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# THE EFFECT OF 15% $CO_2$ INJECTION INTO POME SUPPLEMENTATION ON THE *Euglena* sp. CULTURE'S GROWTH AND LIPID PRODUCTION

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**ABSTRACT**

*The combustion of fossil fuels significantly increases atmospheric carbon dioxide ($CO_2$), contributing to global climate change. Thus, renewable biofuel resources are essential to mitigate $CO_2$ emissions.* Euglena *sp., a microalga which is capable of $CO_2$ fixation, producing biomass that can be utilized for biofuel production. Additionally, microalgae can assimilate nutrients from liquid waste, such as palm oil mill effluent (POME), which is rich in essential elements for growth and lipid synthesis as part of a biorefinery approach. This study investigated the effects of POME supplementation at concentrations of 0%, 15% and 30% under mixotrophic and heterotrophic cultivation with 15% $CO_2$ injection on the morphology, growth, biomass, and metabolite content (lipids and paramylon) of* Euglena *sp. in the laboratory-scale culture. The M. 15% treatment (mixotrophic with 15% POME) yielded the highest performance, with a cell density of 1,458 × $10^4$ cells/mL, optical density of 0.52, biomass of 0.57 g/L and lipid content of 0.42 mg/L. The highest paramylon accumulation occurred in the H. 15% treatment (heterotrophic with 15% POME), reaching 4.516 ± 0.008 mg/L/day on day 15. The morphological analysis revealed that spindle-shaped and elongated cells were found in most treatments, with heterotrophic 15% showing predominantly spindle shapes. The dominant fatty acid methyl esters (FAMEs) identified was methyl palmitoleate.*

**Keywords:** *Euglena* sp., lipid, mixotrophs-heterotroph, palm oil mill effluent, paramylon.

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## INTRODUCTION

Global climate change continues to progress due to multiple factors, including land-use change, environmental degradation, urban development and an increasing consumption of fossil fuels (Abbasi et al., 2022). Elevated atmospheric carbon dioxide ($CO_2$) concentration is a major contributor to this phenomenon (Nunes et al., 2023). Studies indicated that a 1.00% increase in fossil fuel consumption results in $CO_2$ emission increased by 0.48% for petroleum, 0.31% for coal, and 0.17% for natural gas in G7 countries (Canada, France, Germany, Italy, Japan, United Kingdom and United States) (Martins et al., 2021). Approximately 87.00% of the global $CO_2$ emissions originate from fossil fuel combustion, which is primarily used for heat, electricity generation and transportation (Vohra et al., 2021). The continuous use of fossil fuels is environmentally unfriendly, unsustainable and a source of air pollution due to incomplete combustion (Mokhtar, 2014).

The development of renewable energy resources has emerged as a promising strategy to reduce emissions and dependence on fossil fuels. Microalgae have gained significant attention as the third-generation biofuel feedstocks because they can produce high-value compounds such as carbohydrates, proteins, antioxidants and lipids

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(Pal et al., 2019; Zulkarnain et al., 2021). Their high lipid, carbohydrate and protein content makes them highly suitable for biofuel production (Ferreira et al., 2022). Lipid productivity can be optimized through cultivation method engineering that influences metabolic pathways and genetic regulation (Sathya et al., 2023). Stress conditions are also known to enhance the accumulation of storage metabolites such as carbohydrates, proteins, and lipids (Amelia et al., 2023).

The use of wastewater as a cultivation medium for microalgae represents dual strategies in enhancing lipid productivity while mitigating environmental pollution (Cheah et al., 2018). In the palm oil industry, an increased palm oil production correlates with greater volumes of palm oil mill effluent (POME). POME is rich in organic matter, with chemical oxygen demand (COD) vs. biological oxygen demand (BOD) levels reaching up to 100,000 mg/L (Low et al., 2021), posing significant environmental hazards if it is left untreated (Tan et al., 2022). An integrated anaerobic-aerobic bioreactor (IAAB), is a wastewater treatment system that combines sequential anaerobic and aerobic processes to enhance the efficiency of organic load reduction (Mohammad et al., 2021).

Microalgae, including *Euglena* sp., can utilize nutrients present in POME such as nitrogen, potassium, phosphate and sulfate (Mellyanawaty et al., 2018), while simultaneously enhancing lipid accumulation (Kuroda et al., 2018), and has the potential to be utilized for biomass fuel production with high cell extract volume (CEV) and effective POME phycoremediation (Kamyab et al., 2017; Ooi et al., 2023). *Euglena* sp. can thrive in acidic conditions (pH 2–6) (Haraguchi & Zheng, 2022) and tolerate to high nutrient concentrations (Nezbrytska et al., 2022). Moreover, they can adapt to various growth modes (autotrophic, heterotrophic and mixotrophic) (Abreu et al., 2022) and are recognized for their rapid growth even under extreme environmental conditions (O'Neill, 2020). Given these potentials, this study investigated the cultivation of *Euglena* sp. in POME with $CO_2$ injection under mixotrophic and heterotrophic conditions at the concentrations of 0%, 15% and 30%, aiming to optimize cell growth, biomass production, lipid content and paramylon accumulation.

## MATERIALS AND METHODS

### Equipment

Among the equipment and tools which were used in this study including 1 L culture bottles, 1 L Pyrex beakers, 5,000 mL Erlenmeyer flasks, 50 mL Pyrex graduated cylinders, micropipettes, Biologix yellow tips, Biologix blue tips, Olympus CX22LED light microscope, Opti Lab Viewer 4 (PT. Miconos, Indonesia), dropper pipettes, analytical balance (AND GR-200, Japan), OneMED conical tubes (15 and 50 mL), LW Scientific centrifuge, LAQUAact pH meter, laminar airflow cabinet, autoclave (TOMY ES 215), UV-*Vis* spectrophotometer (GENESYS 150), GC-FID gas chromatograph (Agilent Technologies 7890B, Column HP-88), MEMMERT water bath, vortex mixer, vacuum filtration system, magnetic stirrers, 5.0 cm diameter Petri dishes, oven (B-ONE OV-65), hot plate stirrer (H4000-HSE Benchmark), spatulas, well plates, hemocytometer, glass slides and cover slips, pipette pumps, 0.5 cm diameter tubing, tweezers and plastic wrap.

### Materials

The materials used including *Euglena sp.* strain IDN33, distilled water, POME, Cramer-Myers (CM) medium composed of materials including; $(NH_4)_2HPO_4$, $KH_2PO_4$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $Fe_2(SO_4)_3{\cdot}7H_2O$, $MnCl_2{\cdot}4H_2O$, $CoSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, $Na_2MoO_4{\cdot}2H_2O$, $CuSO_4{\cdot}5H_2O$, vitamin B1, vitamin B12, sodium dodecyl sulfate (SDS) 1%, acetone, NaOH, 5% phenol, $H_2SO_4$, KOH, HCl, 70% ethanol, spiritus, methanol, chloroform, 5% phenol, bovine serum albumin (BSA). This experimental design can be seen in *Figure 1*.

