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APPLICATION OF *Euglena* sp. ISOLATED FROM YOGYAKARTA, INDONESIA ON NUTRIENT REMOVAL FROM PALM OIL MILL EFFLUENT (POME)

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ABSTRACT

Palm oil mill effluent (POME) was the main source of liquid waste in Indonesia. It could be utilised as microalgae medium. Euglena *sp., fast-growing microalgae, produces high-value product metabolites. In this research, POME is used as a culture medium because of its rich macronutrients and micronutrient content. In addition, POME also contains high Biological Oxygen Demands (BOD) and Chemical Oxygen Demands (COD). This research was conducted to study BOD and COD in POME after and before treatment, growth rate, biomass and content of carbohydrates, lipids and proteins in* Euglena *sp. that was cultured in a POME medium.* Euglena *sp. cultured in Cramer-Myers (CM) medium was used as control. POME medium concentrations used in this research were 0.50%, 0.75%, 1.00% and 1.25%. The results showed that* Euglena *sp. was able to reduce BOD and COD. Medium 1.25% POME was the best treatment for nutrient removal, growth, and nutritional value. Based on the Logistic modelling, the highest specific growth rate (μ) was achieved in 1.25% POME with μ 0.6894 day and R^2 0.99.*

Keywords: biological oxygen demand, chemical oxygen demand, *Euglena* sp., logistic model, POME.

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INTRODUCTION

Palm oil mill effluent (POME) is an unavoidable byproduct that, if dumped directly into the environment, poses major environmental risks. This is mostly due to its high chemical oxygen demand (COD) and biochemical oxygen demand concentrations (BOD) (Kassim *et al*., 2022). POME has BOD and COD levels up to 100 000 mg L^{-1} (Low *et al.* 2021). POME was a brownish liquid with a pH of 3-4. POME contained N, PO_4, K, SO_4 and Fe elements (Mellyanawaty *et al*., 2018). POME must be effectively pre-treated before disposal (Low *et al*., 2021). Based on the POME characteristic, POME has the potential to be used as a microalgae cultivation medium.

Cultivation of microalgae in POME medium had many promising advantages. Producing microalgae in wastewater reduced the cost of cultivation (Cheah *et al*., 2018). Production of *Euglena gracilis* in wastewater achieved efficient biomass production. Furthermore, the POME medium increased the growth and lipid production of microalgae (Kamyab *et al*., 2018). Besides that, microalgae were also used as nutrient removal agent (Cheirsilp *et al*., 2016; Hambali and Rivai, 2017; Haruna, 2017; Kuroda *et al*., 2018).

In addition, the potential of microalgae to produce biomass and remove nutrients from waste has been intensively investigated. Local mixed

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culture microalgae (*Cyclotella* sp., *Cylindrospermosis* sp., *Golenkinia* sp., *Syracosphaera* sp., *Corethron* sp., *Clamydomonas* sp.) and bacteria (*Corynebacterium ulcerans*, *C. bovis*, *Bacillus cereus*, *B. megaterium*, *Pediococcus parvulus*, *Staphylococcus vitulinus*) from Indonesia decreased BOD and COD in the POME medium (Muttaqin dan Suyono, 2021; Suyono *et al*., 2016; 2018). Previous research reported that *Chlorella vulgaris,* one of the microalgae, had a nutrient removal efficiency of 86% for inorganic nitrogen and 78% for phosphorous (Low *et al*., 2021).

Furthermore, Kuroda *et al*. (2018) reported that *E. gracilis* has the potential as a nutrient removal agent. *Euglena* was used as the phytoremediation agent because *Euglena* grew in the pH range of 2-6 and can stably be grown at acidic pH (Haraguchi and Zheng, 2022). *E. gracilis* was distinguished by its tolerance to high nutrient concentrations in water (Nezbrytska *et al*., 2022). *Euglena* can grow both autotrophic, heterotrophic, and mixotrophic (Rubiyatno *et al.,* 2021). Besides that, Euglenoids were a group of fast-growing algae, capable of reaching very high cell densities, even in extreme environments **(**O'Neill, 2020). Therefore, this study was conducted to determine the ability of *Euglena* sp. in reducing the nutrients present in the POME, to predict biomass production, and to determine the nutritional value contained in the biomass of *Euglena* sp.

