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Research article

The Effect of Tofu Wastewater and pH on the Growth Kinetics and Biomass Composition of *Euglena* sp.

Angga Puja Asiandu[1], Andhika Puspito Nugroho[1], Ahmad Saifun Naser[1], Brilian Ryan Sadewo[2], Mochamad Donny Koerniawan[3], Arief Budiman[4], Ulfah Juniarti Siregar[5], Lucia Tri Suwanti[6] and Eko Agus Suyono[1]*

[1]*Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[2]*Center of Excellent for Microalgae Biorefinery, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[3]*School of Architecture, Planning, and Policy Development, Institut Teknologi Bandung, Bandung, Indonesia*
[4]*Faculty of Engineering, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[5]*Faculty of Forestry, Institut Pertanian Bogor, Bogor, Indonesia*
[6]*Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia*

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Abstract

Media and pH are two crucial factors in microalgal cultivation. Industrial wastewater such as tofu wastewater can be utilized as alternative media for growing microalgae like *Euglena* sp. to produce biomass as feedstock in biorefinery activities. Here, we evaluated combinations of tofu wastewater (L) consisting of 0% (L_1), 75% (L_2), and 100% (L_3) with pH (P) levels consisting of 5.0 (P_1), 5.5 (P_2), 6.0 (P_3), 6.5 (P_4), and 7.0 (P_5). The analyses were carried out on the growth kinetics, biomass, primary metabolite compounds, and pigments of *Euglena* sp. Based on the study, the combinations with the highest cell density, biomass, maximum carbohydrate content, maximum lipid content, and protein content were L_2P_2 (23.13×10^5 cells/mL), L_2P_1 (4.53±0.17 mg/mL), L_1P_5 (0.93±0.02 mg/mL), L_2P_1 (1.27±0.11 mg/mL), and L_3P_4 (256±26.86 ppm), respectively. Moreover, the combinations with the highest chlorophyll-a, chlorophyll-b, and carotenoid were L_2P_4 (33.53±0.13 mg/L), L_2P_2 (17.73±0.50 mg/L), and L_2P_2 (11.65±0.00 mg/L), respectively. The addition of tofu wastewater combined with specific pH level enhanced the growth and biomass composition of *Euglena* sp. ($P<0.05$), with the exception of carbohydrate content. Additionally, each biochemical component of *Euglena* sp. had a different optimum combination of tofu wastewater and pH level. However, this wastewater can potentially be used as an alternative medium for cultivating this microalga in order to cut the production costs of biorefinery activity.

*Corresponding author: (+62) 81328765344 Fax: (+62) 274-439517
E-mail: eko_suyono@ugm.ac.id

1. Introduction

The supply of fossil fuel energy is decreasing due to the release of various kinds of pollutants into the environment. One way to overcome this problem is to produce renewable green energy using microalgae as a potential source of biodiesel. Microalgae contain high amounts of lipids. They grow rapidly and are easy to cultivate [1] using domestic and industrial wastewater [2].

One potential microalga for the use in the production of lipids as raw materials for biodiesel is *Euglena* sp. The lipids produced by this microalga include palmitic, linolenic, and linoleic fatty acids. *Euglena* sp. can accumulate a relatively high lipid content, Furthermore, the biomass production of *Euglena* sp. can also be integrated with the recovery of waste from the environment through the phycoremediation process [2]. Additionally, *Euglena* sp. produces carbohydrates stored as paramylon. These compounds show potential to be used as biofuel feedstock. Another cell compound of *Euglena* sp. is protein. *Euglena gracilis* contains up to 33% protein [3]. *Euglena* sp. can also be used as a pigment source. The primary photosynthetic pigments found in *Euglena* sp. are chlorophyll-a and chlorophyll-b. This microalga also contains accessory pigment carotenoids [4].

Tofu is a food made from soybeans by utilizing soy milk protein coagulated by a certain coagulant. The gelatinization process denatures soybean milk protein, forming a solid structure. [5]. Indonesia is a massive tofu producer. Tofu production releases wastewater into the environment. It is an organic waste containing various kinds of nutrients [6] that are essential to support microalgal growth such as protein, carbohydrate, and lipid. Organic waste can also be a mineral source for plant and microalgal growth [7, 8]. The effect of adding tofu wastewater for microalgal lipid production was reported by Elystia *et al*. [9]. The addition of tofu wastewater increased lipid production in *Scenedesmus* sp. [9]. Widayat and Hadiyanto [10] also reported that the lipid content in *Nannochloropsis* sp. was increased by as much as 34.25% due to the addition of 20% tofu wastewater into the media. Thus, it potentially can be used as an alternative media for the growth of *Euglena* sp. in order to reduce production costs [11].

Euglena gracilis was previously shown to have respiratory activity at pH 5 and 6 with an optimum growth rate at pH 7 [12]. In this study, we examined the effect of combinations of tofu wastewater and pH on the growth rate, biomass, carbohydrates, lipids, protein, and pigmentation of *Euglena* sp. The data were statistically analyzed using Friedman's two-way ANOVA by rank at a 95% confidence level followed by Bonferroni multiple pairwise comparisons.

2. Materials and Methods

2.1 Experimental design and cultivation

The first stage of the study was determining the optimum concentration of tofu wastewater. Wastewater was taken directly from the tofu processing industry located around the Colombo Market, Sleman, Yogyakarta, Indonesia, using a container, then directly transferred to the laboratory and processed in a pretreatment process. Compounds in the wastewater were prior analyzed using proximate analysis by the Center for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia. Based on the analysis, the wastewater composition was water, ash, lipid, protein, and carbohydrate at 99.39%, 0.12%, 0.04%, 0.21%, and 0.30%, respectively. Meanwhile, the tofu wastewater was filtered with a Whatman filter paper (11 microns) and chloramphenicol at a concentration of 35 µg/mL was added before being used to cultivate *Euglena sp.*

In the optimization stage, filtered tofu wastewater samples (0%, 25%, 50%, 75%, and 100% v/v) were added to sterilized Cramer's Myers (CM) medium to a final volume of 450 mL (pH=5.5)

[13]. Then, six-day-old *Euglena* sp. was added to the media at 10% of the final volume [14] and cultivated for 10 days at 28-30ºC with two replications. The observed parameter at this stage was the cell density of *Euglena* sp.

