AL-KAUNIYAH: Jurnal Biologi, 16(2), 2023, 402-411
Website: http://journal.uinjkt.ac.id/index.php/kauniyah
P-ISSN: 1978-3736, E-ISSN: 2502-6720

GROWTH AND LIPID ACCUMULATION OF *Chaetoceros calcitrans* AFTER PHOSPHORUS AND LIGHT INTENSITY OPTIMIZATION

PERTUMBUHAN DAN AKUMULASI LIPID *Chaetoceros calcitrans* SETELAH OPTIMASI FOSFOR DAN INTENSITAS CAHAYA

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Submitted: 6 January 2023; Revised: 7 May 2023; Accepted: 17 June 2023

Abstract

Growth and lipid content of *Chaetoceros calcitrans* are greatly influenced by environmental factors. The aims of this study to optimize phosphorus concentrations and light intensity on the growth and lipid accumulation of *C. calcitrans*. This study used N:P:light intensity concentration from the previous research, namely 441:36.2 µM:2,500 lux (12:1:2,500 lux). Concentrations of P were then optimized to 36.2 µM, 27.5 µM, 18.1 µM, 9.05 µM (1; 0.75; 0.5; 0.25) and light intensity to 2,500; 3,000; 3,500; 4,000 lux. *C. calcitrans* was cultured in medium f/2 guillard, the initial density was 6 x 10^5 cells/mL. Sampling for lipid analysis was conducted in exponential, stationary, and the end of stationary phase by centrifugation, whereas lipid was extracted using the Bligh and Dyer method, and dried lipids were analyzed using gas chromatography-GC. The highest lipid content found at the late stationary phase of the N:P concentrations and light intensity 12:0.5:(4,000 lux), there was 15.46 ± 0.53%-dw with the highest cell density of 5.5 ± 5.56 x 10^6 cells/mL. The analysis result showed that palmitoleic acid (C16:1) was the highest fatty acid produced by each optimization. Nutritional deficiency and high light intensity were triggers for of *C. calcitrans* to accumulate lipids, and influence the fatty acid profile of *C. calcitrans*.

***Keywords*:** *Chaetoceros calcitrans*; Light; Lipids; Phosphorus

Abstrak

Pertumbuhan dan kandungan lipid Chaetoceros calcitrans sangat dipengaruhi oleh faktor lingkungan. Tujuan penelitian ini adalah untuk mengoptimasi konsentrasi fosfor dan intensitas cahaya terhadap pertumbuhan dan akumulasi lipid C. calcitrans. Penelitian ini menggunakan hasil konsentrasi N:P:intensitas cahaya dari penelitian sebelumnya, yaitu 441:36.2µM:2,500 lux (12:1:2,500 lux). Konsentrasi P kemudian dioptimasi menjadi 36,2 µM, 27,5 µM, 18,1 µM, 9,05 µM (1; 0,75; 0,5; 0,25), dan intensitas cahaya menjadi 2.500; 3.000; 3.500; 4.000 lux. C. calcitrans dikultur dalam medium f/2 guilard, densitas awal 6 x 10^5 sel/mL. Pengambilan sampel untuk analisis lipid dilakukan pada fase eksponensial, stasioner, dan akhir stasioner dengan sentrifugasi, sedangkan lipid diekstraksi menggunakan metode Bligh dan Dyer, lipid kering dianalisis menggunakan kromatografi gas-GC. Kandungan lipid tertinggi terdapat pada fase akhir stasioner konsentrasi N:P dan intensitas cahaya 12:0,5:(4,000 lux), yaitu sebesar 15,46 ± 0,53%-dw dengan kerapatan sel tertinggi 5,5 ± 5,56 x 10^6 sel/mL. Hasil analisis menunjukkan bahwa asam palmitoleat (C16:1) merupakan asam lemak tertinggi yang dihasilkan oleh masing-masing optimasi. Kekurangan nutrisi dan intensitas cahaya yang tinggi menjadi pemicu C. calcitrans. mengakumulasi lipid, dan mempengaruhi profil asam lemak C. calcitrans.

***Kata Kunci**: Cahaya; Chaetoceros calcitrans; Fosfor; Lipid*

Permalink/DOI: http://dx.doi.org/10.15408/kauniyah.v16i2.30316

INTRODUCTION

Chaetoceros calcitrans is a widely cultivated microalgae and contains high unsaturated fatty acids (highly unsaturated fatty acid), especially EPA and DHA, has high growth ability, good tolerance to environmental conditions (Guihéneuf & Stengel, 2013; Bastos et al., 2022). Both of these fatty acids are essential fats that are very important for animals and humans, especially in the regulation of the cardiovascular system, reproductive, and vital nutrients in the brain. The phytoplankton groups of diatoms they are excellent producers of EPA and DHA in aquatic food webs (Peltomaa et al., 2019).