### Preparation of POME Media

The POME waste contains particles and microorganisms. To reduce POME waste, filtration was carried out using ordinary filters to reduce particles with larger sizes and then filtration was carried out using 0.45 micron filter paper to reduce smaller particles (primarily removes suspended solids and particulate-associated organic matter) and reduce existing microorganisms. This filtration step was applied to reduce turbidity and improve light penetration, thereby providing more suitable conditions for microalgal cultivation. Dębowski et al. (2025) stated that increased turbidity leads to suboptimal light distribution within the photobioreactor. Similarly, Pena et al. (2020) showed that wastewater with high turbidity limits light penetration and consequently affects the growth rate of microalgae during cultivation. Prior total organic carbon (TOC) analysis shows that the POME used in this study contain 2.425 mg/L TOC. The POME media was then sterilized using an autoclave at a temperature of 121°C for 15 min. Then, the media was formulated according to *Table 1*.

### Cultivation of *Euglena* sp.

*Euglena* sp. culture was put into a 1,000 mL culture bottle, and each culture was mixed with

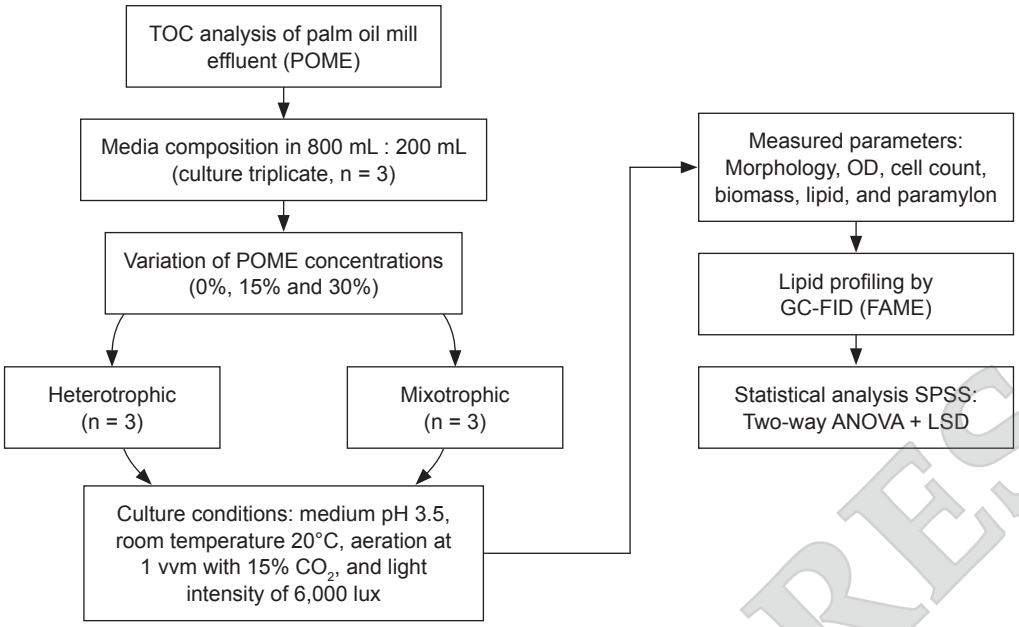



*Figure 1. Overview of the experimental design showing inoculum preparation, POME-enriched media formulation, controlled cultivation conditions and analytical measurements conducted during the study.*

**TABLE 1. POME MEDIA COMPOSITION**

| Treatment | Composition in 1,000 mL medium | | |
|---|---|---|---|
| | CM medium (mL) | POME (mL) | *Euglena* sp. culture (mL) |
| M. Control | 800 | 0 | 200 |
| M. 15% | 680 | 120 | 200 |
| M. 30% | 560 | 240 | 200 |
| H. Control | 800 | 0 | 200 |
| H. 15% | 680 | 120 | 200 |
| H. 30% | 560 | 240 | 200 |

Note: M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent; CM - Cramer-Myers.

800 mL of POME liquid waste at concentrations (0%, 15%, 30%) each by adding 200 mL of culture stock with an average optical density of 0.04. Cultivation of *Euglena* sp. was carried out for 15 days. Environmental factors to support optimal cell growth, such as light (artificial light of 6,000 lux [6,500 K] was used continuously for 24 hr throughout the experiment), aeration (composed of 85% ambient air and 15% $CO_2$), and temperature ranged between 20°C and 35°C and pH of 2.5–3.5. Mixotrophic (light conditions) and heterotrophic (dark conditions) were the cultivation methods used in this study. The calculated POME parameters were the amount of dissolved carbon at the beginning and end to determine the amount of carbon consumed during the growth. Cultures were ensured to have total volume that was not less than 85% from the original culture in the end of the experiment, to ensure the measurement of growth and metabolite from the culture was not biased due to the evaporation of water that could increase the concentration of microalgae in the culture. This method also helped prevent light stress and biological contamination (Guidi et al., 2021).

## Morphology of Cells

The cell size and morphology were examined using a microscope at 400× magnification, assisted by an optilab connected to a laptop. A 1 mL sample was utilized to assess the cell size and morphology. Image Raster 3 software was used to calculate the size of the cells. Measurements were taken on the most extended dimensions (length and width) of each *Euglena* sp. cell within each treatment (Jeon et al., 2017). From the acquired images of cell morphology, random samples were chosen to determine the aspect ratio (AR) (Kim et al., 2021). The AR value was derived by dividing the length of the central axis, which represented the primary axis of the ellipse, by the size of the minor axis or the secondary axis of the ellipse. The cells were then classified into different shapes based on their AR

values (Jeon et al., 2019). AR < 1.5: Classified into rounded shape (spherical), 1.5 ≤ AR ≤ 5: Classified into spindle shape (spindle) and AR > 5: Classified into elongated shape (elongated).