In the second stage, the optimum concentration of tofu wastewater was selected and combined with pH. Tofu wastewater concentrations (L) consisting of 0% v/v (L1, as control), 75% v/v (optimum concentration/L2), and 100% v/v (L3, as control) were added to the CM medium. Meanwhile, the final pH (P) of each medium was 5.0 (P1), 5.5 (P2), 6.0 (P3), 6.5 (P4), and 7.0 (P5) (modified from Danilov and Ekelund [12]) (Table 1). Then, six-day-old *Euglena* sp. was added to each medium at 10% of the final volume [14] and cultivated for 10 days with two replications. Observed variables in the second stage were cell density, growth rate, biomass, lipid content, carbohydrates, protein, chlorophyll-a, chlorophyll-b, and carotenoids.

Table 1. Combinations of tofu wastewater concentrations and pH levels

Concentrations		**pH levels**				
CM Media	**Tofu Wastewater**	**P_1 (5.0)**	**P_2 (5.5)**	**P_3 (6.0)**	**P_4 (6.5)**	**P_5 (7.0)**
L_1 (100% v/v)	L_1 (0% v/v)	L_1P_1	L_1P_2	L_1P_3	L_1P_4	L_1P_5
L_2 (25% v/v)	L_2 (75% v/v)	L_2P_1	L_2P_2	L_2P_3	L_2P_4	L_2P_5
L_3 (0% v/v)	L_3 (100% v/v)	L_3P_1	L_3P_2	L_3P_3	L_3P_4	L_3P_5

2.2 Growth estimation of *Euglena* sp.

The cell density of *Euglena* sp. was estimated using a haemocytometer. The formulas for determining the number of cells [15] and specific growth rate [16] were as follows (equation 1 and equation 2), where μ is the specific growth rate (/day), Xt is the cell density at t time, X_0 is the initial cell density, and t is time (day).

$$\text{Cell density (cell/mL)} = \frac{Number\ of\ cells\ counted\ in\ 5\ corners}{5} 25.10^4 \quad (1)$$

$$\mu = \frac{\ln Xt - \ln Xo}{tx - to} \quad (2)$$

2.3 Growth kinetics modeling

Growth kinetics modeling of *Euglena* sp. was performed using the Logistic and Gompertz model. The Logistics model was calculated using the following formula (equation 3 and equation 4), where X is cell density, X_0 is the initial cell density, X_{max} is the maximum cell density, and μ_{max} is the maximum specific growth rate [17, 18].

$$\frac{dx}{dt} = \mu_{max}\left(1 - \frac{x}{\mu_{max}}\right)x \quad (3)$$

$$x = \left(\frac{Xo.\exp(\mu_{max}.t)}{1-[(\frac{Xo}{X_{max}})(1-\exp(\mu_{max}.t)]}\right. \quad (4)$$

In the Gompertz model, the parameters were maximum cell production (r_m) and lag time (t_L). The determination of the model was carried out using the following formulas (equation 5 and equation 6), where SSR is the sum square residual and SST is the sum square total [17, 18].

$$x = Xo + [X_{max}.\exp[-\exp((\frac{r_m.\exp(1)}{x_{\max}})\,(t_l - t) + 1] \quad (5)$$

$$R^2 = (1 - \frac{SSR}{SST}) \quad (6)$$

2.4 Biomass calculation of *Euglena* sp.

The biomass calculation was carried out by taking 10 mL of the sample and then centrifuging it for 10 min at 3000 rpm. The centrifuged biomass was filtered using filter paper and dried in an oven at 30-50°C. The final biomass was calculated by subtracting the final weight of the sample from the initial weight, and then dividing by the initial sample volume [19]. Biomass productivity was calculated using the following formula (equation 7), where Q_x is biomass productivity (mg/mL/day), X_f is the maximum biomass at day f (mg/mL), X_0 is the initial biomass, T_f is day f (day), and T_0 is day 0 (day) [20].

$$Qx = \frac{Xf - Xo}{Tf - To} \quad (7)$$

2.5 Carbohydrate estimation of *Euglena* sp.

The carbohydrate content (day 1, 3, 5, 7, 9, and 10) was calculated by the Phenol-Sulfate method using glucose-standard curves. Glucose solutions with concentrations of 0.025, 0.05, 0.1, 0.25, and 0.5 g/L were made. Two mL of each solution was mixed with 1 mL of 5% phenol solution and homogenized, then added with 5 mL of concentrated sulfuric acid (H_2SO_4), and incubated for 10 min, shaken and placed in a water bath at 25°C for 15 min. For estimating the carbohydrate content, 10 mL of the sample was taken and centrifuged at 3300 rpm for 15 min. The pellets were then rinsed using distilled water, centrifuged at 3300 rpm for 10 min, added to 0.5 mL of 5% phenol and 1 mL of concentrated sulfuric acid, and then incubated for 30 min. A 2 mL aliquot of each standard solution and sample was taken, and then the absorbance was measured at 490 nm using a UV-Vis spectrophotometer (Genesys 150, Thermo Scientific) [21]. The carbohydrate content was calculated by plotting the sample absorbance against the standard curve absorbance.

2.6 Lipid estimation of *Euglena* sp.

The lipid content was determined using the Bligh and Dryer method by centrifuging 10 mL of the sample for 15 min at 4000 rpm. The pellet was added to 1 mL chloroform and 2 mL methanol, homogenized using a vortex. Then 1 mL of distilled water and chloroform were added, and the samples were homogenized and centrifuged. The lipid layer (bottom layer) was taken using a pipette and placed in a petri dish. The lipid content was calculated by subtracting the final weight of the petri dish minus the initial weight of the petri dish, then divided by the initial volume of the sample [19].