There are many important factors that need to be considered in the *C. calcitrans* cultivation process, both physical and chemical, such as light intensity, nutrition, temperature, pH, and CO2. Among all the factors above, nutrition is the main chemical factor influencing the accumulation of triglyceraldehyde and the fatty acid composition in microalgae. Nitrogen and phosphorus have been reported to have a role in increasing lipid productivity (Khozin-Glodberg & Cohen, 2006; Yaakob et al., 2021). This is evidenced by Mohan et al. (2012) who found the highest lipid content of *Amphiprora paludosa* diatoms was in low NP culture conditions. According to Borowitzka and Borowitzka (1992) simple nitrogen sources such as nitrate and urea can support the growth and production of microalgae PUFAs. While phosphorus is an indispensable element in the regulation of algal metabolism, cell division, and cell membrane stabilizer. It mainly functions in energy transfer to produce ATP and ADP where the direct effect of phosphorus restriction is the reduction of carbon fixation activity resulting in biomass production and growth rate, low cells and increased neutral lipid content especially TAG (Cruz et al., 2015; Mühlroth et al., 2013). Furthermore, Lovio-Fragoso (2019) in his research showed that low concentrations of phosphorus resulted in low cell density and high lipid content in *Chaetoceros muelleri*.

In addition to nutrition, light is the most sensitive factor because it has a great influence on the chemical composition of microalgae including lipids and fatty acids (Liu et al., 2012). According to Chia et al. (2018), generally increasing light intensity can increase the growth of microalgae to the photoinhibition threshold, although the ability of each microalgae species is different. Salisbury and Ross (1995) and Kim et al. (2019) stated that high light-based cultivation can be an effective strategy to increase lipid productivity in microalgae because this process requires a lot of sufficient energy. This is evidenced by Sappewali (2009) who cultured *Tetraselmis chuii* at light intensities of 2,000; 3,000; and 4,000 lux, where the highest lipid was obtained at an intensity of 4,000 lux.

Hasby and Suantika (2016) have previously carried out nitrogen restriction in *C. calcitrans* culture and found the highest lipid content in f/2 media with a ratio of 12:1:2,500 lux (441 M:36.2 M:2,500 lux) which is ±22% of the normal condition. The optimum N concentration in this study became the basis for the N concentration used in this study. Furthermore, optimization of P concentration and light intensity was carried out to optimize the growth and accumulation of *C. calcitrans* lipids.

MATERIALS AND METHODS

The research was conducted at the Inter-University Center (PAU) building, Bandung Institute of Technology.

Experimental Design

The basis concentration of N:P:light intensity in the f/2 medium used was 12:1:2,500 lux (Hasby & Suantika, 2016). Optimization of P and light intensity was carried out in stages to determine the nutritional factors that control the growth and lipids accumulation of *C. calcitrans,* by modifying the P concentration in the f/2 medium according to the desired concentration (Table 1). The study used a completely randomized design (CRD) with 3 replications. After obtaining the optimum P concentration, light intensity was optimized using a Completely Randomized Design (CRD) with 3 replications.

Cultivation of *Chaetoceros calcitrans* on Variation of P and Light Intensity

Cultivation was carried out in a 10 L volume container with 6 L f/2 media, the density of *C. calcitrans* cells inoculated into each treatment was 6 x 10^5 cells/ml as the initial density. The light source used was obtained from LED lamps. The light intensity to the culture was adjusted or reduced by covering the lamp with paper to get the desired light intensity. To keep it constant, light intensity was measured over the culture surface using the *EXTeXH Heavy Light Duty Meter*. The culture was adjusted to salinity, temperature, pH, and photoperiod were set at 30 ppt, 27 °C, ±7.5, and 24:0 (light: dark), respectively.

Table 1. Experimental design.

Optimization (N:P:Light)	
P	Light Intersity
12:1 (2,500)	12:X (2,500)
12:0.75 (2,500)	12:X (3,000)
12:0.5 (2,500)	12:X (3,500)
12:0.25 (2,500)	12: X (4,000)

***Chaetoceros calcitrans* Cell Counting**

Microalgae density was calculated using a Haemacytometer. The relative growth rate (k) was calculated based on the formula according to Hirata et al. (1981), $k = \frac{\log (Nt/No)}{Tt-To}$ 3.22.

The number 3.22 is the constant value, Nt is the population density at time t, No is the cell population density at time 0, To is the initial time, and Tt is the observation time.

Water Quality Measurement

The measured water quality included ammonium, nitrate, nitrite, and phosphate using the spectrophotometric.

Harvesting and Lipid Extraction of *Chaetoceros calcitrans*

Harvesting for lipid analysis was carried out at the exponential, stationary, and final stationary phases by centrifugation, while the Bligh & Dyer method was used during lipid extraction (Nigam et al., 2011). Furthermore, evaporation was carried out to separate the solvent layer from the lipid extract. The lipid extract obtained was weighed and characterized by Gas Chromatography (GC) to determine fatty acid content.

Data Analysis

The data were analyzed using the Analysis of Variance (ANOVA) and further tested with Duncan's test in cases a significant difference found. To determine the correlation between biotic factors (the number of *C. calcitrans* cells) and abiotic factors, the Pearson correlation test was carried out. All Analysis was performed using SPSS software (version 16.0)

RESULTS

Cell Density and Lipid Content on P Optimization

Phosphorus is one of the essential elements for growth, so differences in phosphorus content affect the growth of microalgae. Figure 1 shows that *C. calcitrans* with the lowest phosphorus concentration was still able to survive until the end of the culture period with a relatively lower cell density, although the result was not significantly different from other treatments. This is due to the availability of phosphorus in the media that supports *C. calcitrans* to continue cell division and is still able to maintain its growth rate. However based on Figure 2, the highest lipid content was obtained at a relatively low P concentration of 12:0.5 (2,500) around 14.81%-dw, with a peak cell population of 4.2 ± 7.93 x 10^6 cells/ml, and dry weight of 0.596 ± 0.060 g-L, although the highest cell density was obtained at a P concentration of 36.2 M or 12:1 (2,500).

