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Effect of High Cell Density to Lipid Content Microalgae *Chlorella vulgaris* on Photoautotrophic Cultivation

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ABSTRACT

Chlorella vulgaris is one of microalgae types that has essential ingredients beneficial to humans, such as being a source of good lipid. Chlorella sp. have high lipid content, up to 30%, under autotrophic conditions considered as a promising candidate for commercial lipid production due to its fast growth and easy cultivation. High cell density sedimentation can be investigated as a starving method in cultivation to make microalgae conditions less favorable in obtaining nutrient to lipid accumulation. Starving is one of the stress conditions carried out with the aim of reducing nutrients in the microalgae cultivation process. The aim of this study was to determine the effect of high cell density on increasing lipid content in Chlorella vulgaris. The results of this study showed that high cell density method affected the productivity of Chlorella vulgaris biomass. The data obtained were analyzed using ANOVA α=0.05. The highest productivity and lipid content was obtained from the control sample with a biomass value of 0.36±0.03 g/l and a lipid content of 56.2±4.6% (P<0.05). The conclusion of this study is high cell density may not increase the production of lipid content from Chlorella vulgaris.

Keywords: *Chlorella vulgaris; high cell density; lipid; sedimentation; starvation*

INTRODUCTION

Microalgae are known to have bioactive compounds such as proteins, carbohydrates and lipids (Cheng et al., 2022). Microalgae are organisms that have the potential to be used as a source of alternative materials in various fields of health, food and energy because they have bioactive compounds that can be used as high-value products. Most of several types of microalgae produce certain products among which are enzymes, polymers, bioactive peptides and fatty acids. Microalgae have long been used as dietary supplements for humans. Microalgae in the field of pharmacology are used as antibacterial, antioxidant and antiviral agents. Microalgae have one source of vegetable oil compounds that have the potential to be used as functional food and energy with lipid and fatty acid content. The content of lipids and fatty acids in microalgae is one of the attractions in several microalgae studies (Zhou et al., 2022).

Chlorella vulgaris is one type of microalgae that has essential ingredients that are beneficial to humans (Ru et al., 2020). *Chlorella vulgaris* is a microalgae belonging to the *Chlorophyta* class which has nutritional value and natural bioactive compounds such as carotenoids, phenolic compounds, sulfate polysaccharides and vitamins, one of the functions of these bioactive compounds can affect cell regulation, immune response and as an antioxidant (Novianti et al., 2019). Chlorella has been considered a promising candidate for commercial lipid production due to its fast growth process and easy cultivation (Oh et al.,

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2022). Chlorella sp. have high lipid content up to 30% under autotrophic conditions (Nordin et al., 2022).

The production of lipids and fatty acids in each microalgae depends on the availability of nutrients such as nitrogen, phosphor, kalium and other elements which are affecting factors on the microaglgae's growth and composition of metabolites (Yaakob et al., 2021). Lipids and fatty acids from microalgae biomass have similar content to vegetable oils and are considered as potential sources of food and energy. Under certain conditions, microalgae have 85% lipid of the total dry weight. In addition to the availability of nutrients, cultivation conditions such as light intensity, temperature, pH, media, salinity and homogenization can also affect the growth and production of microalgae lipid content (Khoo et al., 2023).

Of the various methods to increase the accumulation of lipid content, stress treatment is one of the most commonly used methods. Microalgae can change the biosynthetic pathway of membrane compounds into lipid accumulation for storage under unfavorable environmental conditions. Lipid accumulation is a defense mechanism of microalgae cells related to nutritional deficiencies, light intensity, temperature, salinity or other stress conditions such as nitrogen and phosphorus nutrients deficiencies (Nayak et al., 2019). Among various environmental stressors, nitrogen starvation is widely considered as the main strategy to increase lipid accumulation in autotrophic culture due to its high efficiency and ease of implementation (Gojkovic et al., 2020). Starving is one of the stress conditions carried out with the aim of reducing nutrients in the microalgae cultivation process. Starvation can be achieved through several methods such as nutrient reduction or through cell deposition methods during the cultivation process. High cell density sedimentation can be one of the cultivation methods that can be investigated to make microalgae cultivation conditions less favorable in obtaining media nutrients. Therefore, it is necessary to conduct research to optimize productivity by balancing microalgae growth and lipid content. The aim of this study is to determine the effect of high cell density on increasing lipid content in microalgae *Chlorella vulgaris*.