**Cell Count**

Cell density was counted daily during the cultivation period using a Hemocytometer observed under a microscope with the help of Optilab Viewer 4 and counted using a hand counter with a magnification of 4 × 10 (Indahsari et al., 2022). The culture solution in the microtube was fixed with 100 µL of alcohol, then up and down with a micropipette and tip; the solution was dropped 10 µL into the gap between the hemocytometer and the cover glass. The number of *Euglena* sp. microalgae cells were counted using a hand counter by looking at the cells in four blocks containing 16 large boxes. Total cells of *Euglena* sp. were calculated using the Equation (1):

$$\underset{(\text{Cells}/\text{mL})}{\text{Cells number}} = \frac{\text{Dilution factor}}{\text{Number chamber (4)}} \times 10^4 \qquad (1)$$

**Biomass Measurement**

Biomass measurements were carried out every three days during the cultivation period, starting from day 0 to the last day. A sample of 15 mL was taken and placed in a test tube. Previously, the filter paper was weighed using an analytical balance. The sample was then poured into Whatman glass grade GF/C 47 mm microfiber filter paper using a vacuum pump kit (Amelia et al., 2023). The remaining culture that settled around the filter cup was rinsed with sufficient distilled water. The filter paper was then dried in an oven for 60 min at a temperature of 100ºC, after which it was weighed using an analytical balance. Biomass content data was obtained by calculating the difference in filter paper weight. Dry weight measurements were calculated using the Equation (2) (Husna et al., 2020).

$$\underset{(\text{mg}/\text{mL})}{\text{Biomass}} = \frac{\text{final weight} - \text{initial weight}}{\text{sampel volume}} \qquad (2)$$

**Measurement of Lipid Content**

The lipid content test in microalgae aimed to determine the amount of fat or oil produced by microalgae according to the stages of the lipid test according to Bligh & Dyer (1959). Lipid content was measured every three days to capture the major physiological changes associated with different growth phases of microalgae. Empty Petri dishes were weighed using an analytical balance, 15 mL of microalgae samples were centrifuged at 6,000 × g for 15 min, resulting in supernatant (liquid) and pellet (precipitate). The supernatant was discarded and the remaining pellet was added with 2 mL of methanol and 1 mL of chloroform which were homogenized using a vortex for 1 min. Add 1 mL of distilled water and 1 mL of chloroform; the solution was homogenized and centrifuged at 2,700 × g for 15 min. The centrifugation results formed three layers. The bottom layer was separated from the other layers and then placed in a Petri dish. The Petri dish was placed in an incubator for approximately 1 hr until all the chloroform evaporates and the Petri cloud was weighed. Lipid content data was obtained by finding the difference in the Petri dish's weight. The Equation (3) is for calculating total lipid weight (Husna et al., 2020).

$$\underset{(\text{mg}/\text{L})}{\text{Lipid}} = \frac{\text{final weight lipid petri} - \text{initial weight lipid petri}}{\text{sample volume}} \qquad (3)$$

Lipid content represents the amount of lipid accumulated in the microalgal biomass in the average of the 15-day cultivation period, whereas lipid productivity reflects the rate of lipid production per unit time. The lipid productivity values appear similar to lipid content because the cultivation duration was constant and biomass growth was relatively uniform across treatments, making lipid productivity directly proportional to lipid accumulation.

Lipid productivity was calculated through the Equation (4):

$$\underset{(\text{mg}/\text{L}/\text{day})}{\text{Productivity lipid}} = \frac{(\Delta x)}{t} \qquad (4)$$

where $\Delta x$ is the difference between the highest number of days - day 0 and $t$ = the highest day.

Lipid content was calculated through the Equation (5):

$$\underset{(\text{mg}/\text{L})}{\text{Lipid content}} = \frac{L_{highest} - L_0}{t_{highest}} \qquad (5)$$

where, $L_0$ represents the lipids content measured on the first day of each treatment, $L_{highest}$ refers to the maximum lipids content recorded during each treatment and $t_{highest}$ indicates the specific day on which the highest lipids content is observed in each treatment.

**Extraction of Paramylon**

Paramylon extraction was performed every three days following the modified method of Takahashi et al. (2023). A 20 mL aliquot of *Euglena* sp. culture was centrifuged at 6,000 × g for 10 min and the supernatant was discarded. The resulting pellet was mixed with 1.5 mL of acetone and vortexed for 1 min, followed by centrifugation at 9,600 × g for 3 min. The supernatant was removed, and the pellet was washed again with 1.5 mL of acetone, vortexed for 1 min and centrifuged under the same conditions. The pellet was then suspended in 1.5 mL of 1% SDS solution, vortexed for 1 min and incubated in a water bath at 95°C for 30 min. After vortexing for 5 min, the sample was centrifuged at 9,600 × g for 3 min and the supernatant was discarded. The pellet was washed with 1.5 mL of distilled water, vortexed for 1 min and centrifuged at 9,600 × g for 3 min, after which the supernatant was removed. This washing step was repeated three times. The final pellet was suspended in 1 mL of 0.5 N NaOH and incubated at room temperature overnight.

Average paramylon represented the mean level of paramylon accumulated in the microalgal biomass over the 15-day cultivation period, whereas paramylon productivity reflected the rate of paramylon formation per unit time. Although productivity is a rate-based parameter, its value appears nearly identical to the average paramylon because the cultivation period was constant and paramylon accumulation occurred relatively steadily. Under these conditions, paramylon productivity becomes directly proportional to the average paramylon content rather than reflecting large kinetic differences.

**Quantification of Paramylon**

Paramylon quantification was conducted every three days using the method of Dubois et al. (1956). The NaOH-incubated pellet suspension was vortexed for 1 min and 0.5 mL of the sample was transferred into a test tube. An equal volume (0.5 mL) of 5% phenol solution was added, followed by the addition of 2.5 mL of concentrated $H_2SO_4$. The mixture was incubated at a room temperature for 20 min, then gently agitated to ensure proper reaction. After an additional 10 min of incubation, 2 mL of the mixture was transferred into a glass cuvette and absorbance was measured at 480 nm using a spectrophotometer.

Paramylon productivity was calculated through the Equation (6):

$$\text{Paramylon productivity } (\text{g/L/day}) = \frac{(\Delta x)}{t} \quad (6)$$

where $\Delta x$ is the difference between the highest number of days – day 0 and $t$ refers to the highest day.

Pararamylon content was calculated through the Equation (7):

$$\text{Paramylon content } (\text{g/L}) = \frac{P_{highest} - P_0}{t_{highest}} \quad (7)$$

where, $P_0$ represents the amount of paramylon measured on the first day of each treatment, $P_{highest}$ refers to the maximum amount of paramylon recorded during each treatment, and $t_{highest}$ indicates the specific day on which the highest paramylon content is observed in each treatment.

**Fatty Acid Analysis Using GC-FID**

The gas chromatography-flame ionization detector (GC-FID) method is a highly effective and commonly used technique in chemical and industrial laboratories for analyzing the profiles of saturated and unsaturated fatty acids. The GC-FID analysis in this study was conducted at the Integrated Research and Testing Laboratory (LPPT), Gadjah Mada University, Indonesia. The GC-FID procedure consisted of two main stages: hydrolysis and methylation, following method from Breuer et al. (2013) with little modification. Fatty acid methyl esters (FAMEs), including methyl palmitoleate, were analyzed using a GC-FID system. The GC system utilized an HP-88 (or equivalent) capillary column measuring 100 m × 0.25 mm internal diameter × 0.2 µm film thickness. Helium served as the carrier gas at a constant flow rate of 1 mL/min. Samples were injected in split mode with a split ratio of 50:1 and an injection volume of 1 µL. The oven temperature program was set at an initial temperature of 120°C (held for 2 min), increased at 3°C/min to 220°C and then held at 220°C for 5 min. The injector and detector temperatures were maintained at 250°C and 260°C, respectively.