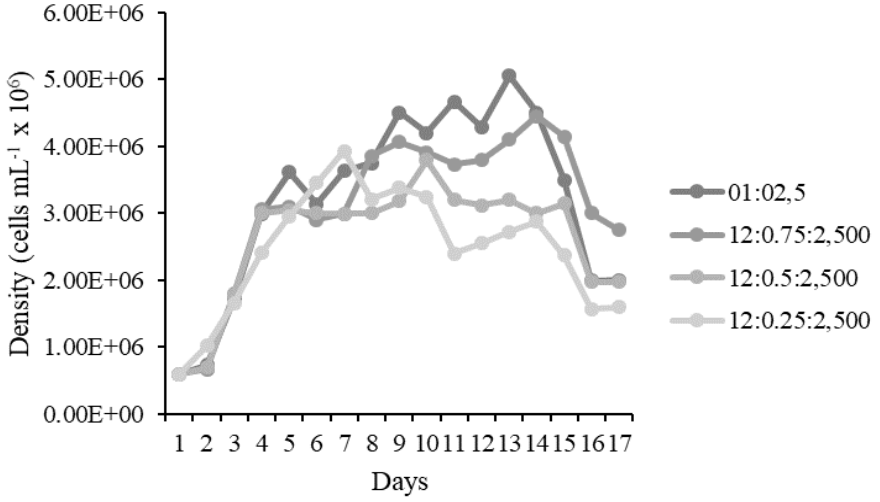

Figure 1. Growth curve of *Chaetoceros calcitrans* after P Optimization

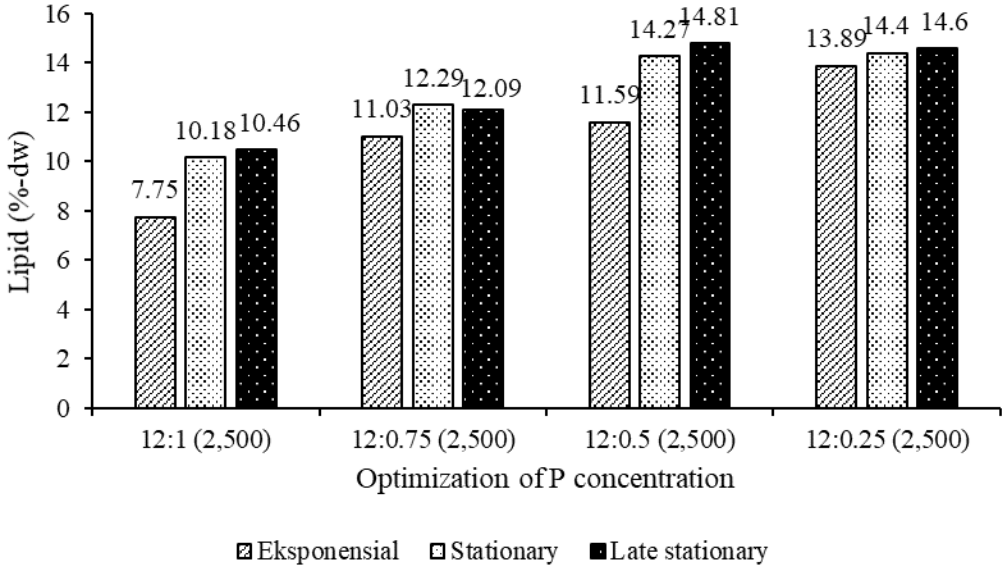

Figure 2. Lipid content of *Chaetoceros calcitrans* after P optimization

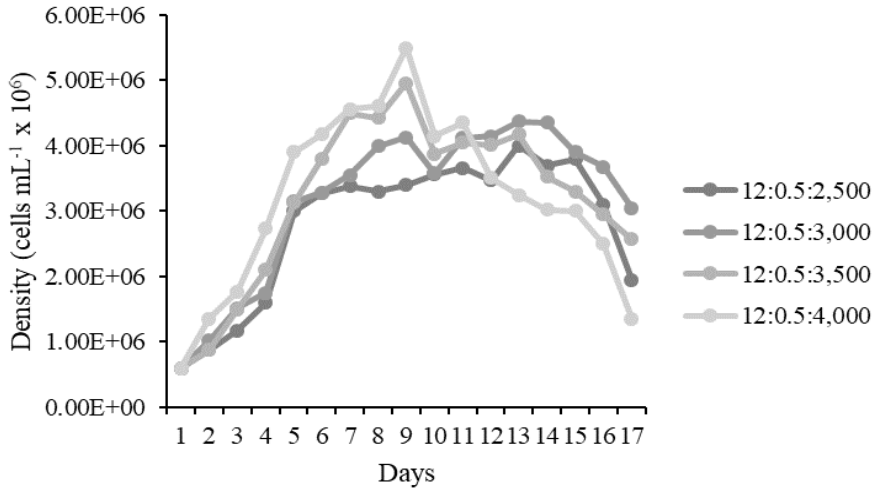

Figure 3. Growth curve of *Chaetoceros calcitrans* after light optimization

Cell Density and Lipid Content after Light Intensity Optimization

Nutrients are essential for microalgae for their growth, light is also needed by microalgae as autotrophs for photosynthesis. Figure 3 shows that at higher light intensities, *C. calcitans* took longer to enter the exponential phase, meaning that high light intensity stimulated *C. calcitrans* to