2.7 Protein estimation of *Euglena* sp.

The protein content was estimated using the Bradford method using the standard Bovine Serum Albumin (BSA) solutions of concentration 0, 20, 40, 60, 80, and 100 ppm [22]. Meanwhile, 2 mL of microalgae samples were taken and centrifuged at 5000 rpm for 3 min [23]. The pellets were added to 1 mL SDS 5%, and incubated at 4°C for 5 min [24]. A 1 mL aliquot of each sample and standard solution were taken and added to 1 mL of Bradford's reagent (modified from Kresnaputra

et al. [22]), and incubated for 5 min. Then the absorbance was measured using a UV-Vis spectrophotometer (Genesys 150, Thermo Scientific) at 595 nm [22].

2.8 Percentage and productivity of *Euglena* sp. primary metabolite determination

The percentage and productivity of each cell compound (carbohydrates, lipids, and proteins) were calculated using equation 8 and equation 9 [25].

$$\%\ cell\ compound = \frac{Total\ cell\ compound}{Biomass} x100\% \quad (8)$$

$$Productivity = Biomass\ productivity\ x\ \%\ cell\ compound \quad (9)$$

2.9 Pigmentation analysis

Pigmentation analysis was performed on chlorophyll-a, chlorophyll-b, and carotenoids. The analysis was carried out by taking 5 mL of sample and centrifuging for 10 min at 6000 rpm. The pellet was taken and added to 10 mL of absolute methanol. The tube containing the mixture of pellets and methanol was wrapped in aluminum foil and incubated at 70°C for 10 min. The mixture was then homogenized and centrifuged at 6000 rpm for 10 min. The supernatant was taken, and the absorbance was measured [26] using a spectrophotometer at 470, 646, and 663 nm. The estimation of chlorophyll-a and chlorophyll-b was done using the Lichtenthaler and Wellburn method with the following formulas (equations 10 and 11) [27]. The carotenoid content was calculated by equation 12 [28].

$$\text{Chlorophyll-a (mg/l)} = 12.21\ Abs\ 663 - 2.81\ Abs\ 646 \quad (10)$$

$$\text{Chlorophyll-b (mg/l)} = 20.13\ Abs\ 646 - 5.03\ Abs\ 663 \quad (11)$$

$$\text{Carotenoid (mg/l)} = \frac{(1000\ \text{Abs}\ 470 - 1.90\ \text{chlorophyll-a} - 63.14\ \text{chlorophyll-b})}{214} \quad (12)$$

2.10 Data analysis

The results of each analysis were analyzed using Friedman's two-way ANOVA by rank at a 95% confidence level followed by Bonferroni multiple pairwise comparisons using IBM SPSS Statistics 26. The total replications for cell density and biomass statistical analysis were 11 replications with the total number of samples being 165. Meanwhile, each statistical analysis of carbohydrate, lipid, protein, and pigment contents were performed with 11 replications and the total number of samples was 90.

3. Results and Discussion

Based on the research, the enrichment of CM media with tofu wastewater generally increased the cell density, biomass, and primary and secondary metabolites in *Euglena* sp. The results of this study provide information regarding the utilization of tofu wastewater in the cultivation of *Euglena* sp. However, further research is needed to determine the potential of metabolites produced by this strain cultivated using tofu wastewater.

3.1 The optimum concentration of the tofu wastewater

Based on tofu wastewater optimization, the highest cell density of *Euglena* sp. was found in the medium containing 75% tofu wastewater ($10.1x10^5$ cells/mL). The second highest cell density was 100% tofu wastewater ($8.7x10^5$ cells/mL). Meanwhile, the cell density of *Euglena* sp. in the other combinations was lower than in the two combinations. Moreover, the lag phase of *Euglena* sp. in the sole CM medium was longer than in other combinations, and the peak exponential phase was reached on day 8. Additionally, the specific growth rates (μ) of *Euglena* sp. cultivated in media containing 75% wastewater (μ=0.49 /day) and 100% (μ=0.30 /day) were higher than sole CM media (μ=0.17 /day).

Tofu wastewater contains essential compounds such as proteins, carbohydrates, and lipids. According to the proximate analysis, the water, ash, lipid, carbohydrate, and protein content of the wastewater used in this study were 99.4, 0.12, 0.04, 0.21, and 0.28%, respectively. In this study, the provision of tofu wastewater in CM medium enhanced the cell density of *Euglena* sp. It was found to be undistinguishable from the organic matter contained in the waste used by microalgae [29]. Sidabutar *et al*. [30] reported that the addition of 85% tofu wastewater in the media increased the cell density of *Chlorella* sp., which reached $18.46x10^6$ cells/mL with a total biomass of 0.70 g/L. Dianursanti *et al*. [29] also reported that *C. vulgaris* cultivated in a medium containing 30% tofu wastewater had a better growth curve than the Walne medium.

Euglena sp. absorbs and utilizes organic compounds in the heterotrophic environment [29] through phagocytosis. The cell surface of *Euglena* sp. forms vesicles to trap food particles [31]. *Euglena* sp. absorbed N and P contained in waste excellently [2]. However, exceeded waste concentration inhibited microalgae growth as reported by Elystia *et al*. [9] who noted that the addition of tofu wastewater at more than 40% inhibited the growth of *Scenedesmus* sp. due to the high concentration of the waste, which caused cell stress. However, in this study, we found that *Euglena* sp. has higher tolerance to high tofu wastewater concentration than *Scenedesmus* sp.

3.2 Growth kinetics of *Euglena* sp.

The cell density of *Euglena* sp. cultivated in tofu wastewater combined with specific pH levels is presented in Figure 1. The highest cell density for the media containing 75% tofu wastewater was found in L_2P_2 (23.13×10^5 cells/mL). Each cell density of *Euglena* sp. in L_2P_3, L_2P_4, and L_2P_5 was more than 15×10^5 cells/ml. The highest cell density in the combination containing 100% tofu wastewater was L_3P_1 ($22.88x10^5$ cells/mL). The lag phase in combinations containing 75 and 100% tofu wastewater was shorter than the sole CM media, and occurred from day 0 to day 2. The exponential phase started on day 3 until it reached the peak exponential on day 7. Moreover, the highest specific growth rate (μ) was for L_3P_5 with 0.81 /day. Meanwhile, all combinations containing 0% tofu wastewater had a lower cell density (less than $10x10^5$ cells/mL) and a lower specific growth rate (less than 0.5/day). Based on Friedman's Two-Way ANOVA, the combination of tofu wastewater and the pH of the media significantly affected the cell density of *Euglena* sp. with $P(0.000)<0.05$.

