Yuzuncu Yil University
Journal of Agricultural Sciences
(Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi)
https://dergipark.org.tr/en/pub/yyutbd

ISSN: 1308-7576 e-ISSN: 1308-7584

Research Article

Tocopherol Content of *Euglena* sp. Isolated from Yogyakarta under Glucose and Ethanol Mixture Treatment

Wynona Zahra AQILLA[1], Dea Putri ANDESKA[2], Tia ERFIANTI[3], Brilian Ryan SADEWO[4] Eko Agus SUYONO*[5]

[1,2,3,5]Universitas Gadjah Mada, Faculty of Biology, Department of Biology, 55223, Indonesia
[4]Universitas Gadjah Mada, Faculty of Engineering, Department of Chemical Engineering, 55284, Indonesia

[1]https://orcid.org/0000-0002-9633-6339, [2]https://orcid.org/0000-0002-6491-1060, [3]https://orcid.org/0000-0002-5462-1131
[4]https://orcid.org/0000-0002-7074-9055, [5]https://orcid.org/0000-0002-9208-4541

*Corresponding author e-mail: eko_suyono@ugm.ac.id

Article Info

Received: 09.12.2023
Accepted: 23.06.2023
Online published: 15.09.2023
DOI: 10.29133/yyutbd.1216693

Keywords

Biomass,
Euglena sp.,
Microalgae,
α-tocopherol

Abstract: *Euglena* sp. is a microalgae with significant potential for utilization as a high-value product because of the presence of protein, lipid, paramylon, and other compounds. Even though these microalgae may be found in freshwater, research on enhancing *Euglena* sp. cultivation is still limited in Indonesia. Tocopherols are antioxidants that can effectively protect against diseases caused by oxidative stress. The isomer of tocopherol with the highest biological activity is α-tocopherol. *Euglena* sp. cells had the highest levels of α-tocopherol compared to other microorganisms. Scientists are continuously trying to determine how to obtain a high α-tocopherol concentration and a significant *Euglena* cell biomass. Photosynthetic organisms culture has been found to boost α-tocopherol content in *Euglena* sp., although heterotrophic culture can potentially increase biomass. This study used photoheterotrophic culture with a mixture of glucose and ethanol to increase the α-tocopherol and biomass concentration inside the culture of the local strain of *Euglena* sp. The addition of treatments in a glucose and ethanol combination with levels of 3:2; 2.5: 2.5; 2: 2; and 0:0 (control) g L^{-1} was used in this study to assess the impact of *Euglena* sp. culture on growth, biomass, and α-tocopherol concentration. According to the findings of this study, the 3:2 treatment produced the most significant specific growth rate and biomass, including 0.992 (OD680/OD680/day) and 8.480 (g L^{-1}). In contrast, the 2.5:2.5 treatment produced the highest α-tocopherol content, specifically 7.09±0.096 mg L^{-1}.

To Cite: Aqilla, W Z, Andeska, D P, Erfianti, T, Sadewo, B R, Suyono, E A, 2023. Tocopherol Content of *Euglena* sp. Isolated from Yogyakarta under Glucose and Ethanol Mixture Treatment. *Yuzuncu Yil University Journal of Agricultural Sciences*, 33(3): 450-460. DOI: https://doi.org/10.29133/yyutbd.1216693

Footnote: This study is based on research data from a graduate program conducted at Gadjah Mada University, which was supervised by Dr. Eko Agus Suyono, M. App. Sc.

1. Introduction

During the Second World War, when Japan, America, and Germany struggled, microalgae cultivation as a global food source was heavily advocated (Potvin et al., 2010). Until now, microalgae are still used by the community as a source of protein, vitamins, and minerals, better known as a functional food. Microalgae outperform other microbes, such as yeasts and molds, regarding food safety.

In terms of efficiency and ease of production, microalgae are superior to mammalian single-cell proteins (Hardiyanto, 2012). Other benefits of microalgae over other microorganisms include a high biomass production per unit (Pradana et al., 2017; Yuarrina et al., 2019). Microalgae can be included in the classification of a functional food because it provides natural sources of protein, carbohydrates, and fats that act as energy sources in the body. More complex, microalgae can also function as a source of vitamins (Grimm et al., 2015). Microalgae as a food source have long been known. Some microalgae are also used as a source of drugs and are used in the pharmaceutical industry. For example, *Nannochloropsis* and *Chaetoceros* are ubiquitous microalgae used as natural feed for aquaculture animals. In addition, these microalgae can produce secondary metabolites utilized as antioxidants (Zulkarnain et al., 2020). Previous research by Tunio et al. (2022) also reported that cyanobacteria *Oscilatoria limosa* produced some bioactive compounds including phenolic acid, flavonoid, proteins, amino acid, and sugar.

Euglena is a freshwater protist that can thrive in various carbon sources, including glucose, glutamate, malate, pyruvate, lactate, and ethanol. These protists can also survive in high-stress environments, such as acidic waters, highly polluted rivers, and mining areas with high heavy metal content. Under extreme conditions, *Euglena* sp. was effectively isolated in the pH range of 2.5 – 3.5. Increasing the production of lipids and fatty acids in *Euglena* sp. through cultivation, metabolic, and genetic engineering are several methods to increase biofuel production (Erfianti et al., 2023). *Euglena* sp. contains paramylon (beta-1,3-glucan), which aids in producing several kinds of chemical components in industrial manufacturing processes. It plays a role in the medical field in synthesizing vitamin E (tocopherol) and 20 amino acids. In addition, *Euglena* is also commonly utilized as a raw material in the manufacture of biofuels, food and feed, and pharmaceutical. Furthermore, it can be used in environmental management, such as CO_2 reduction and water treatment. There is increasing interest in the commercialization of *Euglena* due to its durability and ability to synthesize a wide variety of unique bioproducts.