continue dividing even though it had a high cell density. However, due to high photosynthetic activity, nutrients will be depleted more quickly and eventually enter the death phase. Besides being an important element in the process of photosynthesis, light also plays a major role in the synthesis of lipids and fatty acids. Based on Figure 4, culture at 4,000 lux (12:0.5:4,000 lux) produced the highest lipid of 15.46 %-dw, the peak cell population was 5.5 ± 14.17 x10^6 cells/ml with a dry weight of 0.6136 ± 0.045, followed by light intensities of 3,500; 3,000; and 2,500 lux. The light intensity treatments of 2,500; 3,000; and 3,500 lux were not significantly different but were significantly different from the light intensity of 4,000 lux, this indicates that high light contributed greatly to the increase of lipids.

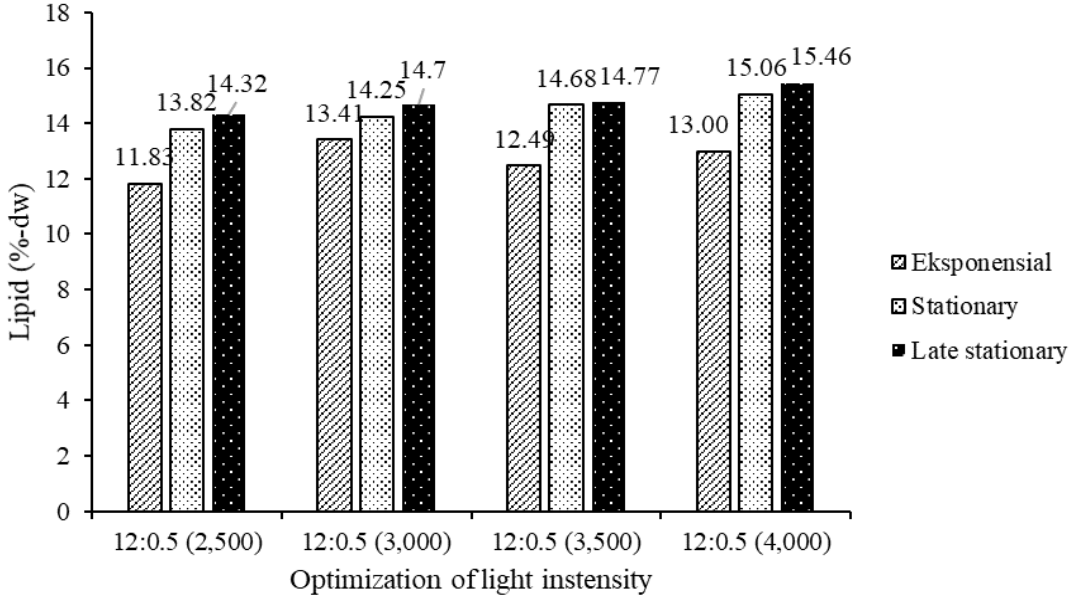

Figure 4. Lipid content of *Chaetoceros calcitrans* after light optimization

Table 2. Fatty acid profile of *Chaetoceros calcitrans* after P and light intensity optimization

Ratio / Fatty acids	12:1 (2,500) Hasby and Suantika (2016)	12:0.5 (2,500)	12:0.5 (4,000)
	% Fatty acid		
C 12:0 (lauric acid)	0.40	0.00	0,00
C 14:0 (mirystic acid)	21.83	0.00	0.00
C 16:0 (palmitic acid)	8.57	21.90	28.88
C 18:0 (stearic acid)	1.33	3.26	2.86
C 20:0 (arachidic acid)	0.66	0.25	0.14
C 22:0 (behenic acid)	0.00	0.00	0.44
C 24:0 (lignoceric acid)	-	-	-
C 16:1 (palmitoleic acid)	28.18	53.62	53.86
C 18:1 (oleic acid)	1.13	0.21	0.74
C 18:2 (linoleic acid)	0.63	1.54	0.48
C 18:3 (linlenic acid)	0.05	0.00	0.18
C20:5 (eicosapentanoate/EPA)	14.28	6.11	2.49
C22:6 (docosahexaenoate/DHA)	1.25	0.81	0.47
Etc.	21.64	12.6	9.52

Fatty Acid Profile after P and Light Intensity Optimization

Under certain environmental conditions, microalgae will usually form their own cell walls in the form of PUFAs as a self-protection mechanism with the help of desaturation enzymes. The

Table 2 shows that the fatty acid content of the SAFA and MUFA groups after optimization of P and light intensity, especially palmitic, palmitoleic, and stearic acids (C16:0, C18:0, C22:0, C16:1) tended to be higher than the previous study, which cultured *C. calcitrans* on nitrate limiting treatment. While the PUFA content, namely eicosapentanoic acid/EPA and docosahexanoic acid/DHA (C20:5, C22:6) tended to be lower.