MATERIALS AND METHODS

Growth Medium and Culture Conditions

The research was conducted in the Biotechnology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia in September 2019 until July 2020. *Euglena* sp. was isolated from ponds, wastewater and rice fields in Yogyakarta, Indonesia. The cultures were kept in a Cramer-Myers (CM) medium at pH 3.5 and 29°C.

The medium concentrations of POME used in this study were 0.50%, 0.75%, 1.00% and 1.25%. The raw POME was stored in a 4°C refrigerator for 2 hr before being placed at room temperature to separate the colloidal suspension from the solids. *Table 1* shows the nutrients for CM medium and POME.

This study was conducted in 250 mL Erlenmeyer flasks containing growth medium, POME and CM at various concentrations and cultures. For this experiment, the working volume was 240 mL for each flask, with the remaining space used to promote gas exchange. The temperature was kept constant at 29°C, with a LED light exposure of 18 mol m^{-2} s^{-1}. The sample was taken daily to measure the growth of *Euglena* sp. in different concentrations of POME medium at the absorbance of 680 nm.

Analytical Determinations

Modelling growth kinetic of* Euglena *sp. POME media. The growth kinetic model of microalgae was widely available (Yang *et al.,* 2011). Non-substrate type limiting, the Logistic model, was the simplest model in microbial growth. Logistic models have commonly been used for illustrating the rapid population growth of the organism (Lam *et al.,* 2017). Furthermore, the Logistic model was highly suitable for microalgae culture (Khavarpour *et al.,* 2011). It was used to predict the number of stable populations using the maximum growth rate per day as its parameter. The Logistic model was calculated using the following Equation (1) and (2).

$$\text{Logistic model: } N(t) = \frac{N_{max}}{1 + \left(\frac{N_{max}}{N_0} - 1\right) exp^{-\mu t}} \quad (1)$$

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

where N(t) was optical density, N_0 was the initial optical density, N_{max} was maximum cell density, t was the time of culture (day), and μ was the specific growth rate (Hanief *et al.,* 2020; Phukoetphim *et al.,* 2017). The determination of the model was carried out using the formula where SSR was the sum of square of residual and SST was the sum of square of total (Ajala *et al.,* 2020).

Biomass production. The biomass of *Euglena* sp. was measured as dry weight. The 5 mL sample was centrifuged for 15 min at 3300 rpm. The supernatant was discarded after that the pellets were placed in the oven at 60°C overnight until the weight was constant, then weighed again on the analytical scales. Experiments were carried out in triplicate. The biomass yield was obtained based on Equation (3).

$$\text{Dry weight (g)} = \text{Conical total weight} - \text{Conical initial weight} \quad (3)$$

Source: Richmond (2003).

Lipids production. A 5 mL sample was centrifuged for 10 min at 4°C at a speed of 4000 rpm. The formed pellets were mixed with 2 mL of methanol and 1 mL of chloroform before being homogenised with a vortex. 1 mL of chloroform and 1 mL of distilled water were added and homogenised again. Pellets were centrifuged at 4000 rpm for 10 min at a temperature of 10°C. The mixture separated into three layers and subsequently, the yellow