Euglena sp. is microalgae that have the potential to be utilized and can be found in various habitats such as fish ponds, rice fields, and polluted waters. As a result, *Euglena* sp. has the potential to be used since it can be isolated from a variety of environments. However, during the utilization process, researchers must recognize the potential negative consequences of by-products created during manufacture. Due to its ability to produce biofuel-synthesizable lipids, *Euglena* sp. has become increasingly popular in the industrial sector. Biodiesel could be made from microalgae, particularly in consortium cultures (Nur et al., 2023).

Tocopherol, often known as vitamin E, is an antioxidant that may help prevent various illnesses caused by oxidative stress (Rizvi et al., 2014). Tocopherol is a vitamin commonly used as a food preservative in the food business (Delgado et al., 2020). Tocopherols are obtained primarily by chemical synthesis and extraction from vegetable oils. However, extraction from vegetable oils comprises a combination of beta and gamma tocopherols. In reality, it must be refined to extract the active form of α-tocopherol for pharmaceutical uses (Ogbonna et al., 2019). Because of their considerable structural similarity, separating homologous mixtures of tocopherols is difficult. However, chromatographic techniques can be used. The tocopherol isomer with the highest biological activity is α-tocopherol. Although many other microbes may accumulate α-tocopherol compared to yeast, moulds, and macroalgae, the α-tocopherol concentration in *Euglena* sp. cells is considered the highest (Gissibl et al., 2019). *Euglena* sp. contains 97% α-tocopherol compared to other tocopherol isomers (Shigeoka et al., 1986). The demand for tocopherols in the market, especially α-tocopherol, is experiencing a rapid increase, so it is necessary to develop an efficient production system for chemically synthesized α-tocopherols by vegetable oils.

Due to a lack of genetic information on metabolic pathways that lead to diverse bioproducts, improved *Euglena* performance depends on improving culture conditions to enable the synthesis of intriguing chemicals, followed by increased culture volume. *Euglena* can be cultivated in heterotrophic, photoautotrophic, or mixotrophic. Depending on the culture method, the final biomass and cellular composition differ. Biomass might be optimized by changing growth determinants such as growth medium, temperature, light intensity, dissolved CO_2 concentration, cultivation procedure, and salinity (Sudibyo et al., 2018). Mix-culture cultivation could also enhance the optimization of algal growth and biomass (Suyono et al., 2016) and also improve the effectiveness of the harvesting process (Irawan et al., 2023). In this study, *Euglena* sp. is grown photoheterotrophically because cell development is not

entirely dependent on photosynthesis since light energy is not the only factor influencing growth; organic carbon substrate also plays a role (Sudibyo et al., 2018). To produce α-tocopherol commercially in *Euglena* sp. it is essential to obtain a high biomass concentration and a high concentration of α-tocopherol per unit cell. Photoheterotrophic culture, when organic carbon supplies and light energy are easily accessible for culture, can overcome problems in cultivation under photoautotrophic and photoheterotrophic circumstances. Glucose has been shown to enhance biomass content, whereas ethanol has been shown to increase the α-tocopherol range (Fujita et al., 2008). As a result, this study employs a combination of glucose and ethanol in various ratios. The amount of biomass is related to the amount of tocopherol. Therefore, if biomass production is high, the probability of tocopherol generation is likewise increased. This study aims to find the optimal glucose-ethanol ratio for enhancing tocopherol synthesis.

2. Material and Methods

2.1.Materials

In 2021, the current investigation was carried out in the Biotechnology Laboratory at Universitas Gadjah Mada in Yogyakarta, Indonesia. *Euglena* sp. strain Indonesia was obtained from the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada (isolated from a wild strain in Yogyakarta). Modified Cramers & Myers was used in the cultivation of *Euglena*, with the following materials: $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $Fe_2(SO_4)_3 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CoSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Na_2MoO_4 \cdot 2H_2O$ (Cramer and Myers, 1952). The medium was sterilized by autoclaving it for 15 minutes at 121ºC. After sterilization, filter-sterilized glucose and ethanol were added to the medium to avoid denaturation by high heat. Afterward, Vitamin B1 and Vitamin B12 were sterilized using a millipore filter.

2.2.Cultivation

Cultivation was carried out in 500 ml flasks using a combination of 300 ml modified CM media and 200 ml pre-cultured *Euglena* sp. cells. The cultivation was carried out in a photoheterotrophic condition, with aeration provided by a combination of 95% air and 5% CO_2 and lighting provided by LED lamps with a vlight intensity of around 1.000 lux). The effects of glucose and ethanol mixture were investigated with ratio: 0 g L^{-1} : 0 g L^{-1}; 3 g L^{-1}: 2 g L^{-1}; 2.5 g L^{-1}: 2.5 g L^{-1}; 2 g L^{-1}: 3 g L^{-1}. Pre-cultivation experiments showed that these concentrations of glucose and ethanol were not inhibitory to cell growth and α-tocopherol production. Three biological repetitions are used in this research (n=3).

2.3.Optical density and biomass

The optical density of the sample was determined by measuring absorbance at a wavelength of 680 nm (Suzuki et al., 2017). The culture biomass was determined by measuring the dry weight of the sample. The samples were centrifuged for 10 minutes at 4000 rpm. The supernatant is removed from the conical tube, leaving just the pellets. The leftover pellets were dried for 12 hours (overnight) in an oven at 36ºC until the weight was consistent (Ben-Amotz et al., 2004). Doubling time was calculated with the following formula:

$$Td = \frac{\ln 2t}{\ln(N_t/N_o)} \quad (1)$$

T = Time interval
Nt = The number of cells at the end of the exponential phase
N0 = The number of cells at the beginning of the exponential phase

The following equation was used to compute the specific growth rate:

$$\mu = \frac{\mathbf{0.693}}{\mathbf{td}} \quad (2)$$

Biomass productivity was calculated with the equation:

$$\text{Productivity (g L}^{-1}\text{ day}^{-1}\text{)} = \Delta x/t \quad (3)$$

Δx = difference in biomass on day t_1 and day t_0 (day 0)
T = time interval (day)