Correlation Between *Chaetoceros calcitrans* Population Growth and Water Quality

Pearson correlation was carried out to determine the relationship between water quality parameters and the increase in the cell population in culture. A positive correlation shows a relationship that is in the direction of growth, while a negative correlation shows an inverse relationship with growth. If the analysis result close to 1, then the correlation is getting closer to growth (Table 3).

Table 3. Person correlation between *Chaetoceros calcitrans* population and water quality

P optimization							
Ratio	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	pH	Temperature	Dissolved oxygen
12:1 (2,500)	0.168	0.238	-0.831*	0.319	0.504	0.277	0.619
12:0.75 (2,500)	0.215	0.656	-0.823*	-0.370	0.210	0.584	0.717
12:0.5 (2,500)	-0.214	-0.146	-0.711	-0.139	0.568	0.284	-0.205
12:0.25 (2,500)	-0.170	0.250	-0.781*	-0.104	0.886*	0.120	0.230
Light optimization							
Ratio	NH_4^+	NO_2^-	NO_3^-	PO_4^{3-}	pH	Temperature	Dissolved oxygen
12:0.5 (2,500)	-0.855*	-0.109	-0.854*	-0.947**	0.777	0.155	0.030
12:0.5 (3,000)	-0.767*	0.834*	-0.738	-0.963**	0.402	0.282	-0.653
12:0.5 (3,500)	-0.186	0.629	-0.422	-0.468	-0.118	0.141	-0.336
12:0.5 (4,000)	-0.258	0.451	-0.202	-0.723	-0.275	-0.132	-0.375

DISCUSSION

Cell Density and Lipid Content on P Optimization

Phosphorus (P) plays critical roles in the metabolism of nitrogen compounds, carbohydrate transportation, carbohydrate metabolism, and fat metabolism (López-Arredondo, 2014). The P deficiency affects nutrient absorption and lowers photosynthesis performance, which might be the crucial cause of the inhibited growth of plant. According to El-Sheek and Rady (1995), the low phosphorus content in the medium will inhibit the cell division process so thus will reduce the chlorophyll content and inhibiting the photosynthesis process, but the resulting lipid content is higher than the normal phosphorus content. In other words, under conditions of limited nutrition, cell division decreases, but the synthesis of lipids and fatty acids is maintained as long as there is sufficient CO_2 and light available for photosynthesis (Figure 2). This is because when growth slows down and the requirements for the synthesis of new membranes are exhausted, the cells will switch fatty acid stores to TAG (Triacylglycerides), thus TAG function as a self-defense mechanism (Sharma et al., 2012).

Li-Beisson et al. (2015) indicated that TAG accumulation under nutrien starvation in microalge is due to increased de novo synthesis of TAG from acyl-CoA, recycling of acyl moieties from the degradation of membrane lipids into TAG and increased carbon flux towards glycerol-3-phosphate and acyl-CoA for fatty acid synthesis (Fan et al., 2011; Goncalves et al., 2013; Miller et al., 2012). According to Mandotra et al. (2016) have studied impact of K_2HPO_4 concentrations on biomass, lipid concentration and fatty acid profile in *Scenedesmus abundans*. The results in this study showed that the phosphorus concentration of 18.1 µM or 12:0.5 (2,500) was significantly different from the ratio of 12:1 (2,500), although not significantly different from the other treatments.

Cell Density and Lipid Content after Light Intensity Optimization

Marine algae from different species respond to stress conditions by altering their metabolism and accumulating high amounts of neutral lipids and other compounds, such as carbohydrates and

secondary metabolites (Merchant et al., 2012; Markou & Nerantzis, 2013). Both P and light optimization in this study showed significant differences in lipid content in each growth phase, where the highest lipid content was generally obtained at the stationary end. The high level of lipids found is due to the production of lipids or the accumulation of fat in this phase, which gradually begins to occur until finally entering the death phase. This is related to the decline of key nutrients such as nitrogen which plays a role in protein synthesis and biomass production (Guzman et al., 2010; Panggabean, 2011).

According to Sitorus (2009) and Liu et al. (2012), a high amount of light intensity can cause metabolic accumulation in the form of lipids. The formation of lipids and fatty acids in light conditions occurs faster than formation in dark conditions. This is due to the availability of energy (2NADPH and ATP) needed for each acetyl group can be available through the photosynthesis process. This was reinforced by Solovchenko (2012) who stated that the synthesis of TAG is activated by high light intensity, especially when there is a nutritional deficiency. Stress conditions will activate the ACC-ase enzyme which acts as a precursor in the formation of lipids, causing an increase in carbohydrates and TAG (Lynn et al., 2000). In addition, under high light stress microalgae accumulate a large amount of reactive oxygen species (ROS) including singlet oxygen (O_2), superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals, resulting in the direct decomposition of the lipids, protein, and nucleic acids (Nzayisenga et al., 2020).