L_2P_2 and L_3P_1 were fitted to the Logistic and Gompertz models (Figure 2 and Figure 3) as these combinations had higher cell densities than others. Based on the Logistic modeling, the maximum specific growth rates (μ_{max}) of L_2P_2 and L_3P_1 were 1.2/day (R^2=0.97) and 1.04/day (R^2=0.97), respectively. According to the Gompertz modeling, the maximum cell production rate (r_m) of L_2P_1 was $12.83x10^5$ cells/mL. The maximum cell production rate (r_m) of L_3P_1 was only 4.8×10^5 cells/mL. The lag time (t_L) of L_1P_2 was 2.41 days (R^2 = 0.99), while L_3P_1 was 1.91 days (R^2 = 0.98). Thus, the adaptation period (the period before the cells entered the exponential phase) of L_2P_2 was longer than L_3P_1.

Based on the growth curve presented in Figure 1 and Figure 2, the adaptation period of *Euglena* sp. in the sole CM medium occurred longer than the medium added with tofu wastewater. In the initial phase of microalgae growth, cells adjust to the new media [32]. Tofu wastewater contains ammonium and phosphate ions that can be directly absorbed and used by microalgae cells. Ammonium is essential in photosynthesis, while phosphate is essential in the metabolism and replication of microalgae cells [29]. Therefore, the organic matter in the tofu wastewater enhanced the adaptation period of *Euglena* sp. and shortened the phase as well.

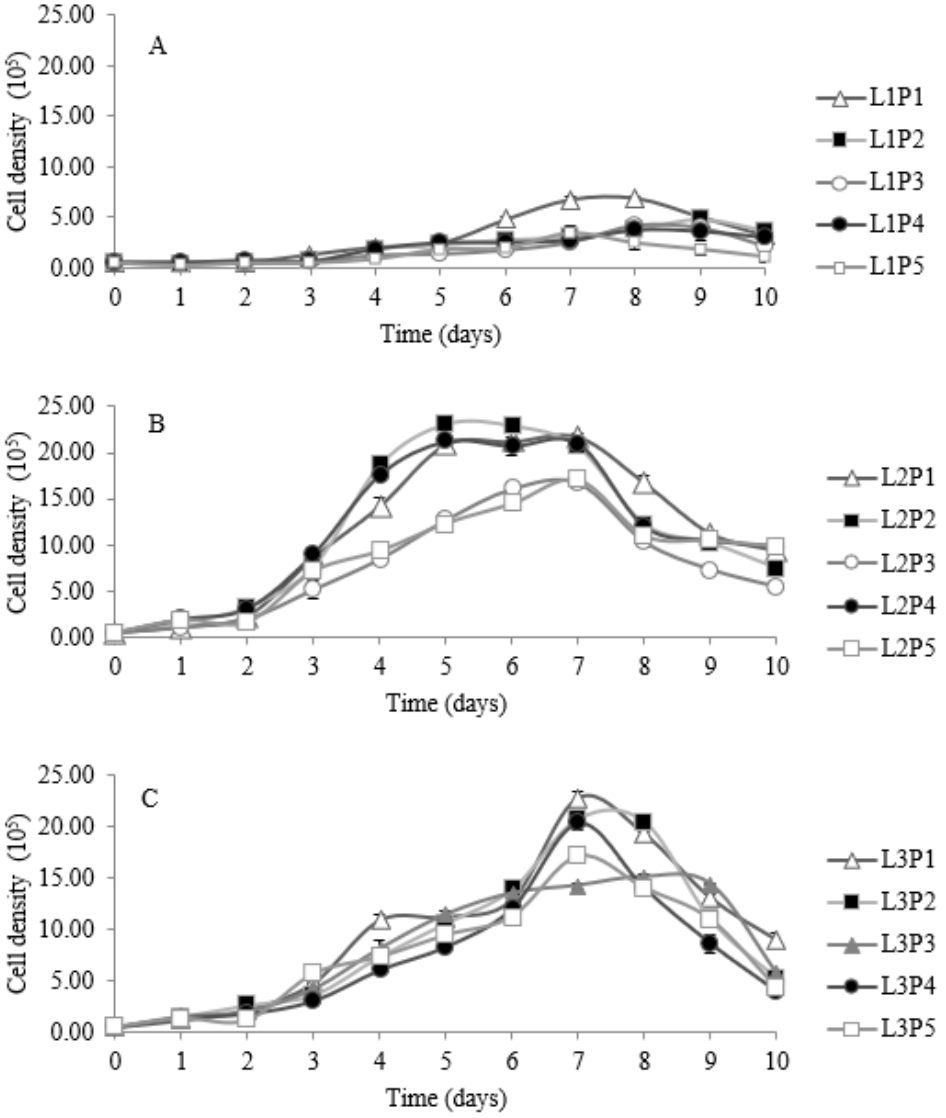

Figure 1. Cell density of *Euglena* sp. (A) cultivated in sole CM media (0% tofu wastewater), (B) cultivated in CM media containing 75% tofu wastewater, (C) cultivated in CM media containing 100% tofu wastewater. Each medium was combined with pH 5.0, 5.5, 6.0, 6.5, and 7.0.