**Statistical Analysis**

The growth rate, biomass, lipid and paramylon content of *Euglena* sp. were analyzed using two-way analysis of variance (ANOVA) and mean differences were analyzed using Duncan Multiple Range Test (DMRT) using IBM Statistical Product and Service Solutions (SPSS) software (Version 26, IBM Corporation, USA). Data significance was determined by a *p*-value of 0.05 at a 95% significance level.

## RESULTS AND DISCUSSION

### Morphology of Cells

As one of the most extensively distributed living things on earth, microalgae can survive in harsh conditions. As the first barrier in constant contact with the surrounding and changing environment, the microalgal cell wall is particularly important in these types of environments. Phenotypic plasticity, or an organism's capacity to exhibit distinct phenotypes in response to varying environmental conditions, is the ability of microalgae to exhibit morphological changes in cold environments (Gonzáles-Hourcade et al., 2023). The morphology of *Euglena* sp. cells after cultivation in various cultivation methods and POME concentrations can be seen in *Figure 2* and *Table 2*.

*Euglena* sp. cells are characterized by a round shape and green color, containing eye spots as photoreceptors. *Euglena* sp. cells cultivated using POME have a length of 20.75 µm. According to morphological observations, our results show that *Euglena* sp. cells are round, with a cell length of around 14.89–24.12 µm with an average range of >1.5–<5 µm, namely at 4.33 µm, which means that *Euglena* sp. cells have a spindle shape (Jeon et al., 2019). These results are similar to the previous findings by Maghfiroh et al. (2023) who showed that *Euglena* sp. cells were cylindrical or spindle-shaped, slightly thin and rounded. In the treatment E, the shape is different compared to other treatments where under heterotrophic conditions, cells become more rounded with high paramylon accumulation (up to 80% of biomass), lack chloroplasts and develop transparent granules from degraded thylakoids due to low photosynthesis (Grimm et al., 2015; Kashiyama et al., 2016). The thylakoid membrane quickly disappears after the cells undergo heterotrophic metabolism. *Euglena* sp. cells become rounded under heterotrophic conditions due to physiological stress caused by the absence of light, leading to the formation of spherical cysts as an adaptive response to environmental pressure (Yoshioka et al., 2020). The growth environment, including the light cycle, also affects *Euglena* sp. morphology and metabolite production, indicating that external stressors can influence the shape (Barsanti et al., 2023). The lowest aspect ratio

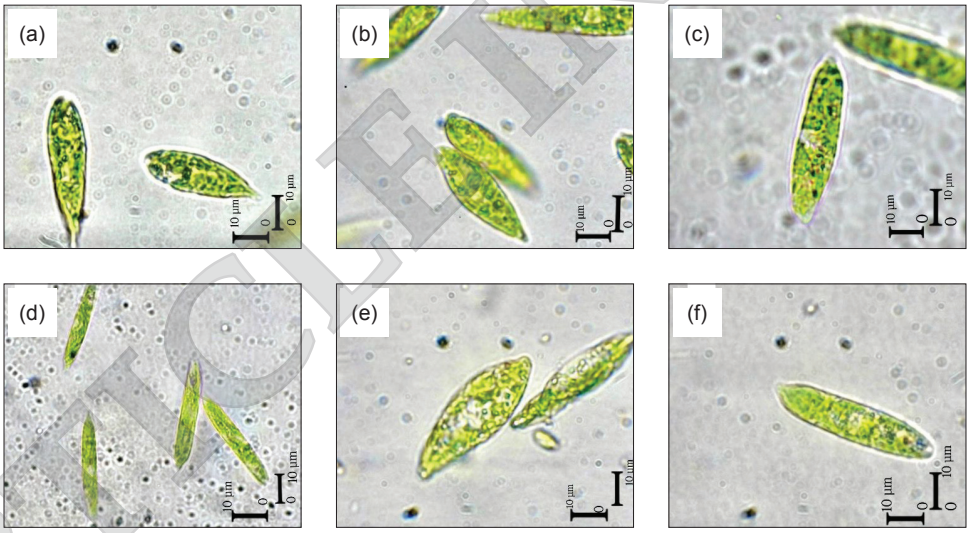



*Figure 2.* Euglena *sp. cell shape. Each treatment was for 15 days of cultivation. In various treatments and POME concentrations respectively, (a) mixotrophic control, (b) mixotrophic POME 15%, (c) mixotrophic POME 30%, (d) heterotrophic control, (e) heterotrophic POME 15% and (f) heterotrophic POME 30%.*

**TABLE 2.** ***Euglena*** **sp. CELL SHAPES IN VARIOUS TREATMENTS FOR 15 DAYS OF CULTIVATION**

| Treatment | Aspect ratio (AR) | Cell shape |
|---|---|---|
| M. Control | 4.62–5.53 | Spindle-elongated |
| M. POME 15% | 3.37–6.69 | Spindle-elongated |
| M. POME 30% | 3.12–5.44 | Spindle-elongated |
| H. Control | 4.10–5.91 | Spindle-elongated |
| H. POME 15% | 2.63–4.25 | Spindle |
| H. POME 30% | 4.25–5.07 | Spindle-elongated |

Note: M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

observed under heterotrophic cultivation with POME indicates a more compact and rounded cell morphology. Under heterotrophic conditions, microalgae rely on dissolved organic carbon rather than light, eliminating the need to increase cell surface area for light absorption and resulting in reduced cell elongation. Within the light intensity range applied in this study, illumination promoted slightly higher aspect ratios under autotrophic or mixotrophic conditions, while no stress-induced morphological changes were observed.

**Growth Rate**

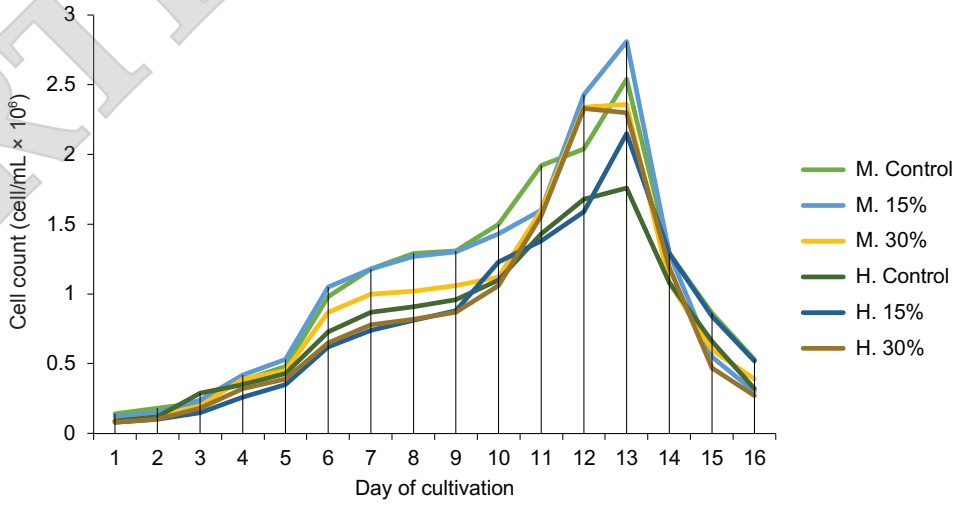
The presence of nitrogen-rich POME promotes cell enlargement in *Euglena* sp. by stimulating cell division, elongation, and differentiation (Khan et al., 2023). Under mixotrophic conditions, cells exhibit greater growth due to the utilization of organic carbon, with increased volume resulting from starch and lipid accumulation (Baldisserotto et al., 2012). As shown in *Figure 3*, the mixotrophic cultures in this study demonstrated a higher growth rate compared to heterotrophic cultures, indicating that *Euglena* sp. can optimize growth under various conditions.