Fatty Acid Profile after P and Light Intensity Optimization

Fatty acids are important as components of the cell membrane, lipid storage or signal transduction pathways, for instance, polyunsaturated fatty acids (PUFAs). In this study, the fatty SAFA and MUFA conten especially palmitic, palmitoleic, and stearic acids (C16:0, C18:0, C22:0, C16:1) after optimization P and ligh intensity tended to be higher than that researched by Hasby and Suantika (2016). While the PUFA content, namely eicosapentanoic acid/EPA and docosahexanoic acid/DHA (C20:5; C22:6) tended to be lower. Vega et al. (2010) reported relatively similar results to this study where there was an increase in some fatty acids from the SAFA (and MUFA groups, while PUPA, especially EPA and DHA tended to be lower. The increase in the content of MUFAs is associated with its central role in the PUFA synthesis system (Maltsev et al., 2021). According to Mühlroth et al. (2013) stress conditions in culture can be the cause of an increase in TAG and a decrease in EPA. This is because when nutrients are available, microorganisms get energy from these nutrients, while when nutrients begin to decline, cell division will decrease and trigger *C. calcitrans* to produce secondary metabolites in the form of fatty acids which in turn will be stored in TAG (Sharma et al., 2012; Shingh & Mallick, 2014).

In addition, the low levels of several fatty acids from the PUFA group found in this study, especially at a high light intensity, were thought to be due to the light intensity that triggered PUFA oxidation. PUFA has more than two double bonds and is more unstable than SAFA and MUFA as PUFAs are more easily metabolized. Under normal conditions, the energy in the form of NADPH and ATP produced during the photosynthesis process will be consumed producing biomass, so that the electron acceptor will be available again. Meanwhile, under conditions of high light intensity, electron acceptors, $NADP^+$ may be depleted (Sharma et al., 2012; Shingh & Mallick, 2014). Therefore, microalgae accumulate fatty acids which are then stored in the form of TAG, and potentially help microalgae to get electron acceptors back (Sharma et al., 2012). In other words, the formation of TAG under stress conditions is a strategy used by *C. caclitrans* as a self-protection mechanism for cells. Liang et al. (2005) also reported that the total number of MUFAs increased while PUFAs decreased over the culture period in *C. gracilis*. Therefore, the culture period also needs to be considered to get optimal EPA DHA.

Correlation Between *Chaetoceros calcitrans* Population Growth and Water Quality

Table 3 showed that ammonium in culture tends to fluctuate, where the increase in ammonium is thought to occur because there is a large number of dead cells causing accumulation of organic substances from *C. calcitrans* which undergoes lysis during the culture period. The dead biomass will be decomposed by bacteria in the culture medium and will release ammonia

compounds which then will be protonated into ammonium. Meanwhile, the decrease in ammonium is thought due to *C. calcitrans* using ammonium ions directly as a substitute for nitrate.

The nitrite content tends to fluctuate during the culture period of all treatments at each optimization. This compound is temporary (inner) because it is a transition between ammonium and nitrate. Fluctuations occur due to the accumulation of organic substances which are a source of ammonium that is rapidly converted to nitrite and then to nitrate by nitrifying bacteria.

In general, nitrate content decreased along with the increasing population of *C. calcitrans* (negative correlation) from all treatments, both on P and light intensity optimization. This is because nitrate is the main source of nutrients needed by microalgae. This compound is derived from the denitrification of NO_2 and N_2 gases by involving anaerobic bacteria under natural conditions. During the culture period, the number of cell populations in the culture increases followed by a decrease in nitrate concentration. While phosphorus tends to decrease (negative correlation). The decrease in phosphorus occurred due to the utilization of phosphorus by *C. Calcitrans*. This is because phosphorus is a limiting factor in the growth of microalgae which is absorbed in the form of orthophosphate (Suantika et al., 2009).

The different results showed by the degree of acidity (pH). According to Kordi and Tancung (2007) pH is inversely proportional to the CO_2 content. The decrease in pH can be caused by the use of nutrients so that the medium tends to be acidic. The increasing contaminants in the culture are thought to be another cause of the decrease in pH. This is in accordance with the results above where at the relatively higher light intensity, the pH is negatively correlated, meaning that nutrient absorption activity is higher due to light stimulation. While the oxygen content in the culture is inversely proportional or tends to decrease. The decrease in oxygen can be caused by various things, including an increase in the number of contaminants and the role of bacteria as organisms that also consume oxygen. In addition, oxygen is used in the process of respiration and the elongation of fatty acids. Meanwhile, the temperature did not show a correlation with the addition of *C. calcitrans* population (relatively stable) in culture. During the culture period, the temperature ranged from ±26–27 °C.

CONCLUSION AND SUGGESTIONS

Concentration (ratio) N:P and light intensity 12:0.5 (4,000) can increase the lipid content. Further optimization is needed to obtain higher lipids. It is necessary to analyze the fatty acid profile in each growth phase to obtain optimal PUFA (EPA and DHA) content.

ACKNOWLEDGEMENTS

Thank you for the Inter-University Center (PAU) building, Bandung Institute of Technology.

REFERENCES

Bastos, C. R. V., Maia, I. B., Pereira, H., Navalho, J., & Varela, J. C. S. (2022). Optimisation of biomass production and nutritional value of two marine diatoms (*Bacillariophyceae*), *Skeletonema costatum* and *Chaetoceros calcitran. biologi*, *II*(4), 594. doi: 10.3390/biology11040594.