TABLE 1. THE COMPOSITION OF CM AND RAW POME MEDIUM

CM medium nutrient element	Concentration (mg L^{-1})	Raw POME nutrient element	Concentration (mg L^{-1})
$(NH_4)_2SO_4$	1 000	Ammoniacal-nitrogen	35
KH_2PO_4	1 000	Suspended solids	18 000
$MgSO.7H_2O$	200	Phosphorus	18
$CaCl.2H_2O$	20	BOD	25 000
$Fe_2(SO_4)_3.7H_2O$	3.0	COD	50 000
$MnCl_2.4H_2O$	1.8		
$CoSO_4.7H_2O$	1.5		
$ZnSO_4.7H_2O$	0.4		
$CuSO_4.5H_2O$	0.02		
$Na_2MoO_4.2H_2O$	0.2		
Vitamin B1	0.1		
Vitamin B12	0.0005		

layer was pipetted and placed on a previously weighed dry Petri dish. The Petri dishes were placed in an oven at 40°C so that the chloroform evaporated and the remaining lipids were left on the petri dish.

$$\text{Total lipids (gr)} = \text{Petri dish total weight} - \text{Petri dish initial weight} \quad (4)$$

Source: Richmond (2023).

Carbohydrate content. Carbohydrate content was determined using a spectrophotometer with at absorbance of 490 nm and a standard curve of glucose concentrations of 25, 50, 75, 100, 125, 150, 175, 200, 225 and 250 mg mL^{-1}. The 5 mL sample was centrifuged for 10 min at 4000 rpm. The formed supernatant was discarded, and the pellets were incubated for 10 min with 5% phenols in a 500 μL volume. Following that, 1 mL of concentrated sulphuric acid was added through the wall before being homogenised and silenced for 20 min (Dubois *et al.*, 1956).

Protein content. Protein content was estimated based on Bradford (1976). Protein concentration was determined using an ELISA Reader BioTek absorbance at 595 nm and a standard curve using BSA with concentrations of 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250 and 2500 μg mL^{-1}. 2 mL sample was inserted into 2 mL tube. The sample was centrifuged at 4000 rpm for 15 min. After forming the pellets, 1 mL of 10% SDS solution was added, and the mixture was heated at 95°C for 5 min. The sample was placed in a refrigerator at 4°C for 5 min. 8 μL of the sample was taken and placed in a 500 μL microplate where 200 μL of Bradford's reagent was added and then homogenised with a micropipette. Protein content was read using a spectrophotometer with 595 nm absorbance.

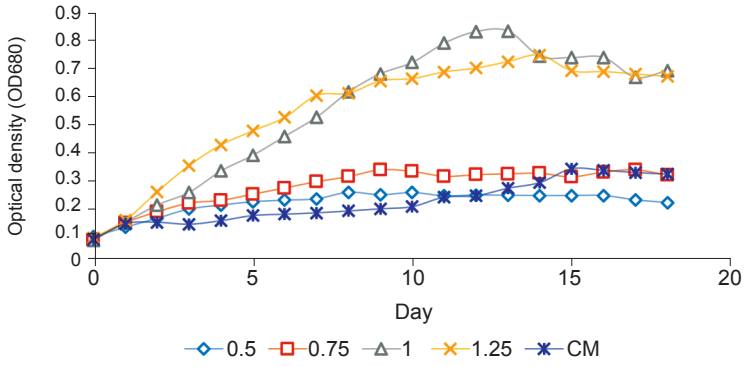
Data Analysis

Data analysis was performed using SPSS v.16 software. The test carried out was One Way Analysis of Variance (ANOVA) to determine the significance of the treatment at the confidence level 95% (α = 0.05) and if the results were significant, it would be followed by Duncan's Multiple Range Test (DMRT) to know the real difference between the concentration of POME Media. The growth model was determined using the following formula: SSR is the sum of square of residual and SST is the sum square total (Ajala *et al.*, 2020).