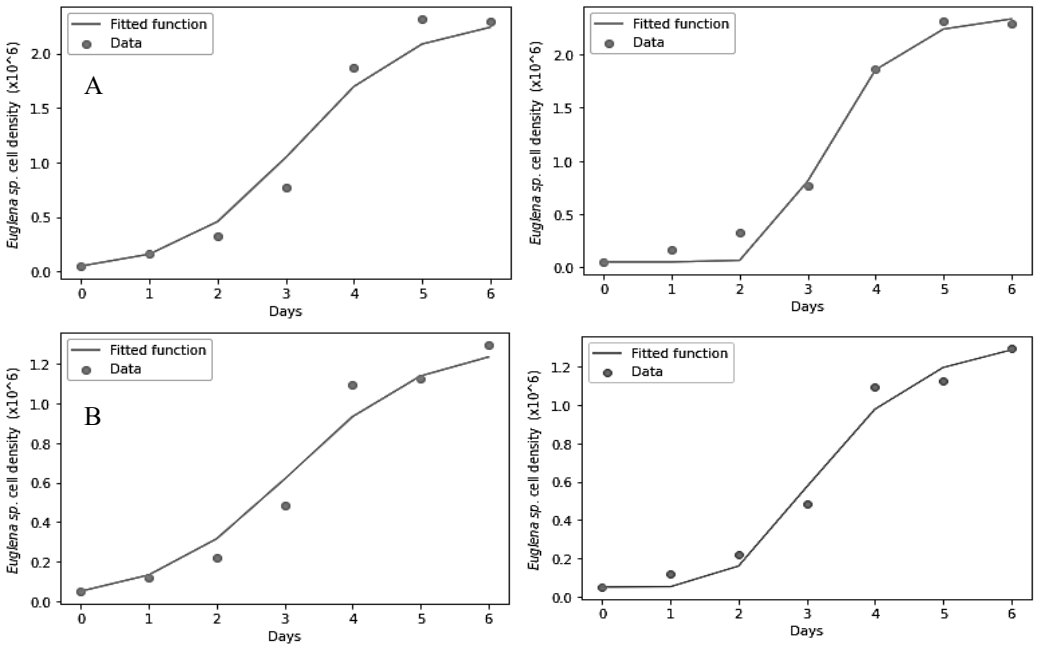

Figure 2. Growth modeling of *Euglena* sp. (A) Logistic (left) and Gompertz (right) model of L2P2. (B) Logistic (left) and Gompertz (right) model of L3P1

The faster the lag phase of the cell is in the medium, the faster the logarithmic or exponential phase will be. In the exponential phase, the division of microalgae cells occurs rapidly and massively due to the high nutrient and CO_2 absorption [32]. At the end of this phase, the number of nutrients in the medium gets depleted and the growth rate of microalgae cells declines. So, the number of living cells will be equal to the number of dead cells [32]. Accumulation of secondary metabolites in the medium also inhibits the growth of the cells [33]. When the cell enters the death phase due to limited nutrients, the number of dead cells becomes higher than the number of living cells [32].

3.3 Biomass of *Euglena* sp.

Biomass of *Euglena* sp. cultivated in all combinations is presented in Figure 3. All combinations containing 0% tofu wastewater with a certain pH level produced a smaller amount of biomass than the combinations containing 75 and 100% tofu wastewater (Figure 3). The maximum biomass of *Euglena* sp. in all combinations of 0% tofu wastewater was less than 3 mg/mL. In the combinations containing 75 and 100% tofu wastewater, each combination had a maximum biomass of more than 3 mg/mL, and the highest biomass was L_2P_1 with a maximum biomass of 4.53±0.17 mg/mL. Furthermore, the highest biomass productivity (Qx) was L_2P_1 (0.41 mg/mL/day). Meanwhile, the lowest biomass productivity was L_1P_4 (0.04 mg/mL/day). Based on Friedman's Two-Way ANOVA, the combination significantly affected the biomass of *Euglena* sp. at $P<0.05$.

The biomass of *Euglena* sp. cultivated in a CM medium enriched with tofu wastewater was higher than in the sole CM medium. Dianursanti *et al*. [29] also reported that the addition of 20 and 30% tofu wastewater increased the accumulation of *C. vulgaris* biomass. A positive result was also

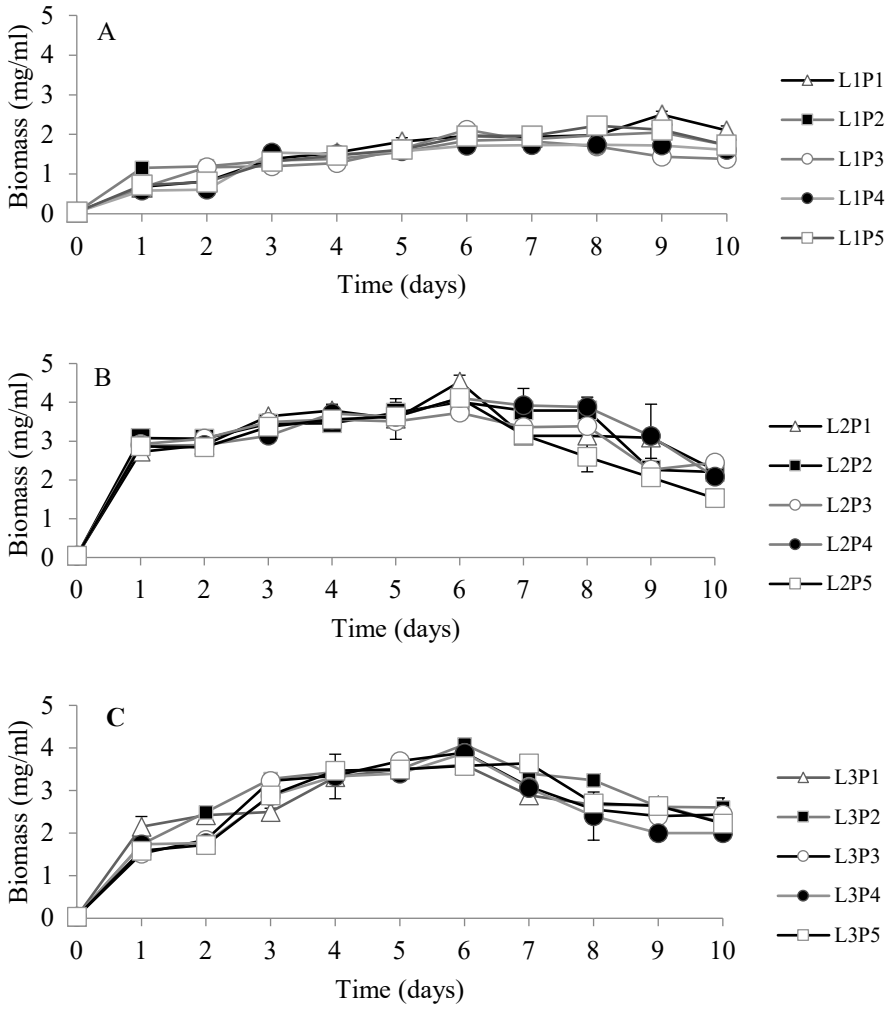

Figure 3. Biomass of *Euglena* sp. (A) cultivated in sole CM media (0% tofu wastewater), (B) 75% tofu wastewater, (C) 100% tofu wastewater

reported by Sidabutar *et al*. [30]. The accumulation of *Chlorella* sp. biomass increased due to the media enrichment with 85% tofu wastewater.