Borowitzka, M. A., & Borowitzka, L. J. (1992). *Mikroalga biotechnology*. Newyork: Cambridge University Press.

Chia, S. R., Ong, H. C., Chew, K. W., Show, P. L., Phang, S-M., Ling, T. C., … Lee, D-J. (2018). Sustainable approaches for algae utilisation in bioenergy production. *Renew Energy*. *129*, 838–52. doi: 10.1016/j.renene.2017.04.001.

Cruz, de C. H., Sun, H-X., Bowler, C., & Chua, N. H. (2015). The *Phaeodactylum tricornutum* long non-coding transcriptome: Mere noise or functionally relevant?. *Conference: Molecular Life of Diatoms*. doi: 10.13140/RG.2.1.3630.6081.

El-Sheek, M. M., & Rady, A. A. (1995). Effect of phosporus stavation on growht, photosynthesis and some metabolic processes in the uniceluller green alga *Clorella kessleri*. *Phyton*, *35*, 139-151.

Fan, J., Andre, C., & Xu, C. (2011). A chloroplast pathway for the *de novo* biosynthesis of triacylglycerol in *Chlamydomonas reinhardtii*. *FEBS Letter, 585*, 1985-1991.

Guihéneuf, F., & Stengel, D. B. (2013). LC-PUFA-enriched oil production by microalgae: Accumulation of lipid and triacylglycerols containing n-3 LC-PUFA is triggered by nitrogen limitation and inorganic carbon availability in the marine haptophyte *Pavlova lutheri*. *Marine Drugs Journal, 11*(11), 4246-66. doi: 10.3390/md11114246.

Goncalves, E. C., Johnson, J. V., & Rathinasabapathi, B. (2013). Converstion of membrane lipid acyl groups to triacylglycerol and formation of lipid bodies upon nitrogen starvation in biofuel green algae *Chlorella* UTEX29. *Planta, 238*, 895-906.

Guzman, H. M., Valido, A., de la Jara., Duarte, L. C., & Presmanes, K. F. (2010). Estimate by means of flow cytometry of variation in composition of fatty scids from *Tetraselmis suecica* in response to culture conditions. *Aquaculture International, 18,* 189-199.

Hasby, R. M., & Suantika, G. (2016). Limitasi nitrat untuk pertumbuhan dan akumulasi lipid *Chaetoceros calcitran*. *Biotika Jurnal Ilmiah Biologi, 14*(1), 66-71. doi: 10.24198/bjib.v14i1.14416.

Hirata, H., Andarias, I., & Yamasaki S. (1981). Effect of salinity temperature on the growth of the marine phytoplankton *Chlorella saccharophila*. *Memoirs Faculty of Fisheries Kagoshima University, 30*, 257-262

Liang, Y., Mai, K., & Sun, S. (2005). Differences in growht, total lipid content and fatty acid compotition among 60 clones of *Cylindrotheca fusiformis*. *Journal of Applied Phycology, 17*, 61-65.

Liu, J., Yuan, C., Hu, G., & Li, F. (2012). Effects of light intensity on the growth and lipid accumulation of microalgae *Scenedesmus* sp.11-1 under nitrogen limitation. *Applied Biochemistry and Biotechnology, 166*, 2127-2137.

Lynn, S., Kilham, S. S., Kreeger, D. A., & Interlandi, S. J. (2000). Effect of nutrient availability on the biochemical and elemental stoichiometry in fresh water diatom *Stephanodiscus minutulus* (*Bacillariophyceae*). *Journal of Phycology, 36*, 10–522.

Khozin-Goldberg, I., & Cohen, Z. (2006). The effect of phosphate starvation on the lipid and fatty acid composition of the fresh water *Eustigmatophyte Monodus subterraneus*. *Phytochemistry, 67*, 696-701.

Kim, K., Jung, J. Y., & Han, H. S. (2019). Utilization of microalgae in aquaculture system: Biological wastewater treatment. *Emerging Science Journal, 3*(4), 209-221 doi:10.28991/esj-2019-01183.

Kordi, M. G. H. K., & Tancung, A. B. (2007). *Pengelolaan kualitas air dalam budidaya perairan*. Jakarta: Rineka Cipta.

Li-Beisson, Y., Beisson, F., & Riekhof, W. (2015). Metabolism of acyl-lipids in *Chlamydomonas reinhardtii*. *The Plant Journal, 82*(3), 504-522. doi: 10.1111/tpj.12787. Epub 2015 Mar 3. PMID: 25660108.

Lovio-Fragoso, J. P., Hayano-Kanashiro, C., & López-Elías, J. A. (2019). Effect of different phosphorus concentrations on growth and biochemical composition of *Chaetoceros muelleri*. *Latin America Journal Aquatiq Research, 47*(2), 361-362. doi: 10.3856/vol47-issue2-fulltext-17.

López-Arredondo, D. L., Leyva-Gonzales, M. A., Gonzalez-Morales, S. I., Lopez-Bucio, J., & Herrera-Estrella, L. (2014). Phosphate nutrition: Improving low-phosphate tolerance in crops. *Annual Review of Plant Biology, 65*, 95-123. doi: 10.1146/annurev-arplant-050213-035949.