METHODS

Strain and Media

Microalgae strain used in this research was *Chlorella vulgaris* obtained from the Culture Collection of Microalgae and Bioprocess Research Grup, Biotechnology research center, Badan Riset dan Inovasi Nasional Cibinong, Bogor, Indonesia. Microalgae *Chlorella vulgaris* was precultured using Tris Acetyl Phosphate (TAP) medium with Tris (hydroxymethyl) aminomethane, macronutrient ($MgSO_4 \cdot 7H_2O$, KH_2PO_4, $CaCl \cdot 2H_2O$, NH_4Cl, K_2HPO_4) and micronutrient ($FeSO_4 \cdot 7H_2O$, H_3BO_3, $Na_2EDTA \cdot 2H_2O$, $MnCl \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Na2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$). After 3 days *Chlorella vulgaris* was cultured in NaOH 60mM medium with macronutrient ($MgSO_4 \cdot 7H_2O$, KH_2PO_4, $CaCl \cdot 2H_2O$, NH_4Cl, K_2HPO_4) and micronutrient ($FeSO_4 \cdot 7H_2O$, H_3BO_3, $Na_2EDTA \cdot 2H_2O$, $MnCl \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$) which has been flowed with CO_2 at speed 1L/minutes. The tools used for analysis included glass tool set, Spectrophotometry UV/Vis (Shimadzu® UV-1700 PharmaSpec), Branson® 8200 Ultrasonic Cleaner United State, Kubota® 6200 Hybrid High Speed Refrigerated Centrifuge, magnetic stirrer, vortex, oven incubator (Binder® RedLine RI-53), pH meter, micropipet, analytical balance, autoclave.

The NaOH solution with distilled water and 1L concentration macronutrients and micronutrients TAP medium in 10ml distilled water were sterilized using autoclave for 15 min

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at 121°C to avoid contamination. Steriled NaOH solution then was flushed using CO_2 gas at a flow rate1L/m under aseptic conditions until pH 6-7.

Stock culture of *Chlorella vulgaris* were precultured in a 500ml *erlenmeyer* with working volume of 300ml medium with an initial OD_{750} of 0.1 using spectrophotometer (Shimadzu® UV-1700 PharmaSpec) which was grown for 3 days carried out under (Tl 36 Watt Philips® White Lamp) lights with intensity of 2000lux and homogenization using shaker at a speed of 120 rpm at room temperature. Pre-culture was carried out under lamp condition with an intensity of 2000lux and homogenization using a shaker at a speed of 120 rpm at room temperature beetwen 30-33°C for 24 hours. Microalgae *Chlorella vulgaris* that had been precultured was transferred into medium (total volume was 1L) with macronutrients and micronutrients TAP, added to NaOH solution with an initial OD_{750} of 0.1 and grown for 7 days. Then, it was cultivated under (Tl 36 Watt Philips® White Lamp) with light intensity about 2000lux, shaken with magnetic stirrer at speed of 600-650rpm for homogenization at room temperature beetwen 30-33°C for 24 hours.

During cultivation process, the cultures were given starvation treatments. Starvation was carried out by stopping the homogenization (magnetic stirrer) to give effect of high cell density or sedimentation. In this treatment, high cell density sedimentation method is carried out with various durations ranging 1 until 5 days. The high cell density treatment for 1 day was carried out by stopping the homogenization on third day. The high cell density treatment for 2 days was carried out by stopping the homogenization in third until fourth day. The high cell density treatment for 5 days was carried out by stopping the homogenization on third until seventh day. In this study there was a control without high cell density treatment to see the difference in the results obtained. Cultures were harvested on seventh day of growth using centrifugation at 8000 rpm for 5 minutes at 5°C. The harvesting aims to obtain *Chlorella vulgaris* biomass which will be analyzed gravimetrically for biomass productivity. The analysis in this study was carried out in **triplicates,** and analyzed using ANOVA with α=0.05.