Mixotrophy, which combines both inorganic and organic carbon sources, effectively enhances cell proliferation and biomass production (Wang et al., 2020). In such cultures, photosynthesis and aerobic respiration operate simultaneously, sharing certain metabolites between pathways (Zhang et al., 2022). Growth rate was not justified solely based on *Euglena* cell shape. Morphological changes (aspect ratio) are interpreted as adaptive responses to metabolic conditions and carbon availability, while growth rate is primarily governed by the metabolic mode and carbon utilization. The cell growth in this study was measured also by cell density and biomass. The cell density profile from day 1 to day 15 is presented below.

Cell count (cell/mL × $10^6$) was determined using a hemocytometer. As shown in *Figure 3*, the highest cell count was observed in the mixotrophic treatment with 15% concentration (1.458 × $10^4$ cells/mL), following by the mixotrophic control (1.061 × $10^4$ cells/mL), mixotrophic 30% (0.944 × $10^4$ cells/mL), heterotrophic 30% (0.920 × $10^4$ cells/mL), heterotrophic 15% (0.920 × $10^4$ cells/mL) and heterotrophic control (0.707 × $10^4$ cells/mL). *Euglena* sp. utilized the phosphate and nitrate contained in the POME as nutrient sources to support cell division. POME contains high levels of nitrate, phosphorus and dissolved carbon, thus cell growth correlates positively with the increasing concentration of POME in the medium.

In the 15% mixotrophic treatment, the growth phase progressed more rapidly because *Euglena* sp. obtained two carbon sources: $CO_2$ aeration at 15% and dissolved organic carbon from POME, resulting in a high growth rate. According to Gao et al. (2022), optimal biomass production can be achieved under mixotrophic cultivation conditions by supplying organic carbon sources to microalgae. The addition of organic carbon enhances biomass productivity by improving growth efficiency and cell density. The $CO_2$ will experience dissolution as the carbon get in contact with water, forming carbonic acid ($H_2CO_3$). Along with time, carbonic acid will also dissociate into $H^+$, $HCO_3^-$ and $CO_3^{2-}$ (Knoche, 1980).

Based on two-way ANOVA, the integration of CM medium with POME wastewater under mixotrophic and heterotrophic cultivation modes showed a statistically significant effect ($p$ = 0.00 < 0.05). This result is supported by the specific growth rate (SGR) values presented in *Table 3*. The




Note: M - mixotroph condition; H - heterotroph condition.

*Figure 3. The cell counts of* Euglena *sp. after cultivation in various cultivation methods and POME concentrations.*

**TABLE 3. SPECIFIC GROWTH RATE (μ) AND DOUBLING TIME (Dt) OF *Euglena* sp. AT VARIOUS POME CONCENTRATIONS AND CULTIVATION METHODS**

| Treatment | Specific growth rate (μ) ± SD* (cell/mL/day) | Doubling time (Dt) ± SD* (cell/mL/day) |
|---|---|---|
| M. Control | $0.480 \pm 0.027^{a}$ | $1.443 \pm 0.084^{c}$ |
| M. 15% | $0.889 \pm 0.054^{d}$ | $0.787 \pm 0.033^{ab}$ |
| M. 30% | $0.611 \pm 0.043^{bc}$ | $1.135 \pm 0.084^{ab}$ |
| H. Control | $0.531 \pm 0.053^{ab}$ | $1.312 \pm 0.132^{bc}$ |
| H. 15% | $0.673 \pm 0.031^{c}$ | $1.030 \pm 0.048^{ab}$ |
| H. 30 % | $0.824 \pm 0.075^{d}$ | $0.844 \pm 0.080^{ab}$ |

Note: * - Values followed by the same letter indicate no significant difference based on Duncan's test in the same column; M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

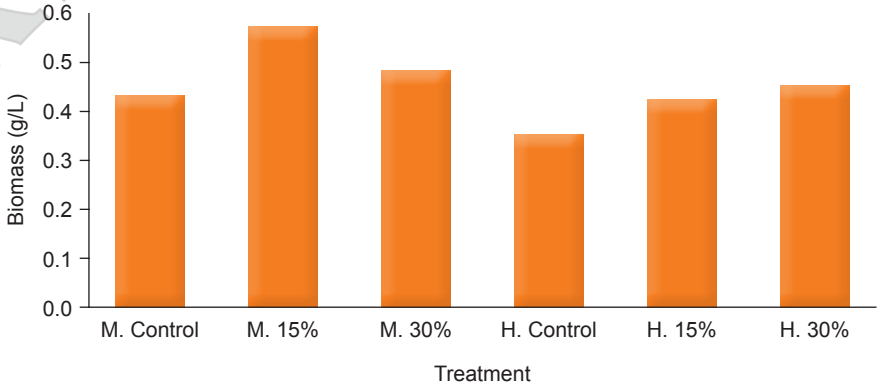
highest SGRs were observed in the M. 15% and H. 30% treatments, reaching 0.889 ± 0.054 cell/mL/day and 0.824 ± 0.075 cell/mL/day, respectively. Consequently, these treatments exhibited the shortest doubling times, with values of 0.787 ± 0.033 cell/mL/day for M. 15% and 0.844 ± 0.080 cell/mL/day for H. 30%. These findings indicate a strong interaction between cultivation mode and the POME concentration in influencing the growth of *Euglena* sp. This interaction was particularly evident under mixotrophic cultivation with 15% POME supplementation, which provided an optimal growth environment. The balanced nutrient composition of the POME supports both photosynthetic and metabolic activities, resulting in a high growth rate and reduced doubling time.

**Biomass**

Cell biomass was determined based on the dry weight over a 15-day cultivation period, as shown in *Figure 4*. The highest biomass yield was observed in the 15% mixotrophic treatment on day 12, reaching 0.57 g/L, followed by the 30% mixotrophic treatment (0.48 g/L), 30% heterotrophic treatment (0.45 g/L), mixotrophic control (0.43 g/L), 15% heterotrophic treatment (0.42 g/L), and heterotrophic control (0.35 g/L) (*Figure 4*). The increase in biomass was directly proportional to the increase in cell density. Accordingly, the dry biomass of the mixed cultures also increased with higher POME concentrations in the medium, consistent with the cell density results.