RESULTS AND DISCUSSION

***Euglena* sp. Growth in POME Medium**

The absorbance represents the cell density of *Euglena* sp. The high value of absorbance described the higher cell density. The highest absorbance occurred at 0.244 ± 0.09 (concentration 0.50%), 0.327 ± 0.12 (concentration 0.75%), 0.840 ± 0.12 (concentration 1.00%), 0.751 ± 0.15 (concentration 1.25%) and 0.331 ± 0.10 (control). The growth phase of *Euglena* sp. cultured in a POME medium was shown in *Figure 1*. The growth phase includes the lag phase, log phase, stationary phase and death phase. The lag phase was the initial period of slow growth. The exponential or log phase was characterised by rapid growth and division (Kawaroe, 2010). The stationary phase was characterised by a balance of cell growth and cell death. The death phase was the stage in which cell death exceeds cell growth (Price and Farag, 2013).



Source: Putri (2020).

Figure 1. Euglena *sp. growth at various POME medium concentrations.*

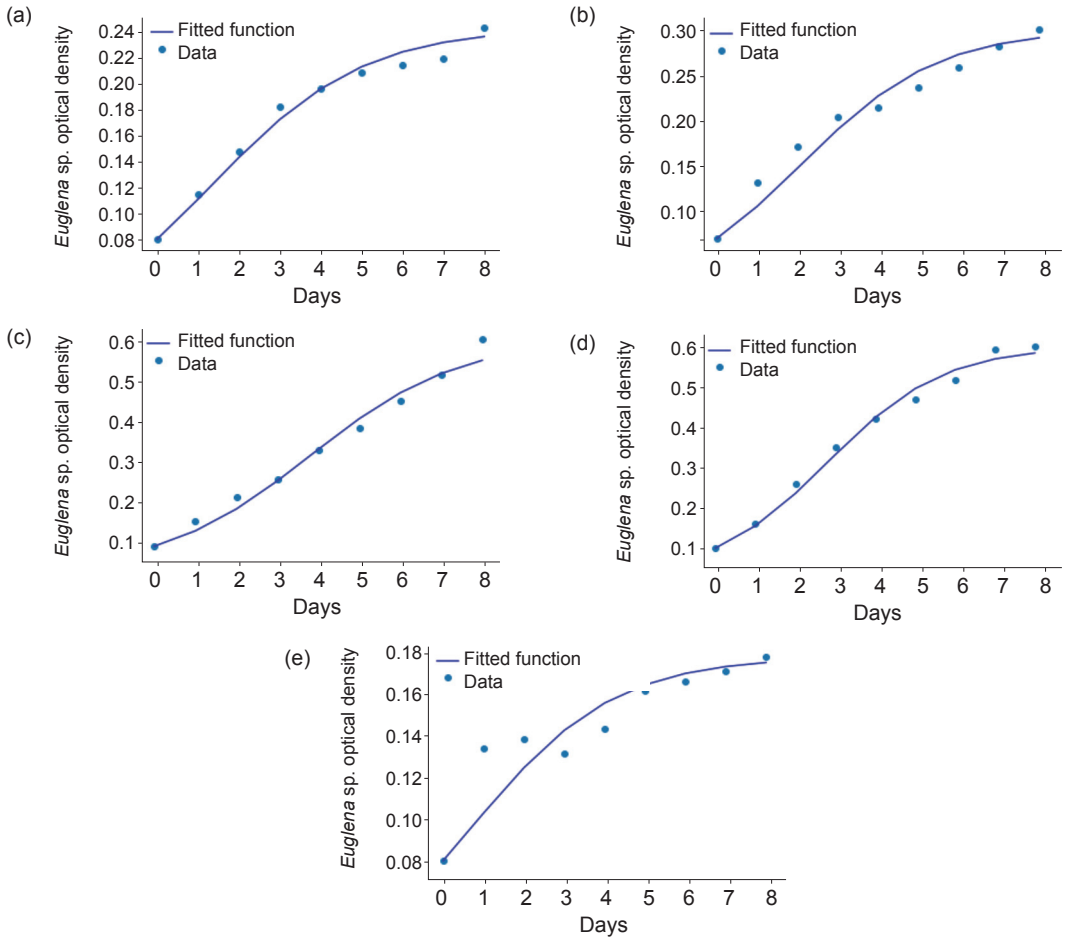


Figure 2. Fitting of growth kinetic of Euglena *sp. in POME media; (a) 0.5% POME concentration; (b) 0.75% POME concentration; (c) 1% POME concentration; (d) 1.25% POME concentration and (e) CM Medium.*