Biomass Productivity (Hawrot-Paw et al., 2020)

Productivity biomass was analyzed by harvesting 40ml of culture on the day of harvest which was placed in 50ml falcon tubes, constant weight (***b*** g). Culture was centrifiged at 8000 rpm for 5 minutes at 5°C. Furthermore, the obtained biomass was dried using oven at 50°C for 24 hours. The dry biomass (***a*** g) obtained will be used for lipid extraction.

$$\boldsymbol{Biomass = a - b}$$

Lipid Extraction (Bligh et al., 2020)

Biomass (***b*** mg) was extracted using a method referring to the (Bligh et al., 2020). Mixture of chloroform and methanol 1:1 (v/v) was used as a solvent in lipid extraction. About 100mg of dried biomass was added with 6ml of solvent in a 15ml falcon tube and sonicated at 40kHz for 2 hours using an Ultrasonic Cleaner (Branson® 8200 Ultrasonic Cleaner, United State). Exctraction was carried out using 2 times twice sonication and proceeded with maceration for 24 hours with the total volume solvent (***c*** ml) used in this extraction was 12ml (6ml chloroform and 6ml methanol). The crude extract from the extraction was separated into a falcon tube with distilled water in a rati of 1:1 (v/v). The tube was then stirred with a vortex for 30 seconds and centrifuged at 4000 rpm for 5 minutes at 5°C. The water layer at the top was removed and 0.5ml (***d*** ml) chloroform layer at the bottom was transferred 0.5ml into a 1.5ml microtube. The solvent was removed by allowing it to evaporate in the open air and the

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total lipid content was measured using gravimetry (***a*** mg). Lipid content was calculated using the following formula (Ratomski et al., 2021):

$$\boldsymbol{Total\ Lipid}\ (\%) = \left(\frac{\boldsymbol{a}}{\boldsymbol{b}} \times \frac{\boldsymbol{c}}{\boldsymbol{d}}\right) \times\ \mathbf{100}\%$$

RESULT AND DISCUSSIONS

Biomass Productivity

Comparison of the biomass productivity of *Chlorella vulgaris* was carried out by calculating the amount of biomass in 40ml and converted to units of g/l harvested biomass on day 7. In Table 1 data show that the highest biomass productivity was obtained from sample A with a value of 0.36±0.03 g/l (P<0.05). Meanwhile, the lowest biomass productivity was obtained in sample D with a value of 0.27±0.01 g/l (P<0.05). This showed that the effect of high cell density stress has an effect on the biomass productivity of *Chlorella vulgaris*. Sample D without homogenization treatment to obtain starvation stress had the lowest biomass productivity value which could be due to stopping homogenization causing the cells to settle and lack nutrients during the cultivation process. Sample B with high cell density treatment for 1 day had a biomass productivity value of 0.30±0.02 g/l lower than the control sample without high cell density treatment. Sample C with high cell density treatment for 2 days had a biomass productivity value of 0.29±0.01 g/l lower than the productivity value of sample B which received high cell density treatment for 1 day. Sample D with high cell density treatment for 5 days had a biomass productivity value of 0.27±0.01 g/l lower than the productivity value of sample C which received high cell density treatment for 2 days. The data showed that the longer the starvation treatment process through the high cell density method, the lower the biomass productivity of *Chlorella vulgaris*. Cultivation conditions without homogenization can cause *Chlorella vulgaris* microalgae cells to settle and not obtain favorable cultivation conditions so that it has an impact on the lack of nutrient absorption by *Chlorella vulgaris* microalgae cells.