Microalgal biomass production can be integrated with waste recovery through the phycoremediation process [34]. The biomass of *Euglena* sp. contains lipids suitable for biodiesel production, its main lipid components being palmitic, linolenic, and linoleic fatty acids [2]. The cultivation of microalgae to produce its biomass also reduces CO_2. Besides containing a high percentage of lipids, its biomass does not overlap with food production. Microalgae have a relatively fast growth rate and life cycle and high productivity per hectare. The cultivation process can be carried out using wastewater. Furthermore, the biorefinery process produces many products based on lipids, carbohydrates, and proteins [35].

In this study, pH level did not seem to affect the growth and biomass of *Euglena* sp. since it is tolerant to pH variation. *Euglena gracilis* also survived at lower pH and acid environments [36].

As for *Euglena gracilis*, respiratory activity occurred actively at pH 5 and 6, with the optimum growth rate at pH 7 [12]. *Euglena* sp. also increased the pH of the domestic waste medium from an initial pH of 6.1±0.1 to 9.16±0.57 [2]. Therefore, the growth of this microalga was not that susceptible to pH.

3.4 Primary metabolite contents of *Euglena* sp.

The maximum metabolite contents and productivity of *Euglena* sp. are presented in Figure 4A, Figure 4B, and Figure 4C.

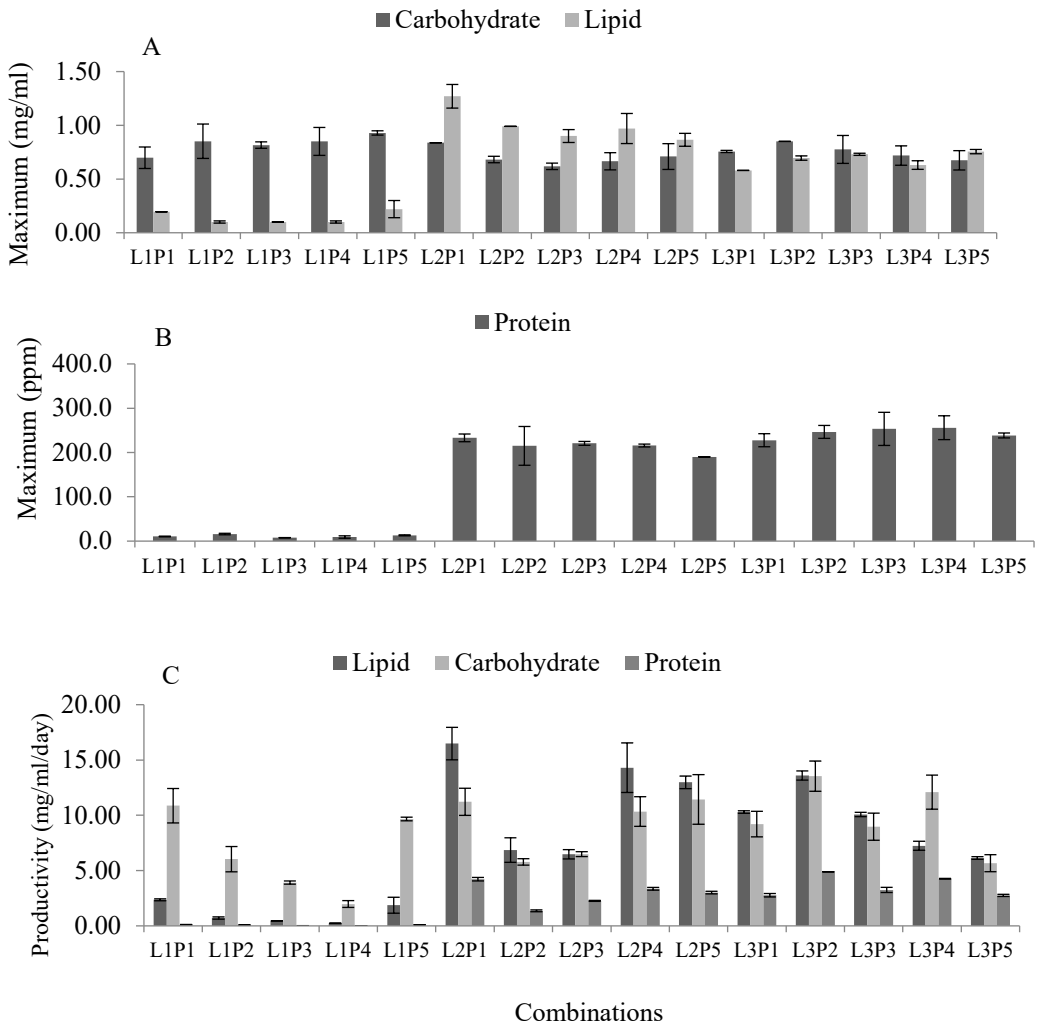

Figure 4. (A) Maximum carbohydrate, lipid, and (B) protein content of *Euglena* sp. (C) Maximum carbohydrate, lipid, and protein productivity of *Euglena* sp.