2.4.α-tocopherol measurement

The cells were extracted from the culture sample by centrifugation. According to Afiukwa et al (2007), the α-tocopherol content of the cell was removed. Quantitative analysis was performed using a spectrophotometric method with spectrophotometer UV-Vis and α-tocopherol standard. α-tocopherol quantification was carried out with a wavelength of 450 nm. Standard curves (r^2 value = 0.999) were made with standard α-tocopherol solutions with graded concentrations of 1 mg L^{-1}, 2 mg L^{-1}, 4 mg L^{-1}, 8 mg L^{-1}, and 16 mg L^{-1}. The absorbance of the *Euglena* sp. sample was calculated by the standard curve. Tocopherol productivity was calculated with the following equation:

$$\text{Productivity (mg/mL/day)} = \frac{\Delta x}{t} \quad (4)$$

Δx = difference in α-tocopherol on day t_1 and day t_0 (day 0)
T = time interval (day)

2.5.Statistical analysis

SPSS software was used to conduct all statistical analyses. Analysis of variance (ANOVA) and Duncan's multiple range tests at $p<0.05$ was used to compare the significant level between values. Statistical significance was defined as $p<0.05$ or above.

3. Results

3.1. Effect of glucose and ethanol on cell growth

Growth characteristics include specific growth rates and doubling time. The specific growth rate is the rate at which microalgae cells grow per unit of time and may be used to calculate the carrying capacity of nutrients for microalgae cell growth and division. Doubling time is the time necessary for microalgae cells to double in number. The fastest doubling time occurs in the logarithmic phase, which is the phase where the cells divide rapidly and constantly. Low doubling time values are correlated with high specific growth rate values and vice versa (Nurhanifah et al., 2019; Liu et al., 2011). Microalgae strains with a short doubling time and high specific growth rate are ideal for developing in-scale production. This is because the time from cultivation to harvest can be achieved with a shorter duration. Therefore the product can be more efficient.

According to Figure 1. It can be seen that the *Euglena* sp. culture, which had the highest absorbance, was the 3 g glucose: 2 g ethanol treatment. In contrast, the lowest absorbance was the control treatment—adding a glucose and ethanol combination enhanced cell development. Cultures with 3:2 and 2:3 treatment had the highest absorbance on the 4th day, respectively, namely 1.272 and 1.091, cultures with treatment 2.5:2.5 had the highest absorbance on the 5th day, which was 1.132, while the control with 0:0 treatment had the highest absorbance on the 6th day, which was 0.679. This information does not need to be deleted entirely; it is not interested in the results. These data and explanations describe the relationship between biomass productivity and cell growth, doubling time. These data lead to the conclusion that *Euglena* sp. in this study can be harvested quickly and effectively for further development (production scale).

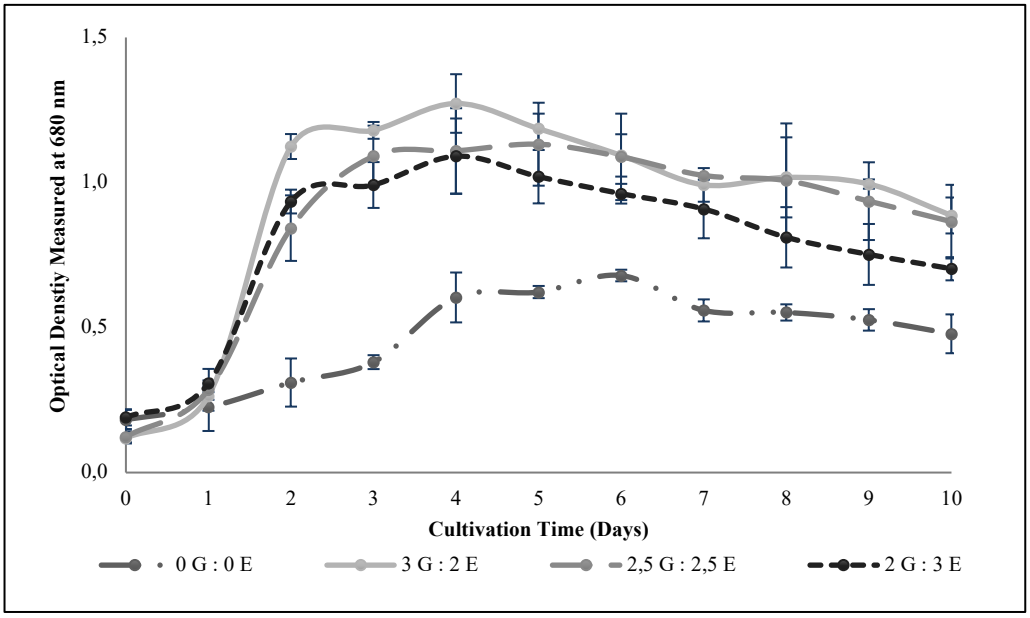

Figure 1. Effects of glucose and ethanol mixture with different ratios to cell growth of *Euglena* sp.

The treatment of a combination of glucose and ethanol with a ratio of 3:2 0.608 ± 0.049 resulted in the most incredible Specific growth rate (OD680 day-1), followed by 2.5:2.5 treatment with a value of 0.533 ± 0.008; treatment 2:3 worth 0.489 ± 0.005; and the last is control (0:0) worth 0.224 ± 0.014. The value obtained in this study is directly proportional to the absorbance results obtained, where the highest absorbance was achieved by the 3:2 treatment, and the lowest absorbance was performed by the control treatment (0:0).