The result showed that the lag phases in all treatments occurred in the 1st-2nd days. The factor that determines the lag phase were the age and cell number of the inoculum. The lag phase would be shorter or even invisible if the inoculated cells were from cultures that were in the exponential phase (Kurnianto and Suyono, 2021). The logarithmic phase occurred from 3rd-8th in 0.50%, 3rd-9th in 0.75%, 3rd-13th in 1.00%, 3rd-14th in 1.25% and, the control was on the 3rd-5th day. The stationary phase occurred from 9th-10th at 0.50%, 10th at 0.75%, 13th-16th at 0.10%, 15th-16th at 1.25%, and the stationary phase of the control started on 16th day. The death phase started on the 11th day at 0.50% and 0.75% whereas 1.00%, 1.25%, and control treatment entered the death phase on the 17th day. 0.50%, and 0.75% had shorter logarithmic phases and the control had lower optical density because the concentration of nutrients available was less than the concentration of 1.00% and 1.25%. The POME media concentration affected the growth

phase and cell density. The growth phase in 1.00% and 1.25% treatments were longer than in 0.50% and 0.75% treatments. Besides that, the result suggested that treatments 1.00% and 1.25% increased the cell density of *Euglena* sp. compared to the control, 0.50% and 0.75% treatments. Nutrient availability in 1.00% and 1.25% media was sufficient for growth compared to the control, 0.50% and 0.75%. Furthermore, Chowdury *et al*. (2020) reported that microalgae growth was affected by nutrients.

Modelling Growth Kinetic of *Euglena* sp. POME Media

The result of the specific growth rate (μ) of *Euglena* sp. in various POME concentrations by logistic modelling was shown in *Table 2*. Based on the Logistic modelling, the highest specific growth rate (μ) was achieved in 1.25% POME with μ 0.6894 day^{-1} and R^2 0.99. The Logistic model fits the microalgae growth curves better for all POME concentrations. The values of the coefficient of determination $R^2 >$ 0.95 established the goodness of fit of the Logistic model in the study. On the contrary, Control with CM Media indicates that the Logistic model could not be used due to low R^2.

Biomass and Lipids Production of *Euglena* sp.

According *to Figure 3a* biomass changed during cultivation. The highest biomass was shown at a concentration of 1.25% while the lowest biomass occurred at the control treatment. The result showed that increased concentration of POME has increased the production of biomass. The ANOVA showed that the Biomass treatment with 1.25% POME concentration was significantly different from the other treatments, including the control treatment. A previous study by Cheah *et al.* (2018) showed that *C. sorokina* cultured on POME media had higher biomass than the control treatment on BBM media. Furthermore, wastewater was a better medium for *E. gracilis* culture than a modified medium (Kuroda *et al.*, 2018). Biomass productivity is influenced by several factors, such as CO_2, nutrients N and P, and light. The content of nitrogen at certain concentrations increases the growth of microalgae and CO_2 fixation (Viena, 2014).

TABLE 2. GROWTH RATE PARAMETER OF LOGISTIC MODEL

Variation	μ max	R^2
0.50% POME	0.5345	0.98
0.75% POME	0.5865	0.95
1.00% POME	0.5517	0.98
1.25% POME	0.6894	0.99
Control	0.5640	0.79

Source: Putri (2020).