The maximum carbohydrate content of *Euglena* sp. in the combinations containing 0% tofu wastewater was higher than in other combinations, with the highest carbohydrate content in L_1P_5

(0.93 0.02 mg/mL). The carbohydrate percentage of the combination containing 0% tofu wastewater (L_1P_1-L_1P_5) was also higher than other combinations, and the highest carbohydrate percentage was L_1P_3 (59.12±2.17%). However, the highest carbohydrate productivity was L_3P_2 (13.54±1.37 mg/mL/day). Meanwhile, the maximum carbohydrate percentages of *Euglena* sp. cultivated in the combinations containing 75 and 100% tofu wastewater were L_2P_5 (46.35±9.09%) and L_3P_4 (35.92±4.58%). Based on the Friedman's Two-Way ANOVA, each combination significantly affected the carbohydrate content and carbohydrate productivity (P<0.05), except for the carbohydrate percentage (P(0.273)>0.05)) of *Euglena* sp.

According to the cell compound analysis, all combinations containing 0% tofu wastewater had higher carbohydrate accumulation than other combinations due to the active photosynthesis activity in a medium without additional nutrients. Besides, nitrogen limitation also initiated the synthesis of carbohydrate in *C. zofingiensis* [1] and *Euglena* sp. [13]. Based on Wang *et al.* [3], *Euglena* sp. also acted as a photoautotrophic organism. It carried out photosynthesis to produce glucose stored in the form of carbohydrates [37]. The optimum pH for photosynthesis in *Euglena* sp. was 6.0. [12]. The higher the rate of photosynthesis, the higher the amount of carbohydrates accumulated in the cell.

In this study, the highest carbohydrate percentage of *Euglena* sp. was higher when compared to previous studies conducted by Suzuki *et al*. [13] using *Euglena gracilis* and *Euglena anabaena* var. *minor*. In their study, the percentage of carbohydrates only reached 40 and 45%, respectively. The carbohydrate compounds in those microalgae were stored as β-1,3 glucan and known as paramylon. The accumulation of paramylon in *Euglena gracilis* was enhanced by nitrogen limitation [13]. Paramylon is located in the cell's cytoplasm or the pyrenoid [38]. Paramylon can be used as the feedstock for biofuels, biomaterials, biomedicine, pharmaceuticals, and functional nutrition [39].

In contrast to carbohydrate contents, the combinations containing 75% tofu wastewater accumulated higher lipid content than those containing 0 and 100% tofu wastewater, and the highest lipid content was found in L_2P_1 (1.27±0.11 mg/mL). In the media containing 100% tofu wastewater and certain pH levels (5.0-7.0), the maximum lipid content was L_3P_5 (0.75±0.02 mg/mL). Meanwhile, the combinations containing 0% tofu wastewater accumulated a lower lipid content that ranged from 0.10±0.00 mg/mL to 0.22±0.08 mg/mL. Furthermore, the highest lipid productivity was also seen in L_2P_1 with 16.48±1.47 mg/mL/day. Furthermore, the highest lipid percentage was L_2P_5 (52.6±2.31%). Among the combinations containing 100% tofu wastewater, the maximum lipid percentage was L_3P_5 (32.7±0.63%). Meanwhile, the lipid percentages of *Euglena* sp. cultivated in the medium containing 0% tofu wastewater were also lower than in the combination containing 75 and 100% wastewater, and the highest lipid percentage was L_1P_5 (10.38±4.00%). Therefore, the combinations of tofu wastewater and pH were significantly affected the lipid content, lipid productivity, and lipid percentage of *Euglena* sp. at P<0.05.

Tofu wastewater also enhanced the lipid percentage of *Euglena* sp. This result was the same as Elystia *et al*. [9]. Based on their report, the addition of 20% tofu wastewater increased the lipid percentage in *Scenedesmus* sp. by 27.12%. [10]. Widayat and Hadiyanto [10] reported that medium supplemented with 20% tofu wastewater enhanced lipid accumulation in *Nannochloropsis* sp. by 34.25%. Dianursanti *et al*. [29] also found that *C. vulgaris* cultivated in medium supplemented with 30% tofu wastewater accumulated a higher lipid percentage than in the sole Walne medium, which was 23.25% [29]. Moreover, the highest lipid accumulation of *Euglena* sp. in this study was higher than the lipid percentage of *Euglena gracilis* reported by Wang *et al*. [3]. *Euglena gracilis* accumulated lipids at up to 42% in photoautotrophic conditions. The maximum lipid percentage in this study was also higher than the maximum lipid accumulation of *Euglena* sp. cultivated in domestic waste (25.6%) [2].

Lipid synthesis in *Euglena* sp. occurs in mitochondria with the main component being phosphoglycerol, and it occurs in plastids with the main composition in the form of

glycosyldiacylglycerols [40]. The fatty acid composition of *E. gracilis* lipids is dominated by C16 to C18 (93.46%), and palmitic acid (C16:0) was the main fatty acid at high level as 24.17%. Other fatty acids were oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) at 22.20%, 13.68%, and 11.61%, respectively [41]. Meanwhile, *Euglena* sp. cultivated in domestic waste contained 13 kinds of fatty acid methyl esters that were dominated by 52% unsaturated fatty acids. The main components of these fatty acids were palmitic acid (46%), α linolenic acid (23%), linoleic acid (22%), and stearic acid (3%). Meanwhile, PUFA (poly-unsaturated fatty acids) content was 46.6% [2].

The lipid produced by *E. gracilis* had a Cetane Number of 73.08%. This percentage exceeded the standards set by the United States (51%) and Europe (47%). The iodine value (IV) of lipids from *E. gracilis* was 9,680 gl/100 g biodiesel. This amount was much lower than the standard of 120 gl/100 g biodiesel [41]. Therefore, lipid produced by *Euglena* sp. is a promising feedstock for the production of biofuel as future renewable energy.

Moreover, the addition of tofu wastewater combined with a certain pH level increased protein accumulation in *Euglena* sp., where the maximum protein content was 256±26.86 ppm (L_3P_4). The highest protein productivity and protein percentages were L_3P_2 (4.89±0.00 mg/mL/day) and L_2P_5 (12.20±0.48%). Meanwhile, all combinations of pH and 0% tofu wastewater accumulated lower maximum protein content (less than 16 ppm), protein productivity (less than 0.2 mg/mL/day), and protein percentages (less than 1%). According to Friedman's Two-Way ANOVA, the combinations were significantly different in the protein content, protein percentage, and protein productivity of *Euglena* sp. (P<0.05).