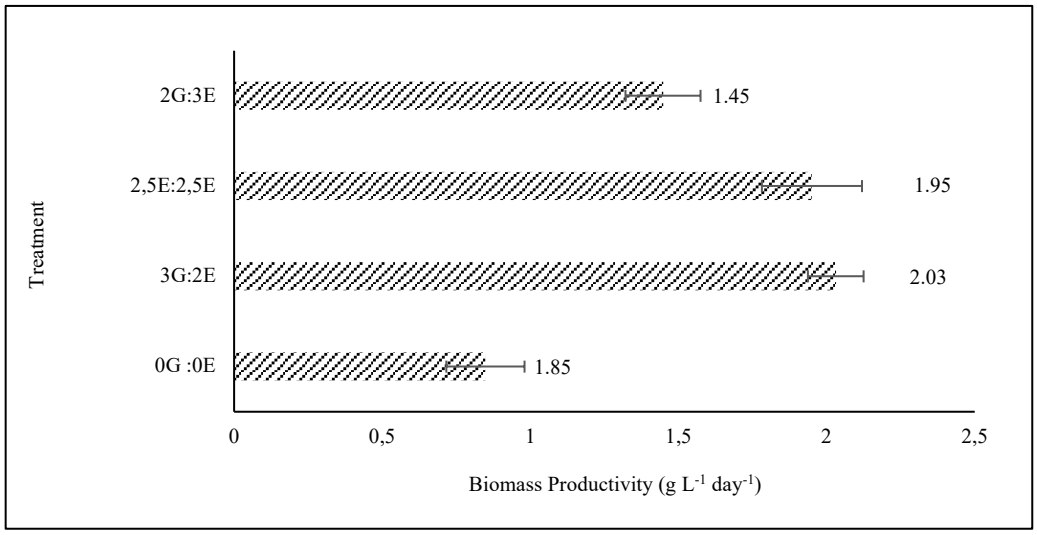

Figure 2. The specific growth rate of *Euglena* sp. in glucose and ethanol mixture with different ratios.

According to the data on Figure 2, the highest biomass productivity was achieved by 3:2 treatment of glucose: ethanol (2.03 g L^{-1} day^{-1}), followed by 2.5: 2.5 of glucose: ethanol (1.95 g $L^{-1}day^{-1}$), 2:3 of glucose: ethanol (1.45 g L^{-1} day^{-1}), and the lowest biomass productivity was 0:0 of glucose: ethanol (1.85 g L^{-1} day^{-1}). This result showed that microalgae successfully consumed the carbon source through glucose and ethanol. This finding was supported by a previous study by Afiukwa and James (2007) that the growth of *E. gracilis* was higher under the mixed carbon culture (ethanol and glucose), reaching 2.34 ± 0.109 x 10^7 mL for the cell density. Furthermore, the previous results indicated that the diverse carbon culture promoted cell growth, but antioxidant vitamin concentrations were insufficient.

Thus, the combined substrate system has a high potential for producing *Euglena* biomass on a large scale.

3.2. Effect of glucose and ethanol on dry weight (dry biomass)

Overall, from Figure 3, it can be seen that the highest trend in biomass was found in the 3 grams of glucose: 2 grams of ethanol treatment, while the lowest glucose trend was found in the control treatment. This trend is directly proportional to the increase in *Euglena* sp. cell density, where the mixture of glucose and ethanol enhanced growth. In all treatments, it was seen that the highest biomass was achieved on the 6th day, and on the 8th day, the biomass in all treatments decreased.

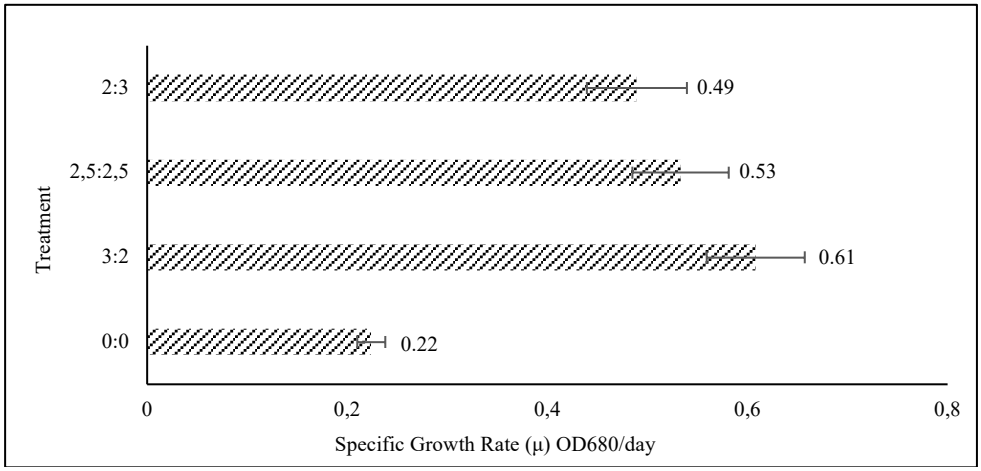

Figure 3. Effects of glucose and ethanol mixture in different ratios to the biomass of *Euglena* sp.

Figure 4 showed the biomass productivity of *Euglena* sp. in glucose and ethanol mixture with different ratios. The highest average of biomass in each treatment (3:2; 2.5:2.5; 2:3; and control) was 12.94 g L^{-1}, 12,32 g L^{-1}, 9.98 g L^{-1}, respectively. 6,36 g L^{-1}. The biomass produced on the last day of observation in each treatment (3:2, 2.5:2.5; 2:3, and control) was 9.40 g L^{-1}, and 10.32 g L^{-1}, respectively. 5,69 g L^{-1}, 5,69 g L^{-1}. The treatment of a combination of glucose and ethanol yielded the total biomass (g L^{-1} day^{-1}) with a ratio of 3:2 which was 8.48 ± 0.28, then 2.5:2.5 treatment which was 8.12 ± 0.55, treatment 2:3, which is 6.18 ± 0.7, and the lowest is the control treatment (0:0) which is 4.38 ± 0.23. Thus, this observation indicates that the 3:2 treatment is optimal for obtaining the highest biomass yield. This is in line with the productivity of the resulting biomass. These results indicate that the higher the glucose ratio increases the cell growth and biomass content.