The efficacy of using POME for microalgae cultivation was determined by biomass concentration and lipid content (Cheah *et al.*, 2018). The highest lipid production occurred at 1.00% POME medium with a value of 0.333 ± 0.015 g L^{-1}. This result was significantly different from other treatments, except for 1.25% of the POME Medium. The highest lipid content occurs on the 15th day. It can be seen that manipulating the environment's lack of nutrient content (N) can increase lipid production in microalgae. Kamyab *et al*. (2017) reported that lipid production could be increased under nutrient starvation conditions.

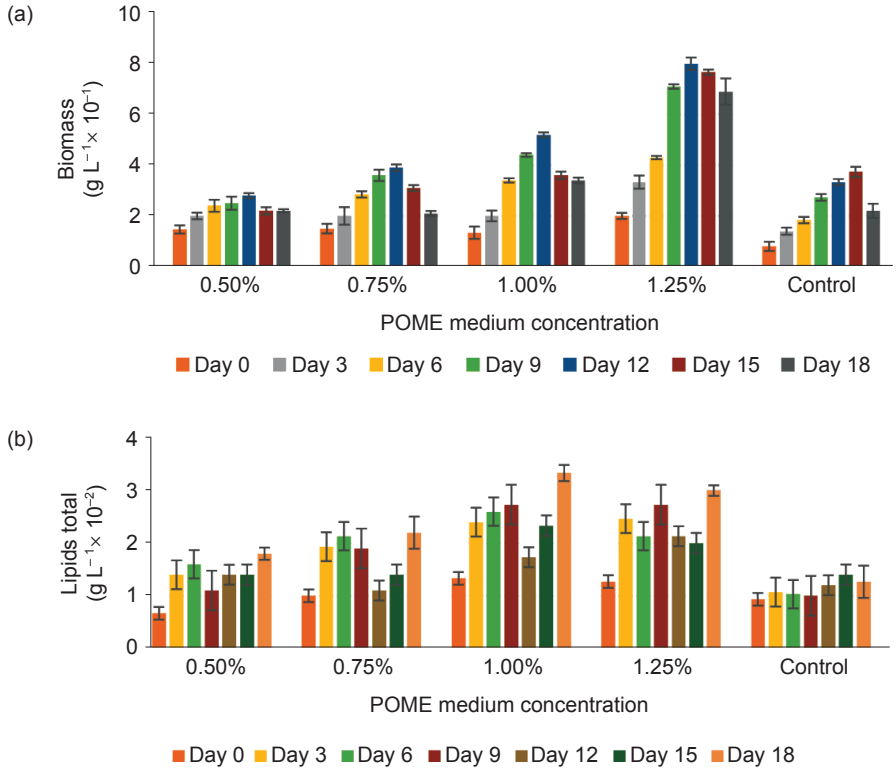
Carbohydrates and Proteins Content of *Euglena* sp.

The highest carbohydrates content produced was on the 18th day, except in the control treatment, which produced the highest carbohydrates on the 15th day, which was 1.87 ± 0.29 mg mL^{-1} (concentration 0.50%), 1.94 ± 0.28 mg mL^{-1} (concentration 0.75%), 3.32 ± 0.50 mg mL^{-1} (concentration 1.00%), 4.20 ± 0.35 mg mL^{-1} (concentration 1.25%) and 2.17 ± 0.29 mg mL^{-1} (control) (*Figure 4a*). Carbohydrate is used as the short-term energy source for microalgae. Carbohydrates production was related to environmental stress factors such as light, temperature and nutrient supply (Hariz *et al*., 2019). Microalgae changed their biomass composition under stress conditions to accumulate more carbohydrates (Suyono *et al*., 2016).

The highest protein content was reached at the concentration of 1.25% with a value of 5153 ± 2.69 μg mL^{-1}. The protein content in each treatment was 3.27 ± 1.32 μg mL^{-1} (concentration 0.50%), 4.78 ± 2.15 μg mL^{-1} (concentration 0.75%), 4.83 ± 0.94 μg mL^{-1} (concentration 1.00%), 5153 ± 2.69 μg mL^{-1} (concentration 1.25%), and 4.45 ± 1.80 μg mL^{-1} (control) (*Figure 4*). This suggests that high nitrogen content in the medium promotes cell chlorophyll pigment synthesis. Carbohydrates and proteins will increase as photosynthesis progresses.