Based on O'Neill *et al*. [42], *Euglena gracilis* has 30,000 genes coding the formation of proteins as well as the biosynthesis of lipids, amino acids, carbohydrates, vitamins, polyketides, and non-ribosomal peptides. However, the highest protein percentage of *Euglena* sp. in this study (14%) was much lower when compared to *Euglena gracilis* reported by Wang *et al*. [3], which reached 34.2% when the cells had reached their exponential phase. Microalgae species have different abilities in accumulating proteins [43]. Additionally, protein synthesis in microalgae requires a higher energy demand than sugar synthesis, so the accumulation of protein in microalgae cells will be lower when compared to other biochemical products [44]. Moreover, the excess nitrogen content in the medium inhibits the rate of protein synthesis in microalgae [45].

3.5 Pigment contents estimation of *Euglena* sp.

The pigment contens of *Euglena* sp. are presented in Figure 5. Based on the pigmentation analysis, chlorophyll-a in *Euglena* sp. was higher than chlorophyll-b and carotenoids (Figure 5). In this study, *Euglena* sp. cultivated in media containing 75 and 100% tofu wastewater produced higher chlorophyll-a than the combination in sole CM media. The highest chlorophyll-a content was L_2P_4 with 33.53±0.13 mg/L. Chlorophyll-a content of combinations containing 100% tofu wastewater ranged from 21.98±0.70 mg/L to 29.01±0.90 mg/L. *Euglena* sp. cultivated in media containing 0% tofu wastewater produced chlorophyll a at 1.15±0.03 mg/L to 9.14±0.03 mg/L.

Moreover, *Euglena* sp. cultivated in combinations containing 75% tofu wastewater produced a higher chlorophyll b content than other concentrations. The highest chlorophyll b was L_2P_2 with 17.73±0.50 mg/L. Similar to chlorophyll a and chlorophyll b, the content of carotenoid was also higher in the combinations containing 75% wastewater, and the highest carotenoid content was L_2P_2 (11.65±0.00 mg/L). Based on Friedman's Two-Way ANOVA, the combinations were significantly different in the content of chlorophyll a, chlorophyll b, and carotenoids of *Euglena* sp. due to each P<0.05.

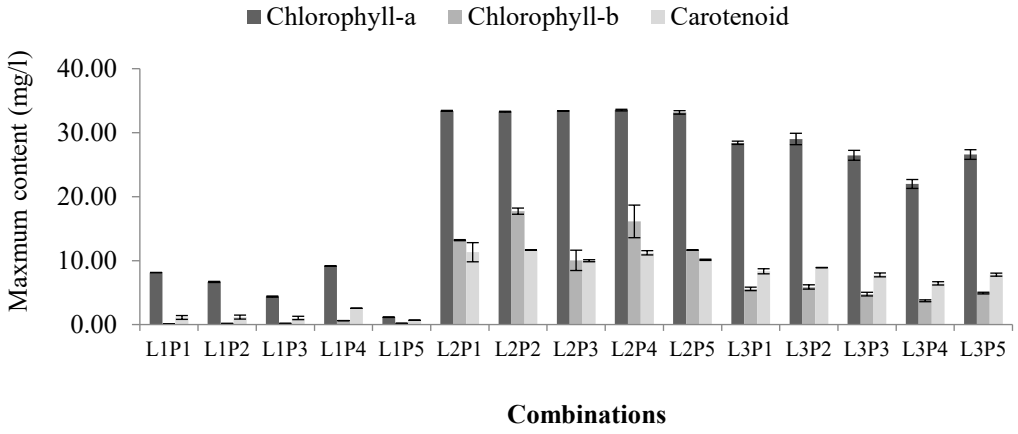

Figure 5. Maximum chlorophyll a, chlorophyll b, and carotenoid contents of *Euglena* sp. cultivated in each combination

Tofu wastewater enhanced the production of pigments in *Euglena* sp. The effect of supplementing tofu wastewater on photosynthetic pigments of microalgae was previously reported by Apriati [46] who reported that the addition of 25% tofu wastewater increased the levels of chlorophyll-a (3.53 mg/l) and chlorophyll-b (3.72 mg/l) in *Chlorella pyrenoidosa*. It could be triggered by ammonium content in tofu wastewater. Ammonium contained in tofu wastewater is essential in photosynthesis and chlorophyll formation [29]. The photosynthetic activity was closely related to the content of photosynthetic pigments produced by these microalga cells. Meanwhile, according to Danilov and Ekelund [12], the photosynthesis of *E. gracilis* was optimum at pH 6.

The primary photosynthetic pigments in *Euglena* sp. are chlorophyll a and b [47]. *Euglena* sp. also contains carotenoid pigments [4] such as xanthophyll, astaxanthin (euglenorhodone), zeaxanthin, carotene, and β-carotene. Chlorophylls and carotenoids are fat-soluble molecular pigments. Both pigments are found in thylakoid chloroplasts in *Euglena* sp. cells and can be isolated using organic solvents such as acetone and methanol. Xanthophyll, which is the form of astaxanthin, is a pigment causing a reddish color [48, 49]. Astaxanthin is one of the essential pigments in pharmaceuticals as it has anticancer activity [50]. Other xanthophylls found in *Euglena* sp. are diadinoxanthin and diatoxanthin.

4. Conclusions

Based on this study, we concluded that tofu wastewater can be used as an alternative nutrient source for the growth of *Euglena* sp. Some essential nutrients contained in tofu wastewater such as C, N, and P, enriched the nutrient availability for this strain thus enhancing the metabolism of this microalga. Generally, the optimum concentration of tofu wastewater (75%) and the sole tofu wastewater (100%) combined with a specific pH level produced a higher growth rate, biomass, and primary and secondary metabolite content in *Euglena* sp. than the sole CM media. Our study indicated that tofu wastewater can be used to economically replace synthetic commercial media in mass-scale cultivation to reduce the production cost in producing a high amount of biomass used as feedstock in biorefinery activities.

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