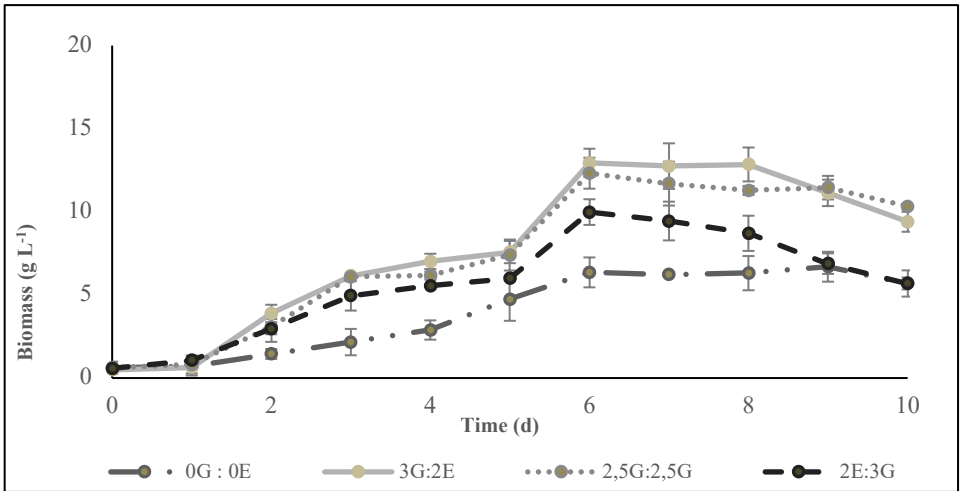

Figure 4. Biomass productivity of *Euglena* sp. in glucose and ethanol mixture with different ratios.

3.3. The effect of glucose and ethanol on the amount of α -tocopherol

Figure 5 showed that the α-tocopherol content increased from the beginning of cultivation until it reached the highest range on the 6th day when *Euglena* was at the end of the logarithmic phase. On the 8th day, α-tocopherol content decreased as the biomass and cell density of *Euglena* sp. The 2.5 glucose:2.5 ethanol culture treatment generally had the greatest α-tocopherol concentration. In the 3:2 and 2:3 treatments, the productivity of α-tocopherol was inversely proportional to the biomass produced by *Euglena* sp.

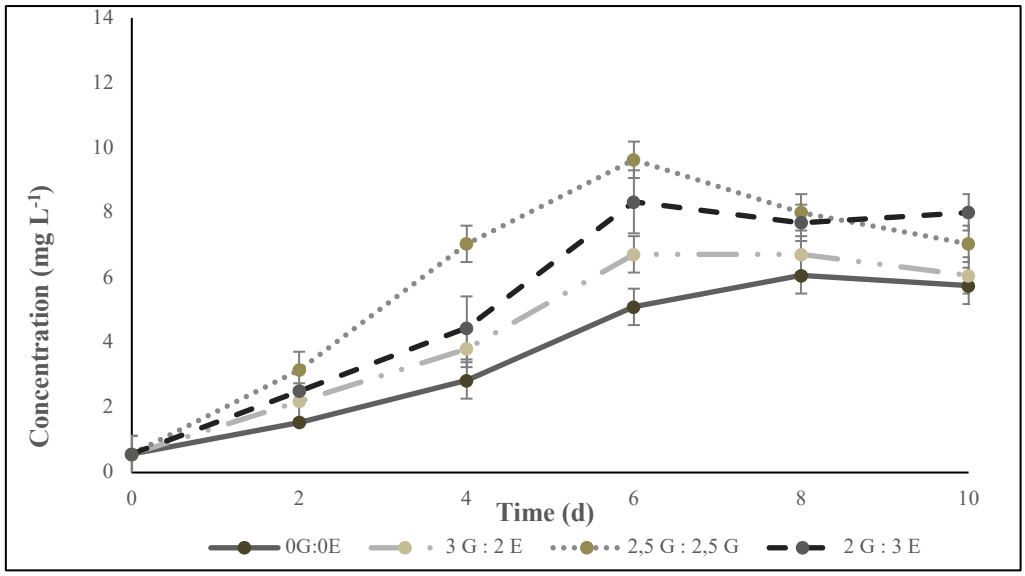

Figure 5. Effect of glucose and ethanol mixture with different ratios to α-tocopherol content of *Euglena* sp.

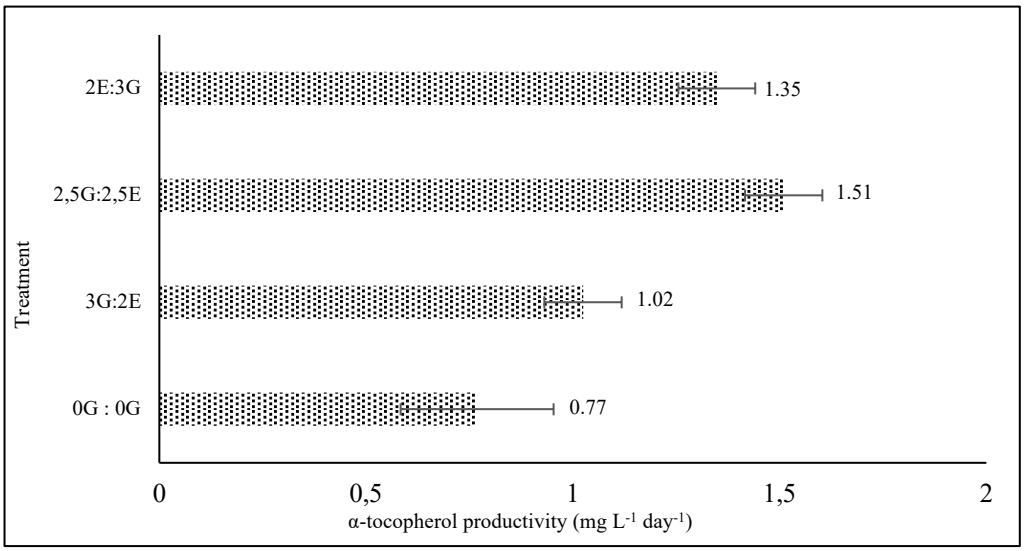

Figure 6. α-tocopherol productivity of *Euglena* sp. in glucose and ethanol mixture with different ratios.