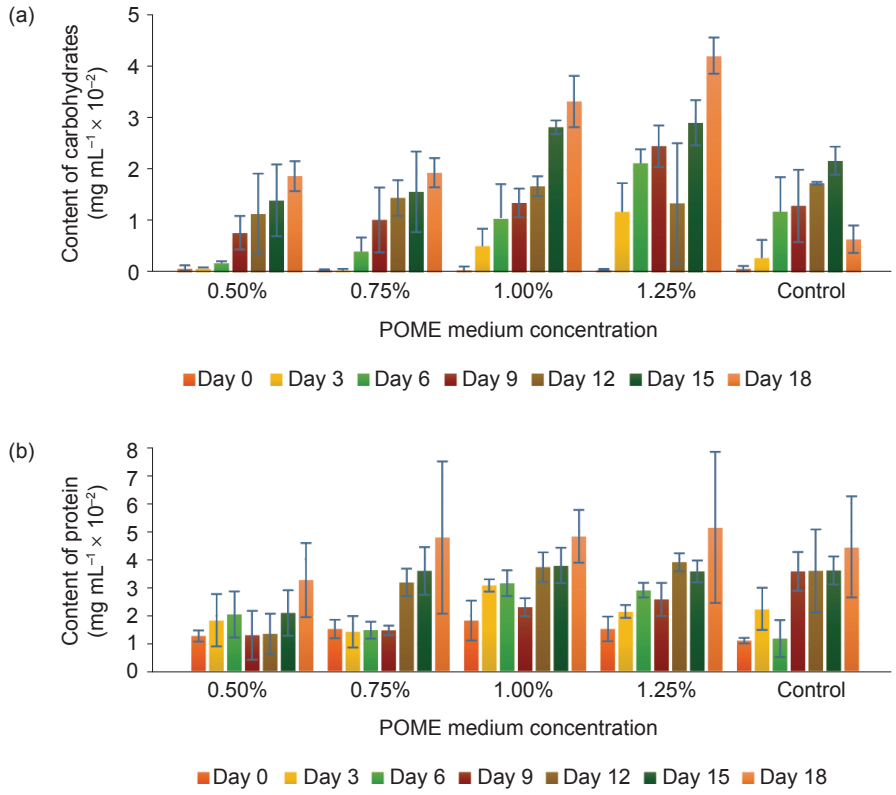
Productivity Biomass, Lipids, Carbohydrates and Proteins of *Euglena* sp.

According to *Table 3*, the treatment with a medium concentration of POME at 1.25% reached the highest biomass productivity (6.67 ± 0.85 g L^{-1}), carbohydrates (2.34 ± 0.35 mg mL^{-1}), and protein (2.86 ± 2.69 g L^{-1}). This indicates that the higher the concentration of POME medium, the higher the content of carbon, nitrogen and phosphorus, allowing *Euglena* sp. to optimally utilise it for the formation of biomass, carbohydrates, and proteins. Muttaqin and Suyono (2021) reported that mixed culture microalgae bacteria could grow up to



Source: Putri (2020).

Figure 3. (a) Biomass, and (b) lipids production of Euglena *sp. at various POME medium concentrations.*



Source: Putri (2020).