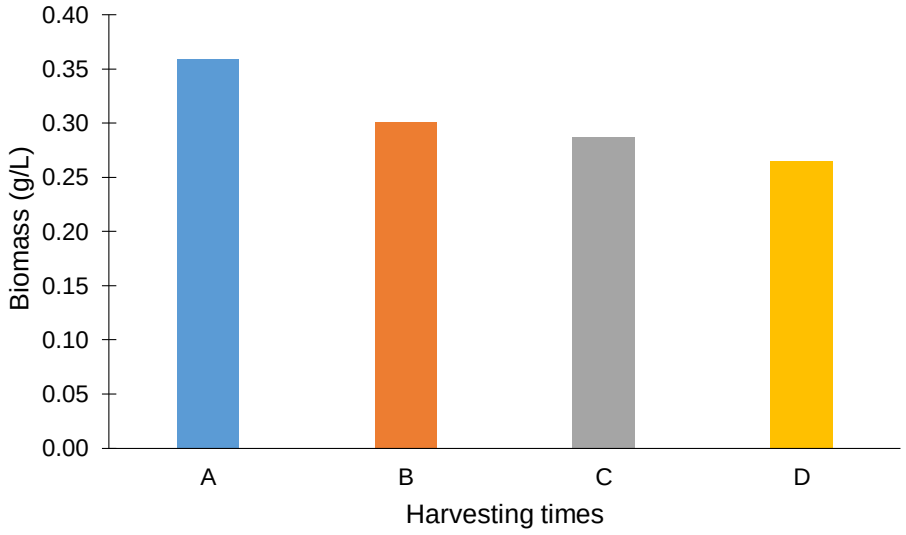

Figure 1. Average Weight of Biomass *Chlorella vulgaris*

Note for harvesting time : A control harvest 7th day, B high cell density 1 days, C high cell density 2 days, D high cell density 5 days. (n=3)

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Optical density is one of the methods used to observe the growth and condition of microalgae cells using a spectrophotometer by measuring cell density. Optical density of microalgae *Chlorella vulgaris* was measured using a wavelength of 750nm (Zahir et al, 2011). Optical density is usually used to observe microalgae growth patterns by observing cell density using certain wavelengths. The optical density obtained can reflect the cell density and the number of cells associated with the productivity of microalgae biomass. From the optical density data, the highest cell density value was obtained in sample A with an absorbance value of 1.64±0.02 abs (P<0.05). The lowest optical density value was obtained in sample D with a value of 0.96±0.08 abs (P<0.05). The optical density value data has the same graphic pattern as the *Chlorella vulgaris* biomass productivity value. This indicates that the starvation treatment with the high cell density method has an effect on the cell density of *Chlorella vulgaris*. Sample B with high cell density treatment for 1 day has an absorbance value of 1.51±0.08 abs which is lower than the control value. Sample C with high cell density treatment for 2 days had an absorbance value of 1.39±0.11 lower than sample B with 1 day high cell density treatment. Sample D with high cell density treatment for 5 days had an absorbance value of 0.96±0.08 abs lower than sample C with high cell density treatment 2 days. This value showed that the longer the high cell density stress treatment affects the density of *Chlorella vulgaris* microalgae cells.

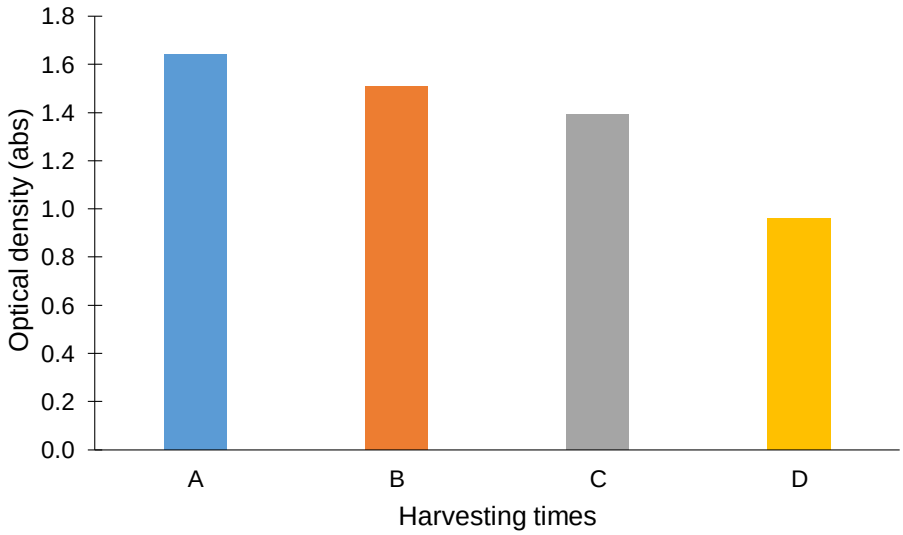

Figure 2. Average of Optical Density *Chlorella vulgaris*
Note for harvesting times : A control harvest 7th day, B high cell density 1 days, C high cell density 2 days, D high cell density 5 days. (n=3)