Based on Figure 6, it can be seen the productivity of α-tocopherol produced by *Euglena* sp. in various treatment cultures. The highest α-tocopherol productivity was obtained in cultures treated with 2.5:2.5, which was 1.51 ± 0.09, followed by 2:3 treatment which was 1.35 ± 0.09, and 3:2 treatment was 1.02 ± 0.09. The lowest α-tocopherol productivity was obtained by the control treatment (0:0) of 0.77 ± 0.05. The value of α-tocopherol productivity was divided into three clusters, indicating significant differences between treatments, except for treatments 2.5:2.5 and 2:3, which were in the same notation.

Treatment of 2.5:2.5 resulted in the highest α-tocopherol productivity and average total tocopherol. The highest average total α-tocopherol per gram of cell biomass was produced in the

treatment 2.5:2.5, then followed by the control treatment (0:0); 3:2, and the last one is 2:3 with a successive content of 1.133 ± 0.215; 1.126 ± 0.078; 1.124 ± 0.134; and 0.812 ± 0.032 mg/g-cell. The mixed culture treatment of glucose and ethanol in a ratio of 2.5:2.5 produced the highest total α-tocopherol per gram of cell biomass because the total α-tocopherol produced had the highest content compared to other treatments, and the biomass produced was also high. The control (0:0) treatment delivered high total α-tocopherol per biomass; this was due to the low α-tocopherol content and low biomass obtained by this treatment, so the α-tocopherol content per gram cell was high, but the biomass produced was low. The treatment with the lowest total α-tocopherol per gram of biomass was the 2:3 treatment; in this treatment, the α-tocopherol content made was not too high compared to other treatments but produced the highest biomass in other treatments.

Table 1. The effect of a glucose-ethanol combination on biomass and α-tocopherol synthesis by *Euglena* sp.

Treatment	Average Biomass Production (g L^{-1})	Average α-tocopherol Production (mg L^{-1})	α-tocopherol per Cell Biomass (mg g^{-1}-cells)
0g glucose: 0g ethanol	$4.38^{a} \pm 0.02$	$4.37^{a} \pm 0.06$	$1.13^{b} \pm 0.08$
3g glucose: 2g ethanol	$8.48^{d} \pm 0.04$	$5.21^{b} \pm 0.09$	$0.81^{a} \pm 0.03$
2.5g glucose: 2.5g ethanol	$8.12^{c} \pm 0.05$	$7.09^{d} \pm 0.10$	$1.13^{a} \pm 0.04$
2g glucose: 3g ethanol	$6.18^{b} \pm 0.02$	$6.31^{c} \pm 0.09$	$1.12^{b} \pm 0.06$

[1]Significance at 95%.

4. Discussion

In this study, the growth of *Euglena* sp. was more significant when mixed carbon sources (glucose and ethanol) were added to the culture medium than when the carbon sources were not combined. In this study, adding carbon sources has enhanced cell development (Ogbonna et al., 1988; Afiukwa et al., 2007; Fujita et al., 2008). The highest cell density value was obtained in the 3:2 treatment, where the glucose ratio was higher than in ethanol. This follows the previous research, which states that glucose increases cell density up to 4 times compared to culture with ethanol. The previous study also proved that *Euglena* culture with a higher glucose ratio than ethanol produced higher cell density (Fujita et al., 2008). The occurrence of catabolite repression causes an increase in cell density by glucose. A previous study stated that glucose could affect the plastid *Euglena* sp. in the same way as bacteria and eukaryotes with the repression of these catabolites. Catabolite inhibition establishes the level of carbon resource utilization; before the energy required by the synthetase is consumed, the more efficient carbon source will be fully utilized to use the less efficient carbon source. Extensive research has been conducted on inhibiting catabolic metabolites in prokaryotes and eukaryotes (such as yeast), in which the synthesis of specific enzymes and all organelles, mitochondria, and microorganisms are synthesized inhibited by glucose (a fermentable carbon source). In photosynthetic autotrophic cultures, light acts through two photoreceptors to induce the formation of the enzymatic machinery necessary to use light and carbon dioxide as the sole source of carbon and energy for growth. This process is called chloroplast development (Görke et al., 2008). The regulation of catabolite inhibition involves the phosphotransferase system (PTS). Inactivation of PTS can reduce inhibition by increasing cAMP levels (Zhang et al., 2009). Glucose and Light allow CO_2 to be the only carbon source and energy source for the growth of *Euglena* sp.

Light induces enzymes for chloroplast development that occurs in photoautotrophic growth. Other carbon sources, such as ethanol, will form glyoxysomes when added. Glyoxisomes are organelles that contain enzymes needed for photoheterotrophic growth. Glucose content in the growth medium specifically represses photoinduction enzymes such as chloroplast valyl-t-RNA. The presence of glucose correlates with reduced adenosine 3':5'-monophosphate cAMP in cells. *Euglena* has cAMP and enzymes for its metabolism. The carbon dioxide produced by glucose metabolism is subsequently utilized in photosynthesis (Ogawa et al., 1981). The presence of a preferred carbon source inhibits the expression and, in many cases, the functioning of the catabolic system that facilitates the utilization of secondary substrates.

Adding a glucose and ethanol combination to the culture medium enhanced the α-tocopherol concentration more than the culture without the mixture. The activity of mitochondrial and chloroplasts

influences the formation of α-tocopherol. The mitochondria and cytoplasm contain most enzymes involved in the glycolytic pathway and ethanol metabolism in *Euglena* sp. In general, the light energy absorbed by chlorophyll is utilized for cell development and metabolite formation throughout the photosynthesis process (Schwelitz et al., 1978).