Two-way ANOVA revealed that cultivation mode, substrate concentration and their interaction significantly affected biomass production ($p = 0.00 < 0.05$). This indicates that biomass accumulation was determined by both individual factors and their combined effect. Duncan's test showed that the mixotrophic treatment with 15% substrate (M. 15%) produced the highest biomass on day 12 (3.973 ± 0.146 g/L), significantly different from all other treatments. This suggests that mixotrophic metabolism at moderate substrate levels optimizes carbon utilization by combining photosynthetic and organic carbon assimilation (*Table 4*).

The heterotrophic treatment at 30% (H. 30%) resulted in 3.570 ± 0.09 g/L, significantly lower than M. 15% but higher than the control. Meanwhile, the heterotrophic control of 2.903 ± 0.15 g/L showed the lowest biomass, indicating limited growth under insufficient carbon




Note: M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

*Figure 4. The dry weight of* Euglena *sp. cell biomass in various cultivation methods and POME concentrations.*

availability. Overall, mixotrophic cultivation at 15% substrate concentration was the most effective condition for enhancing biomass production on day 12.

**TABLE 4. BIOMASS CONTENT OF *Euglena* sp. CELLS IN VARIOUS CULTIVATION METHODS AND POME CONCENTRATIONS**

| Treatment | Biomass productivity (g/L/day) ± SD* |
|---|---|
| M. Control | 3.356 ± 0.101[b] |
| M. 15% | 3.973 ± 0.146[c] |
| M. 30% | 2.900 ± 0.153[a] |
| H. Control | 2.903 ± 0.156[a] |
| H. 15% | 3.380 ± 0.201[b] |
| H. 30 % | 3.570 ± 0.091[b] |

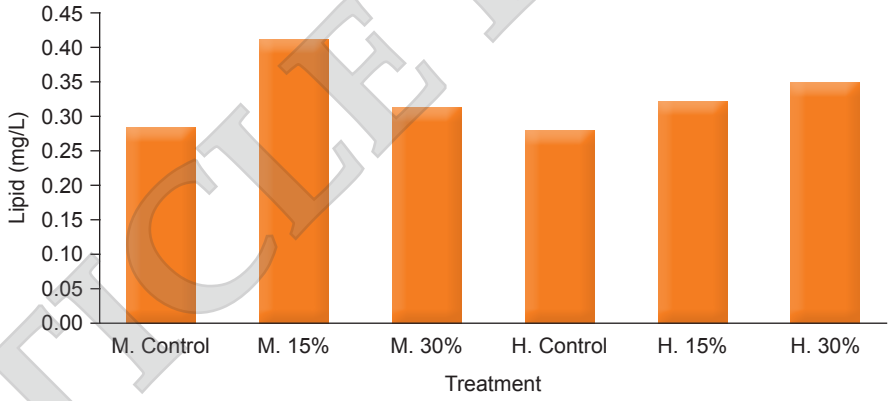
Note: * - Values followed by the same letter within the same column are not significantly different according to Duncan's Multiple Range Test (DMRT); M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

## Lipid

In this study, the effect of the POME supplementation on the lipid productivity was found to be optimal under the 15% mixotrophic treatment compared to other treatments, reaching 0.406 ± 0.011 mg/L/day, followed by the 30% mixotrophic treatment (0.340 ± 0.010 mg/L/day), 15% heterotrophic treatment (0.316 ± 0.014 mg/L/day), mixotrophic control (0.300 ± 0.010 mg/L/day) and the lowest lipid production observed in the 30% heterotrophic treatment (0.273 ± 0.006 mg/L/day) and heterotrophic control (0.273 ± 0.015 mg/L/day). Based on two-way ANOVA and DMRT analyses, the total lipid content in the medium supplemented with 15% POME under the mixotrophic method was significantly different from the control. Similarly, heterotrophic treatments supplemented with the POME also showed significant differences compared to the control. These results are presented in *Figure 5*.

The biomass and lipid content were used to identify strains with the most efficient nutrient uptake to optimize the lipid production. In other words, high biomass yield was positively correlated with high lipid production and directly proportional to cell density and biomass accumulation. In *Euglena* sp., lipid production naturally increases under stress conditions. Furthermore, sufficient nutrient availability for growth also influences the production of metabolites.

*Table 5* presents the mean for lipid content during 15 days of observation for each treatment, showing significant differences based on ANOVA




Note: M - mixotrophic condition; H - heterotrophic condition.

*Figure 5. Lipid content of* Euglena *sp. in various cultivation methods and POME concentrations.*

**TABLE 5. TOTAL LIPID CONTENT AND LIPID PRODUCTIVITY OF *Euglena* sp. UNDER VARIOUS CULTIVATION METHODS AND POME CONCENTRATIONS DURING 15 DAYS OF CULTIVATION**

| Treatment | Mean lipid content (mg/L) ± SD* | Lipid productivity (mg/L/day) ± SD* |
|---|---|---|
| M. Control | 0.320 ± 0.01[b] | 0.300 ± 0.010[b] |
| M. 15% | 0.426 ± 0.011[d] | 0.406 ± 0.011[d] |
| M. 30% | 0.360 ± 0.01[c] | 0.340 ± 0.010[c] |
| H. Control | 0.293 ± 0.015[a] | 0.273 ± 0.015[a] |
| H. 15% | 0.336 ± 0.015[b] | 0.316 ± 0.014[b] |
| H. 30 % | 0.293 ± 0.005[a] | 0.273 ± 0.006[a] |

Note: * - Values followed by the same letter within the same column are not significantly different according to Duncan's Multiple Range Test (DMRT); M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

analysis. Based on *Table 5* the highest average of total lipid content was obtained in mixotrophic cultivation with 15% POME (0.426 ± 0.01 mg/L), whie the lowest was observed in the heterotrophic control and H. 30% (0.293 ± 0.005 mg/L), with no significant difference between them. The mixotrophic cultivation at all concentrations had a significant and optimal effect on the lipid levels, supported by higher $CO_2$ and light supply, which enhanced acetyl-CoA synthesis, lipid productivity and growth (Qiu et al., 2017; Wu et al., 2022). Organic carbon and nitrogen sources from wastewater also contributed to increased biomass and lipid production (Coronado-Reyes, 2022; Singh et al., 2014).

Conversely, the heterotrophic cultivation resulted in a lower lipid content due to energy allocation for cell division, low light intensity and reduced expression of key lipid synthesis genes (Huang et al., 2022; Wang et al., 2022). Furthermore, limited carbon precursors (acetyl-CoA and glycerol-3-P) under the heterotrophic conditions restricted lipid accumulation compared to photoautotrophic cultivation.