Maltsev, Y., Maltseva, K., Kulikovskiy, M., & Maltseva, S. (2021). Influence of light conditions on microalgae growth and content of lipids, carotenoids, and fatty acid composition. *Biology (Basel), 10*(10), 1060. doi: 10.3390/biology10101060.

Mandotra, S. K., Kumar, P., Suseela, M. R., Nayaka, S., & Ramteke, P. W. (2016). Evaluation of fatty acid profile and biodiesel properties of microalgae *Scenedesmus abundans* under the influence of phosphorus, pH and light intensities. *Bioresource Technology, 201*, 222-229

Markou, G., & Nerantzis, E. (2013). Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions. *Biotechnology Advances, 31*, 1532-1542. doi.org/10.1016/j.biotechadv.2013.07.011.

Merchant, S. S., Kropat, J., Liu, B., Shaw, J., & Warakanont, J. (2012). TAG, you are it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. *Current Opinion in Biotechnology, 23*, 352-363. doi.org/10.1016/j.copbio.2011.12.001.

Miller, M. R., Siew-Young, Q., Kathrin, S., Nalder., & Michael, A. P. (2012). Changes in oil content, lipid class and fatty acid composition of the microalga *Chaetoceros calcitrans* over different phases of batch culture. *Aquaculture Research, Plant Physiology, 154*, 1737-1752. doi: 10.1111/are.12107.

Mohan, N., Rajaram, M. G., Boopathy, A. B., & Rengasamy, R. (2012). Biomass and lipid production of marine diatom *Amphiprora paludosa* W. Smith at different nutrient concentrations. *Journal of Algal Biomass Utilization, 3*(4), 52-59.

Mühlroth. A., Keshuai, L., Gunvor, R., Per, W., Yngvar, O., Martin F. H-M, … Atle, M. B. (2013). Pathways of lipid metabolism in marine algae, co-expression network, bottlenecks and candidate genes for enhanced production of EPA and DHA in species of Chromista. *Marine Drugs Journal*, *11*(11), 4662-4697. doi: 10.3390/md11114662.

Nigam, S., Rai, M. P., & Sharma, R. (2011). Effect of nitrogen on growth and lipid content of *Chlorella pyrenoidosa*. *American Journal of Biochemistry and Biotechnology*, *7*(3), 124-129. doi: 10.3844/ajbbsp.2011.124.129.

Nzayisenga, J. C., Farge, X., Groll, S. L., & Sellstedt, A. (2020). Effects of light intensity on growth and lipid production in microalgae grown in wastewater. *Biotechnology for Biofuels, 13(*4), 1-8. doi: 10.1186/s13068-019-1646-x.

Panggabean, L. M. G. (2011). Koleksi kultur mikroalga. *Oseana*, *XXXII*(2), 11-2011.

Peltomaa, E., Hallfors, H., & Taiple, S. J. (2019). Comparison of *Diatoms* and *Dinoflagellates* from different habitats as sources of PUFAs. *Marine Drugs Journal*, *17*(4), 233. doi: 10.3390/md17040233.

Salisbury. F. B., & Ross, C. W. (1995). *Fisiologi tumbuhan*. Bandung: ITB press.

Sappewali. (2009). Penentuan intensitas cahaya optimum pada pertumbuhan dan kadar lipid mikroalga *Tetraselmis chuii* (Master's thesis). Institut Teknologi Sepuluh Nopember (ITS), Surabaya, Jawa Timur, Indonesia.

Sharma, K. K., Holger, S. & Peer, M. S. (2012). High lipid induction in microalgae or biodiesel production. *Article*, 5, 1532-1552.

Shingh, D. K., & Mallick, N. (2014). Accumulation potential of lipids and analysis of fatty acid profile of few microalgal species for biodiesel feedstock. *Journal of Microbiology and Biotechnology Research*, *4*(1),37-44.

Solovchenko, A. E. (2012). Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses: Review. *Russian Journal of Plant Physiology*, *59*(2), 167-176.

Sitorus, M. (2009). Hubungan nilai produktivitas primer dengan konsentrasi klorofil-a, dan faktor fisika kimia di perairan Danau Toba, Balige, Sumatra Utara (Master's thesis). Universitas Sumatra Utara, Medan, Indonesia.

Suantika, G., Pingkan, A., Dea, I. A., & Yusuf, S. (2009). Pengaruh kepadatan awal inokulum terhadap kualitas kultur *Chaetoceros gracilis* (Schütt) pada sistem batch. *Jurnal Matematika dan Sains*, *14*(1), 1-8.

Vega, J. M. P., Roa, M. A. C., Saavedra, M. del P. S., Ramírez, D. T., & Davalos, y. C. R. (2010). Effect of culture medium and nutrient concentration on fatty acid content of *Chaetoceros muelleri. Revista Latinoamericana de Biotechnologia Ambiental Y Algal, 1*(1), 6-15.

Yaakob, M. A., Mohamed, R. M. S. R., Al-Gheethi, A., Gokare, R. A., & Ambati, R. R. (2021). Influence of nitrogen and phosphorus on microalgal growth, biomass, lipid, and fatty acid production: An overview. *Cells*, *10*(2), 393. doi: 10.3390/cells10020393.