When ethanol is added to the culture medium as a carbon source, it activates the electron transport system in mitochondria and chloroplasts, producing Reactive Oxygen Species (ROS). Superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO) are reactive oxygen species consisting of radical and non-radical oxygen species generated by partial reduction of oxygen. Cellular ROS can be created endogenously, as in mitochondrial oxidative phosphorylation, or by contact with external sources. When ROS invades the cellular antioxidant defense system by raising ROS levels or decreasing cellular antioxidant capability, this is referred to as oxidative stress (Ray et al., 2012). In yeast, ROS is linked to damage to the mitochondrial and cell membranes (Jarboe et al., 2013). Under these conditions, α-tocopherol is synthesized as an antioxidant to prevent damage caused by these reactive oxygen species. Under photoheterotrophic conditions, the chlorophyll concentration was much lower than in photoautotrophic states. This is because adding a carbon source can inhibit the photoautotrophic mechanism so that light energy is not the primary energy source in the metabolism of *Euglena* sp. Thus the synthesis of α-tocopherol in the cultivation of *Euglena* sp. is more dependent on mitochondrial activity than chloroplast activity. Adding ethanol supports the production of α-tocopherol in *Euglena* sp. because it can increase mitochondrial activity, whereas when glucose becomes the primary carbon source, mitochondrial activity decreases sharply (Fujita et al., 2008; Rodriguez-Zavala et al., 2010). This study employs a combination of glucose, favorable for cell development, and ethanol to achieve practical- tocopherol synthesis.

Conclusion

Based on the findings of this study, it is possible that adding a glucose and ethanol combination to a photoheterotrophic culture might enhance the cell growth rate, biomass, and tocopherol content of *Euglena* sp. culture. The 3:2 treatment yielded the highest specific growth rate and biomass, with values of 0.99 (OD680/OD680/h) and 8.48 g L^{-1}, respectively. Meanwhile, the 2.5:2.5 treatment had the greatest tocopherol concentration, with a value of 7.09±0.096 mg L^{-1}.

Acknowledgments

The authors are grateful for the financial support from the Ministry of Education, Culture, Research & Technology, and Higher Education of Indonesia. This research is part of the thesis of the first author, authors are also thankful to the Laboratory of Biotechnology, Faculty of Biology, Universitas Gadjah Mada for allowing us to use the laboratory facilities.

References

Afiukwa, C. A,. & Ogbonna, J. (2007). Effects of mixed substrates on growth and vitamin production by *Euglena* gracilis. *African Journal of Biotechnology*, *6*(22), 2612-2615. https://doi.org/10.5897/AJB2007.000-2417.

Aqilla, W. Z. (2021). *Pengaruh Campuran Glukosa Dan Etanol Terhadap Pertumbuhan Dan Kandungan A-Tocopherol Pada Kultur Fotoheterotrofik Euglena* sp. *Strain 2 Skala Laboratorium*. (Undergraduate thesis), Universitas Gadjah Mada, Faculty of Biology, Yogyakarta, Indonesia.

Ben-Amotz, A. (2004). *Industrial Production of Microalgal Cell-Mass and Secondary Products—Major Industrial Species*: *Dunaliella. In: Richmond, A., Ed., Handbook of Microalgal Culture: Biotechnology and Applied Phycology,* Blackwell Science, Oxford, 273-280.

Cramer, M., & Myers, J. (1952). Growth and photosynthetic characteristics of *Euglena gracilis*. *Archiv für Mikrobiologie, 17,* 384-402.

Delgado, A., Al-Hamimi, S., Ramadan, M. F., Wit, M. D., Durazzo, A., Nyam, K. L., & Issaoui, M. (2020). Contribution of tocols to foof sensorial properties, stability, and overall quality. *Journal of Food Quality,* 1-8. https://doi.org/10.1155/2020/8885865.

Erfianti, T., Maghfiroh, K. Q., Amelia, R., Kurnianto, D., Sadewo, B. R., Marno, S., Devi, I. Dewayanto N., Budiman, A., & Suyono, E.A. (2023). Nitrogen sources affect the growth of local strain *Euglena* sp. isolated from Dieng Peatland, Central Java, Indonesia, and their potential as bio-avtur. *IOP Conf. Series: Earth and Environmental Science.* 1151 (2023) 012059. http://doi:10.1088/1755-1315/1151/1/012059.

Fujita, T., Aoyagi, H., Ogbonna, J. C., & Tanaka, H. (2008). Effect of mixed organic substrate on α-tocopherol production by *Euglena gracilis* in photoheterotrophic culture. *Applicative Microbiology Technology*, *79*, 371–378. https://doi.org/10.1007/s00253-008-1443-0.

Gissibl, A., Sun, A., Care, A., Nevalainen, H., & Sunna, A. (2019). Bioproducts from *Euglena gracilis*: synthesis and applications. *Frontiers in Bioengineering and Biotechnology*, *7*, 108. https://doi.org/10.3389/fbioe.2019.00108.

Görke, B., & Stülke, J. (2008). Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol, 6*, 613–624. https://doi.org/10.1038/nrmicro2032.

Grimm, P., Risse, J. M., Cholewa, D., Muller, J. M., Beshay, U., Friehs, K., & Flaschel, E. (2015). Applicability of *Euglena gracilis* for biorefineries demonstrated by the α-tocopherol and paramylon followed by anaerobic digestion. *Journal of Biotechnology*, *215*, 72 – 79. https://doi.org/10.1016/j.jbiotec.2015.04.004

Hadiyanto, H., & Nur, M. A. (2012). *Mikroalga Sumber Pangan Dan Energi Masa Depan*. Undip Press, Semarang, Indonesia.

Irawan, A.P., Rahmawati, A., Fahmi, U.A., Budiman, A., Maghfiroh, K.Q., Erfianti, T., Andeska, D.P., Putri, R.A.E., Nurafifah, I., Sadewo, B.R., Suyono, E.A. 2023. Studies on bioflocculant exopolysaccharides (EPS) produced by *Anabaena* sp. and its application as bioflocculant for low-cost harvesting of *Chlorella* sp. *Asian J Agric & Biol*. (3). doi:10.35495/ajab.2022.150.

Jarboe, L. R., Royce, L.A., & Liu, P. (2013). Understanding biocatalyst inhibition by carboxylic acids. *Frontiers in Microbiology*, *4*(272), 1-8.

Liu, J., Huang, J., & Chen, F. (2011). Microalgae as Feedstocks for Biodiesel Production. In: Biodiesel - Feedstocks and Processing Technologies. *Croatia*, 58-78.

Nurhanifah, N. D., Padil, & Muria, S. R. (2019). Kultivasi Mikroalga Menggunakan Media AF6 Berdasarkan Perbedaan Intensitas Cahaya. *JOM FTEKNI, 6*(1), 1-5.