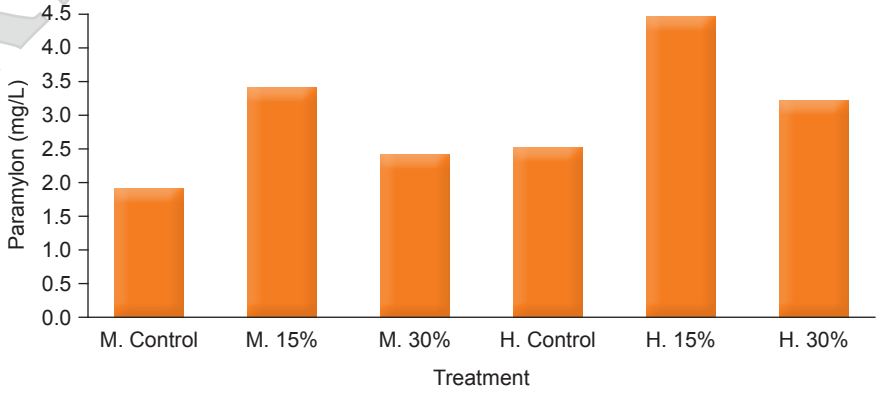
**Paramylon**

Paramylon content was measured every three days over a 15-day cultivation period, starting from day 0. As shown in *Figure 6*, the highest paramylon productivity in *Euglena* sp. was observed under the heterotrophic conditions, reaching 4.477 mg/L/day on day 12 of cultivation. Generally, paramylon accumulates during the exponential growth phase and is subsequently utilized by the cells upon entering the stationary phase (Kim et al., 2022).

The mixotrophic control treatment had the lowest value, namely 1.924 mg/L/day. The heterotrophic treatment provided a significant and optimal effect on obtaining the highest paramylon levels because, when compared, the results were significantly different from other treatments. *Euglena* sp. tends to accumulate paramylon in dark conditions and consume paramylon under the light (Zeng et al., 2016). Higher paramylon content can be obtained under heterotrophic conditions (up to 85% paramylon with organic carbon sources, $CO_2$ and in the absence of light) (Wang et al., 2023). In light cultures, paramylon content tends to be lower (Grimm et al., 2015).

Based on *Figure 6* the average total paramylon content of *Euglena sp.* cultivated with POME reached its highest level on day 12. The heterotrophic treatment yielded significantly higher paramylon levels compared to mixotrophic cultivation, as confirmed by ANOVA analysis (*Table 5*). The highest productivity was obtained in mixotrophic POME 15% (4.466 ± 0.012 mg/L/day), followed by mixotrophic POME 30% (3.426 ± 0.008 mg/L/day) and heterotrophic POME 30% (3.746 ± 0.018 mg/L/day). The lowest was observed in the mixotrophic control (1.904 ± 0.003 mg/L/day).

ANOVA analysis revealed a significant difference ($p = 0.00 < 0.05$) among treatments on paramylon production in *Euglena* sp., indicating that cultivation mode and POME concentration significantly influenced paramylon accumulation and productivity. This is evidenced by the results of paramylon production and paramylon productivity in *Table 6*. The highest paramylon production results were found in the 15% POME mixotrophic treatment, reaching 4.516 ± 0.008 mg/L/day. The next highest paramylon yield was followed by the 15% POME mixotrophic treatment of 3.449 ± 0.0005$^c$ mg/L/day, then the 30% POME heterotrophic treatment reached 3.267 ± 0.0007 mg/L/day, followed by the heterotrophic control reaching 2.567 ± 0.014 mg/L/day, the 30% POME mixotrophic 2.456 ± 0.0019 mg/L/day and the lowest treatment to produce paramylon was the mixotrophic control of 1.924 ± 0.0002 mg/L/day.




Note: M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

*Figure 6. Paramylon content of* Euglena *sp. in various cultivation methods and various POME concentrations.*

The higher paramylon content under the heterotrophic conditions was supported by sufficient organic carbon availability, such as glucose, which serves as a precursor for paramylon synthesis from UDP-glucose (Sveráková et al., 2021, Uliesther et al., 2023). Previous studies reported that *Euglena* under heterotrophy can accumulate up to 67% paramylon (Ivusic et al., 2022), whereas in light-exposed cultures, levels are lower because paramylon is utilized for protein and membrane lipid synthesis (Feuzing et al., 2022; Grimm et al., 2015).

Furthermore, paramylon accumulation is closely related to the exponential growth phase, when high metabolic activity promotes the synthesis of energy reserves (Kim et al., 2023). In this study, the heterotrophic treatment achieved the highest paramylon content in parallel with optimal cell growth, as increased availability of organic carbon sources enhances carbon assimilation and storage pathways in *Euglena* species (Xiao et al., 2025). $CO_2$ injection further contributed to increased paramylon accumulation, since elevated $CO_2$ concentrations enhance carbon fixation efficiency, leading to greater biomass and storage carbohydrate accumulation (Yuan et al., 2024). Therefore, the combination of heterotrophic conditions, POME supplementation as an organic carbon source, and $CO_2$ injection effectively maximized paramylon accumulation by increasing intracellular carbon availability and flux toward storage metabolites (Erfianti et al., 2025).

$CO_2$ availability not only influences microalgal growth but also plays a crucial role in regulating metabolic pathways associated with primary and secondary metabolite productions. Elevated $CO_2$ supply enhances carbon fixation and biomass accumulation through intensified photosynthetic metabolism and gene expression related to carbon assimilation (Yuan et al., 2024). Simultaneously, an increased intracellular carbon flux can be redirected toward the synthesis of storage and secondary metabolites, such as lipids and paramylon, especially under mixotrophic or heterotrophic conditions where both inorganic and organic carbon sources are available (Xiao et al., 2025).

Paramylon cover larger percentage of microalgal biomass compared to lipid in this study. Even so, the majority of metabolites in this research still need significant improvements, as paramylon used to be found in dry biomass of *Euglena gracilis* for up to 55% (Yan et al., 2023). While lipid content should be around 20%–30% of dry biomass from *E. gracilis* (Jung et al., 2021). Therefore, mode of metabolite production and extraction need to be improved in near future to balance the potentials of *Euglena* as bioremediation agents and valorization of products.