Figure 4. (a) Content of carbohydrates, and (b) content of proteins production of Euglena *sp. at various POME medium concentrations.*

TABLE 3. PRODUCTIVITY BIOMASS, LIPIDS, CARBOHYDRATES, AND PROTEINS OF *Euglena* sp. AT VARIOUS POME MEDIUM CONCENTRATIONS

Concentration medium	Productivity (x 10^{-1})			
	Biomass ($g\ L^{-1}\ day^{-1}$)	Lipids ($g\ L^{-1}\ day^{-1}$)	Carbohydrates ($mg\ mL^{-1}\ day^{-1}$)	Proteins ($\mu g\ mL^{-1}\ day^{-1}$)
Concentration 0.50%	2.33 ± 0.46	1.00 ± 0.12	1.04 ± 0.29	1.82 ± 1,32
Concentration 0.75%	3.25 ± 0.63	1.22 ± 0.31	1.08 ± 0.28	2.66 ± 2.15
Concentration 1.00%	4.33 ± 0.46	1.85 ± 0.15	1.84 ± 0.50	2.68 ± 0.94
Concentration 1.25%	6.67 ± 0.85	1.67 ± 0.10	2.34 ± 0.35	2.86 ± 2.69
Control	2.22 ± 0.22	0.93 ± 0.31	1.45 ± 0.26	2.47 ± 1.80

Source: Putri (2020).

TABLE 4. BOD AND COD BEFORE AND AFTER CULTIVATION *Euglena* sp. AT VARIOUS POME MEDIUM CONCENTRATIONS

Concentration medium	BOD ($mg\ L^{-1}$)		COD ($mg\ L^{-1}$)	
	Before cultivation	After cultivation	Before cultivation	After cultivation
Concentration 0.50%	12.4	4.26	32.2	9.48
Concentration 0.75%	6.67	2.23	34.8	10.01
Concentration 1.00%	15.8	8.86	34.8	14.5
Concentration 1.25%	14.9	6.76	36	15.2

Source: Putri (2020).

15% concentration of POME. Furthermore, *Chlorella vulgaris* and *C. pyrenoidosa* showed a higher absorbance at concentration of 50%. The POME medium concentration treatment of 1% produced the highest lipid productivity of 1.85 ± 0.15 g L^{-1}. This suggests that microalgae produce lipids when nutrient, energy, and carbon sources are limited and cellular photosynthesis mechanisms are active.

Biological Oxygen Demands (BOD) or Chemical Oxygen Demands (COD)

Biological Oxygen Demands (BOD) or Chemical Oxygen Demands (COD) decreased in the POME as the medium at the concentration of the treatment. The highest reduction of BOD was reached at concentration of 0.75% with a value of 66.70%. Whereas the lowest reduction of BOD was reached at a concentration of 1.00% with a value of 43.92%. The highest reduction of COD was reached at concentration of 0.75% with a value of 71.40%. Whereas the lowest reduction of COD was reached at a concentration of 1.00% with a value of 57.78% (*Table 4*). COD reduction in this research is higher than in the previous study by (Cheah *et al*., 2018). On the other hand, the efficiency of reducing nutrients is lower than in previous studies. Previous research reported that *C. vulgaris*, one of the microalgae, had a nutrient removal efficiency of 78.00%-86.00% for organics and inorganics, respectively (Low *et al*., 2021). Nutrient removal of microalgae occurs because microalgae can absorb nutrients and phosphorous to perform photosynthesis (Low *et al*., 2021). Furthermore, POME can be used as a suitable medium for the growth and nutrient removal of mixotrophic microalgae (Haruna *et al*., 2017; Nur and Hadiyanto, 2015).

CONCLUSION

Cultivation of *Euglena* sp. decreased BOD and COD levels in POME medium. It has the potential for the development of nutrient removal in POME using *Euglena* sp. The 1.25% POME was the best treatment because it has the highest growth rate, metabolite production, and efficient nutrient removal. Based on the Logistic modelling, the highest specific growth rate (μ) was achieved in 1.25% POME with μ 0.6894 day^{-1} and R^2 0.99. The biochemical composition of biomass, especially protein, carbohydrate and lipid, was evaluated to suggest future uses of biomass as new sources for industrial applications.

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