mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFAs) dropped with high salinity, while the amount of saturated fatty acids (SFAs) increased. However, a wide range of PUFAs were discovered in our study, with a methyl linoleate level of 12% or less. The European B100 biodiesel standard (EN 14214) sets a limit of 12% (wt) for components containing allylic and, in particular, bis-allylic double bonds in the FAME profile (Islam et al., 2013). Due to the increased viscosity of the fuel and the creation of gums, sedimentation, and engine deposits, as well as the degree of unsaturation of biodiesel FAMEs, poor oxidative stability is a key biodiesel criterion (Barabás & Todoruþ, 2011; Hoekman et al., 2012).

Conclusion

The freshwater microalga *Euglena* sp. is a potential source of feedstock for the generation of biofuels. More SFA and less UFA were produced by *Euglena* sp. grown in high salinity, resulting in higher-quality lipids for the production of biodiesel. Changes in physiology, morphology, and biochemistry can be induced by cultivating *Euglena* sp. in high salinity using NaCl and KCl. For instance, salinity stress affected a decrease in growth rate and photosynthetic pigment (chlorophyll-a, chlorophyll-b, secondary carotenoid). Additionally, salt stress changed how cells functioned, leading to an increase in biomass and the build-up of storage molecules such as carbohydrates, proteins, and lipids.

Table 3. Fatty acids methyl esters (FAME) produced by *Euglena* sp. grown in salinity treatment.

No	Parameter	C. No	Control	NaCl 200 mM	KCl 200 mM
1	Methyl decanoate	**C10:0**	**<0.1**	**0.21**	**<0.1**
2	Methyl undecanoate	**C11:0**	**<0.1**	**0.38**	**0.20**
3	Methyl laurate	**C12:0**	**1.06**	**1.16**	**1.02**
4	Methyl tridecanoate	**C13:0**	**2.62**	**5.57**	**4.90**
5	Methyl myristate	**C14:0**	**8.22**	**9.01**	**9.00**
6	Methyl pentadecanoate	**C15:0**	**1.23**	**2.62**	**2.48**
7	Methyl cis-10-pentadecenoate	**C15:1**	**1.93**	**1.62**	**1.43**
8	Methyl palmitate	**C16:0**	**11.97**	**9.35**	**10.15**
9	Methyl palmitoleate	**C16:1**	**1.53**	**2.28**	**2.11**
10	Methyl heptadecanoate	**C17:0**	**4.18**	**4.09**	**5.57**
11	Methyl cis-10-heptadecenoate	**C17:1**	**10.14**	**9.02**	**10.04**
12	Methyl stearate	**C18:0**	**<0.1**	**0.65**	**0.76**
13	Methyl elaidate	**C18:1**	**0.88**	**5.30**	**5.01**
14	Methyl oleate	**C18:1**	**10.85**	**1.43**	**1.67**
15	Methyl linolelaidate	**C18:2**	**3.38**	**5.17**	**5.95**
16	Methyl linoleate	**C18:2**	**15.79**	**12.70**	**12.69**
17	Methyl arachidate	**C20:0**	**0.12**	**0.14**	**0.25**
18	Methyl linolenate	**C18:3**	**11.89**	**10.38**	**9.72**
19	Methyl heneicosanoate	**C21:0**	**1.34**	**2.16**	**1.70**
20	Methyl cis-11,14-eicosadienoate	**C20:2**	**1.19**	**0.28**	**1.98**
21	Methyl docosanoate	**C22:6**	**<0.1**	**1.82**	**<0.1**
22	Methyl cis-8,11,14-eicosatrienoate	**C20:3**	**2.05**	**0.31**	**0.31**
23	Methyl cis-11,14,17-eicosatrienoate	**C20:3**	**<0.1**	**1.31**	**0.95**
24	Methyl erucate	**C22:1**	**<0.1**	**0.81**	**0.47**
25	Methyl cis-5,8,11,14-eicosatetraenoate	**C20:4**	**3.16**	**3.19**	**3.90**
26	Methyl tricosanoate	**C23:0**	**0.41**	**0.71**	**0.65**
27	Methyl cis-13,16-docosadienoate	**C22:2**	**0.93**	**1.10**	**0.99**
28	Methyl cis-5,8,11,14,17-eicosapentaenoate	**C20:5**	**2.25**	**2.92**	**2.84**
29	Methyl lignocerate	**C24:0**	**0.87**	**0.48**	**0.14**
30	Methyl nervonate	**C24:1**	**0.28**	**1.79**	**1.36**
31	Methyl cis-4,7,10,13,16,19-docosahexaenoate	**C22:6**	**1.73**	**2.03**	**1.77**
SFA			**32.02**	**36.53**	**36.82**
MUFA			**25.61**	**22.25**	**22.09**
PUFA			**42.37**	**41.22**	**41.09**
%Relative Area			**100.00**		

Acknowledgments

The authors thank Ministry of Research, Technology, and Higher Education Indonesia for the scholarship of this study with the grant number 122/E5/PG.02.00.PL/2023; 3079/UN1/DITLIT/DitLit/PT.01.03/2023. This manuscript is a part of the first author's dissertation.

Supplementary Materials

Supplementary materials is not available for this article.

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