**TABLE 6. TOTAL PARAMYLON CONTENT AND PARAMYLON PRODUCTIVITY OF *Euglena* sp. UNDER DIFFERENT CULTIVATION METHODS AND POME CONCENTRATIONS**

| Treatment | Average paramylon (mg/L) ± SD* | Paramylon productivity (mg/L/day) ± SD* |
|---|---|---|
| M. Control | $1.924 \pm 0.0002^{a}$ | $1.904 \pm 0.003^{a}$ |
| M. 15% | $3.449 \pm 0.0005^{c}$ | $3.426 \pm 0.008^{c}$ |
| M. 30% | $2.456 \pm 0.0019^{b}$ | $2.436 \pm 0.026^{b}$ |
| H. Control | $2.567 \pm 0.014^{b}$ | $2.545 \pm 0.22^{a}$ |
| H. 15% | $4.516 \pm 0.008^{d}$ | $4.466 \pm 0.12^{e}$ |
| H. 30 % | $3.267 \pm 0.0007^{c}$ | $3.746 \pm 0.18^{d}$ |

Note: * - Values followed by the same letter within the same column are not significantly different according to Duncan's Multiple Range Test (DMRT); M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

**TABLE 7. PERCENTAGE OF LIPID AND PARAMYLON COMPARED TO BIOMASS OF *Euglena* sp. UNDER DIFFERENT CULTIVATION METHODS AND POME CONCENTRATIONS**

| Treatment | Lipid content (%) | Paramylon content (%) |
|---|---|---|
| M. Control | 0.0007 | 0.0045 |
| M. 15% | 0.0007 | 0.0060 |
| M. 30% | 0.0008 | 0.0051 |
| H. Control | 0.0008 | 0.0073 |
| H. 15% | 0.0008 | 0.0107 |
| H. 30 % | 0.0007 | 0.0072 |

Note: M - mixotrophic condition; H - heterotrophic condition; POME - palm oil mill effluent.

In this study, $CO_2$ injection promoted both cell growth and metabolite production in *Euglena* sp., indicating a close coupling between carbon assimilation and metabolic allocation. These findings are consistent with previous reports showing that optimal $CO_2$ concentrations enhance not only growth but also the accumulation of valuable metabolites by improving intracellular carbon balance and metabolic efficiency (Suzuki et al., 2017; Wu et al., 2022).

**Fatty Acids Profile**

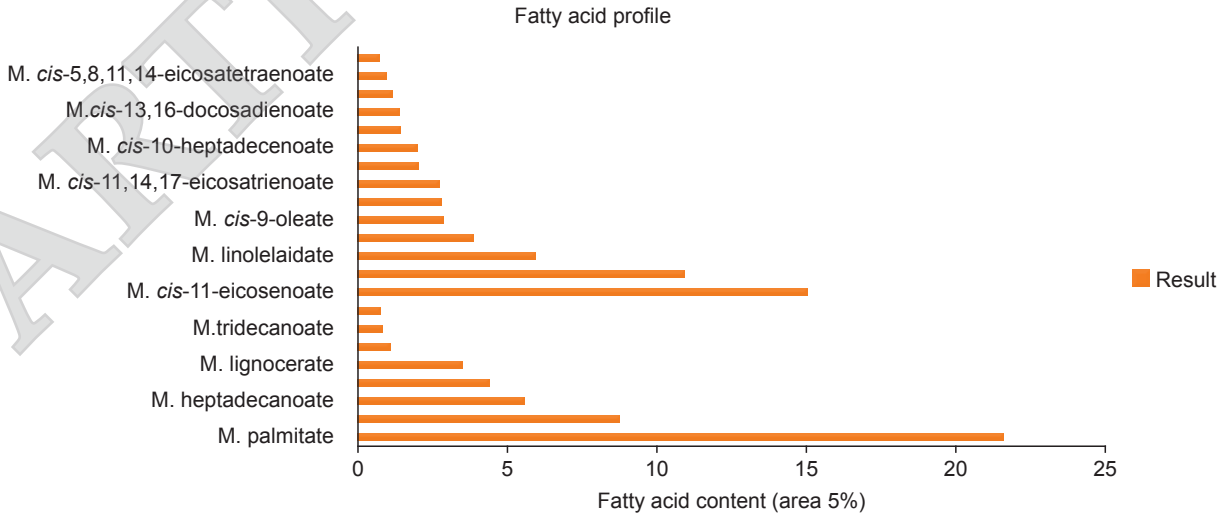
The mixotrophic treatment with 15% POME produced 22 types of fatty acids, consisting of 46.39% saturated fatty acids (SFA), 25.23% monounsaturated fatty acids (MUFA) and 27.81% polyunsaturated fatty acids (PUFA). The highest content was found in methyl palmitoleate (21.60%), while the lowest was methyl pentadecanoate (0.74%), with several fatty acids detected at concentrations below 0.1% (*Figure 7*). A higher lipid production occurred under light conditions, which also influenced the fatty acid profile. Factors such as species, nutrient availability, temperature, and stress conditions also affect lipid accumulation (Zhang et al., 2022).

Under the mixotrophic conditions, *Euglena* sp. achieved the highest lipid productivity of 4.516 ± 0.008 mg/L. The use of POME as a culture medium significantly enhanced fatty acid production due to its nutrient richness. In the mixotrophic cultivation, *Euglena* sp. can produce PUFA such as eicosapentaenoic acid (EPA) and arachidonic acid (ARA). The addition of glucose or glycerol to the medium has been reported to significantly increase lipid synthesis compared to photoautotrophic conditions (He et al., 2022).

The absence of certain methyl fatty acid derivatives, including methyl butyrate, methyl hexanoate, and methyl *cis*-11,14-eicosadienoic, may be attributed to the lack of specific micronutrients serving as their biosynthetic precursors. Several identified SFAs (such as methyl palmitate, methyl myristate, methyl *cis*-11-eicosenoate and methyl linoleate) are promising biodiesel feedstocks due to their high stability and productivity (Kilic, 2025). Fatty acids considered ideal for biodiesel include palmitic acid, stearic acid, methyl *cis*-10-heptadecenoate, linoleic acid and linolenic acid (Jafarihaghighi et al., 2022). Biodiesel is typically produced through the catalytic transesterification of triglycerides with short-chain alcohols under specific reaction conditions.

## CONCLUSION

This study highlights the innovative utilization of POME as a cost-effective substrate to enhance the cultivation of *Euglena* sp. The most favorable growth response was observed under the mixotrophic conditions with 15% POME, yielding a cell density of $1.458 \times 10^4$ cells/mL, an optical density of 0.52, a biomass concentration of 0.57 mg/mL, and a lipid content of 0.42 mg/mL. In contrast, the highest paramylon accumulation was recorded under the heterotrophic conditions with 15% POME, reaching 4.516 ± 0.008 mg/L/day on day 15. Morphological analysis revealed that spindle-shaped and elongated cells were found across all mixotrophic treatments, spindle-shaped and elongated forms under the heterotrophic conditions at 0% and 30%, and predominantly spindle-shaped cells at 15% POME. Furthermore, methyl palmitoleate was identified as the most dominant fatty acid. Collectively, these




Note: M - methyl.

*Figure 7. Fatty acid profile of* Euglena *sp. at the optimal concentration, showing lipid metabolites produced under mixotrophic cultivation with 15% palm oil mill effluent (POME).*

findings underscore the dual function of POME supplementation in promoting microalgal growth and metabolite production, while also establishing its potential as a sustainable feedstock for advancing bioenergy development.

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