Nur, F., Erfianti, T., Andeska, D. P., Putri, R. A. E., Nurafifah, I., Sadewo, B. R., & Suyono, E. A. (2023). Enhancement of Microalgal Metabolite Production through *Euglena* sp. Local Strain and Glagah Strain Consortia. *Biosaintifika*, *15* (1). 36-47.

Ogawa, T., & Aiba, S. (1981). Bioenergetic analysis of mixotrophic growth in Chlorella vulgaris and Scenedesmus acutus. *Biotechnology and Bioengineerin. Bioeng*., *23*, 1121-1132. https://doi.org/10.1002/bit.260230519.

Ogbonna, J. C., Tomiyama, S., & Tanaka, H. (1998). Heterotrophic Cultivation of *Euglena gracilis* Z for Efficient Production of α-Tocopherol. *Journal of Applied Phycology, 10*(1), 67–74. https://doi.org/10.1023/A:1008011201437.

Potvin, G., & Zhang, Z. (2010). Strategies for high-level recombinant protein expression in transgenic microalgae: A review. *Biotechnology Advance, 28*, 910-918. https://doi.10.1016/j.biotechadv.2010.08.006.

Pradana, Y. S., Sudibyo, H., Suyono, E. A., Indarto, & Budiman, A. (2017). Oil Algae Extraction of Selected Microalgae Species Grown in Monoculture and Mixed Cultures for Biodiesel Production. *Energy Procedia, 105*, 277–282. https://doi.org10.1016/j.egypro.2018.03.314.

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signaling*, *24*(5), 981–990. https://doi.org/10.1016/j.cellsig.2012.01.008.

Rizvi, S., Raza, S. T., Ahmed, F., Ahmad, A., Abbas, S., & Mahdi, F. (2014). The role vitamin E in human health and some diseases. *Sultan Qaboos University Medical Journal, 14,* e157-65.

Rodriguez-Zavala, J. S., Ortiz-Cruz, M. A., Mendoza-Hernández, G., & Moreno-Sánchez, R. (2010). Increased synthesis of α-tocopherol, paramylon and tyrosine by *Euglena* gracilis under conditions of high biomass production. Journal of Applied Microbiology, 109(6), 2160-2172. https://doi.org/10.1111/j.1365-2672.2010.04848.x.

Schwelitz, F. D., Cisneros, P. L., & Jagielo, J. A. (1978). The effect of glucose on the biochemical and ultrastructural characteristics of developing *Euglena* chloroplasts. *Journal Protozooogyl, 3*(2), 398-403. https://doi.org/10.1111/j.1550-7408.1978.tb03914.x.

Shigeoka, S., Onishi, T., Nakano, Y., & Kitaoka, S. (1986). The contents and subcellular distribution of tocopherols in *Euglena gracilis*. *Agric. Biol. Chem., 50*, 1063–1065. https://doi.org/10.1080/00021369.1986.10867518.

Sudibyo, H., Purwanti, Y., Pradana, Y.S., Samudra, T. T., Budiman, A., & Suyono, E. A. (2018). Modification of growth medium of mixed-culture species of microalgae isolated from southern java coastal region. *MATEC Web of Conferences*, *154*, 1-6 https://doi.org/10.1051/matecconf/201815401001.

Suyono, E. A., Fahrunnida, Nopitasari, S., & Utama, I. (2016). Identification of microalgae species and lipid profiling of Glagah consortium for biodiesel development from the local marine resource. *Journal of Engineering and Applied Sciences, 11*, 9970-9973.

Suzuki K. (2017). Large-scale cultivation of *Euglena*, In Schwartzbach, S.D., Shigeoka, S. (Eds). *Euglena*: Biochemistry, Cell, and Molecular Biology. *Springer International Publishing, Cham*., 285-293. https://doi.org/10.1007/978-3-319-54910-114.

Tunio, A.A., Naqvi, S.H., Tunio, Q.N., Charan, T.R., Bhutto, M.A., Mughari, M.H. 2022. Determination of Antioxidant, Antimicrobial Properties with Evaluation of Biochemicals and Phytochemicals Present in *Oscillatoria limosa* of District Jamshoro, Pakistan. *Yuzuncu Yil University Journal of Agricultural Sciences.* 32 (3); 538-547.

Yuarrina, W. P., Pradana, Y. S., Budiman, A., Majid, A. I., Indarto, & Suyono, E. A. (2018). Study of cultivation and growth rate kinetic for mixed cultures of local microalgae as third generation (G-3) bioethanol feedstock in thin layer photobioreactor. *Journal of Physics: Conference Series*, *1022,*1-9. https://doi.org/10.1088/1742-6596/1022/1/012051.

Zavala, J. S. R., Ortiz-Cruz, M. A., Mendoza-Hernandez, G., & Moreno-Sanchez, R. (2010). Increased synthesis of α-tocopherol, paramylon, and tyrosine by *Euglena gracilis* under conditions of high biomass production. *Journal of Applied Microbiology*, *109*(6), 2160-2162. https://doi.org/10.1111/j.1365-2672.2010.04848.x.

Zhang, X., Jantama, K., Moore, J. C., Jarboe, L. R., Shanmugam, K. T., & Ingram, L. O. (2009). Metabolic evolution of energy-conserving pathways for succinate production in *Escherichia coli*. *Proc Natl Acad Sci USA*, *106*(48), 20180-20185. https://doi.org/10.1073/pnas.0905396106.

Zulkarnain, I. M., Rahayu, H. T., Herida, A. P., Erfianti, T., Kusumaningrum, H. P., Zainuri, M., Endrawati, H., Widowati, I., Haryanti, W. D. U., & Mahendrajaya, R. T. 2020. Improvement of the potency of microalgae Nannochloropsis and Chaetoceros through antioxidant analysis and optimization of DNA isolation. *Journal of Physics: Conference Series. 1943. 012080.* https://doi:10.1088/1742-6596/1943/1/012080.