Lipid

Microalgae lipid accumulation can be increased by environmental stress conditions which include media growth conditions such as lack of nitrogen and phosphorus nutrients (Nayak et al., 2019). Under certain stress conditions, the formation of protein and carbohydrate synthesis enzymes in Chlorella decreases and carbon is directed to form lipids. The high cell density method was given with the aim of stopping homogenization which had an impact on cell deposition so that the cultivation conditions without stirring were expected to inhibit nutrient absorption by *Chlorella vulgaris* microalgae cells. The results of the research data showed that the highest lipid content was obtained in sample A with a lipid content of 56,2±4.6% (P<0.05). While the lowest lipid content value was obtained in sample D with high cell density treatment for 5 days with a value of 43,9±4.2% (P<0.05). Sample B using the high cell density method for 1 day had a lipid content value of 54,0±3.3%. Sample

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C with high cell density treatment for 2 days had a lipid content value of 43,9±4.2%. The high-density treatment aimed at giving starvation effect to microalgae cells under growth conditions was not proven to accumulate more lipids than the control treatment. Nutrient starvation can decrase biomassa productivity but increase the lipid content of the microalgae (Rugnini et al., 2020). In this study, nutrient starvation treatment approach with the high cell density method reduced the biomass productivity but was not proven to increase the lipid acumulation of *Chlorella vulgaris*. This showed that the high cell density method used as a starvation stress approach did not increase the lipid accumulation of the *Chlorella vulgaris*. *Chlorella vulgaris* microalgae in this study may still be able to absorb media nutrients well even without a homogenization process so that the high cell density treatment carried out was not proven to accumulate higher lipids in *Chlorella vulgaris*. Nitrogen starvation treatment is the most used method to increase lipid acumulation under autotrophic cultivation condition (Gojkovic et al., 2020).

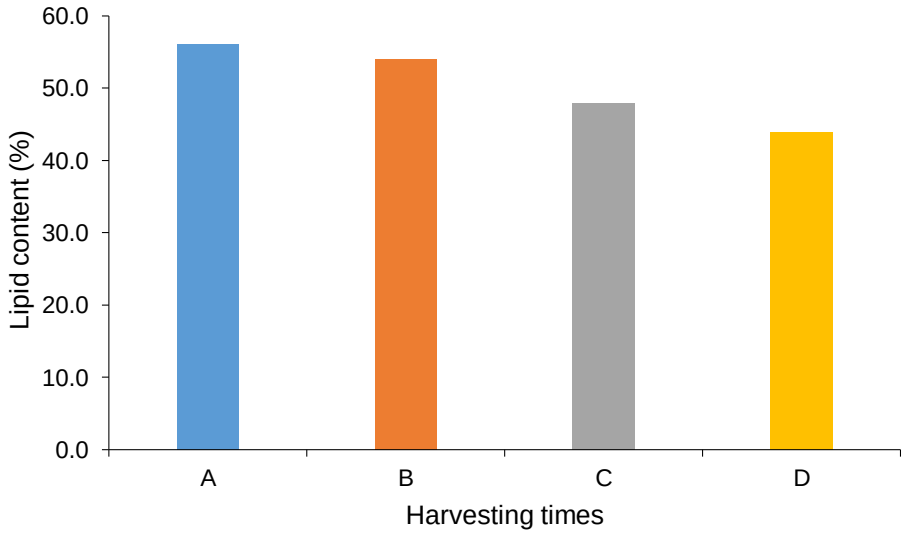

Figure 3. Lipid of *Chlorella vulgaris*
Note for harvesting times : A control harvest 7th day, B high cell density 1 days, C high cell density 2 days, D high cell density 5 days. (n=3)

CONCLUSION

The high cell density method affects biomass productivity, optical density, and lipid content of the microalgae *Chlorella vulgaris*. The longer high-density stress, the lower the productivity of biomass, cell density and also the lipid content of the microalgae *Chlorella vulgaris*. Therefore, the starvation stress approach using the high cell density method was not proven to accumulate higher lipids than the control treatment.

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