Effect of Light Intensity, CO_2 Gas Concentration, Culturing Period and Walne Nutrient Concentrations on Biomass and Lipid Productivity of *Chlorella vulgaris* in Sea Water Media

Timotius Candra Kusuma[1], *Anggun* Rindang Pratiwi[1], *Septiandre*[1], *Siti* Zulaikah[1*]

[1] Department of Chemical Engineering, Sepuluh Nopember Institute of Technology,Surabaya

Abstract. The biomass and lipid productivity of Chlorella vulgaris cultured in sea water media were conducted in this study. The effect of light intensity (5000 and 10000 lux), CO_2 gas concentration (0.03%, 1% and 2%), culturing period (7 and 17 days) and walne nutrient concentrations (0%, 0.05%, 0.1% and 0.3%) on biomass and lipid productivity of C. vulgaris cultured in photobioreactor were studied systematically. The biomass and lipid productivity were increased with increasing light intensity and CO_2 gas concentration. Longer culturing period, C. vulgaris produced more biomass and lipid content. However, biomass and lipid productivity at shorter cultured period were higher than longer cultured period. The highest biomass productivity of 139 mg/L/d was obtained under the following condition: light intensity = 10000 lux, CO_2 gas concentration = 2%, culturing period = 7 days, and walne nutrient concentration = 0.3%. The highest lipid productivity of 40.68 mg/L/d was obtained under the following condition: light intensity = 10000 lux, CO_2 gas concentration = 2%, culturing period = 7 days, and walne nutrient concentration = 0.005%. This study shows that a microalga C. vulgaris was a potential candidate as a source of biodiesel production.

1 Introduction

The availability of energy from non-renewable sources was limited. Therefore an innovation is needed to overcome this problem. Microalgae are known as photosynthetic microorganisms that can combine solar, water and CO_2 energy to serve as biomass. Microalgae is a highly efficient biomass capable of taking waste (zero energy) in the form of carbon (CO_2) and converting it into a high density of liquid energy form (petroleum) (Widjaja et al, 2008). According to some estimates, the yield (per hectare) of algae oil can produce 200 times greater than the yield of vegetation or other vegetable oils (Dermibas, 2008). The type of microalga to be used in this study was *Chlorella vulgaris*. Miao and Wu (2006) reported that heterotrophic growth of *Chlorella protothecoides* was able to produce lipid content of 55% and it could also turn into biodiesel (Miao and Wu, 2004, 2006; Miao et al., 2004; Wu et al., 1992, 1994). The effect of CO_2 concentration on lipid productivity can be seen from the previous research which obtained the result that at 50 mL/min CO_2 flowrate, the lipid productivity after 17 days of nutrient deficiency reaches more than 50% which is the highest value when compared to lipid content using a lower CO_2 flowrate (Widjaja et al, 2008).

Therefore, the objective of this research is to study the effect of light intensity, CO_2 gas concentration, culturing period and walne nutrient concentration on biomass and lipid productivity from *Chlorella vulgaris* on sea water medium. The expected benefits of this study are expected to show a comparison of growth rates and lipid production of *Chlorella vulgaris* on optimal amounts of nutrients and light for biofuel production.

2 Materials and methods

2.1. Materials

Chlorella vulgaris microalgae and walne nutrient obtained from Cultivation Centre of Air Payau Situbondo. Technical methanol and aquadest were obtained from local market. Filter paper 2.5μm grade 42 diameter 125mm was obtained from GE Healthcare Life Sciences U. Sea water with salinity of 24.7 ppt (2.47%) was obtained from Centre of decorative fish Gunungsari, Surabaya, Indonesia.

* Corresponding author: szulle@chem-eng.its.ac.id, szullehakim@gmail.com

2.2 Algae cultivation

Chlorella vulgaris (200 mL) obtained from Cultivation Centre of Air Payau Situbondo, Indonesia had cell concentration of ± 5000 cells/ml was added into sterilized seawater (800 mL) with salinity of 24.7 ppt (2.47%). Salinity of seawater was measured use Salinometer EC 10 from ρH on Lab. Cultivation was performed on a lab scale used light intensity of 5000 and 10.000 lux was measure using Lux digital LX1010B from Dekko with a lighting ratio of 12 h : 12 h (dark: light) and added (0%, 0.05%, 0.1% and 0.3% of walne nutrient, where each 1 mL walne nutrient contains 17.26 mg of nitrogen. Aeration was carried out with different flow rate and CO_2 concentration (2 mL/min (0.03%), 62.5 mL/min (1%) and 125 mL/min (2%)).

During the culturing period, each day monitor and maintain the water level of the photobioreactor by adding a sterilized aquadest. The added aquadest volume varies depending on the amount of water lost. UV-VIS spectrophotometer (GENESYSTM 10S, Thermo Scientific USA) was used to measure the absorbance of Chlorella vulgaris culture every 24 hours. Harvesting was done after 7 and 17 days of cultivation

2.4 Extraction and Distillation

The extraction was carried out by soxhlet extractor using methanol as solvent. Algae extraction was done until the paper filters used wrapped microalgae turn back to the original colour. Separation of microalgae oil and methanol was carried out using a rotary vacuum evaporator, Yamato, USA.

2.5 Analysis

Chlorella vulgaris growth analysis was observed daily through absorbance observation using UV-VIS spectrophotometer. The wavelength used was 685 nm. The relationship between absorbance with the number of cells for *Chlorella Sp.*(Hadiyanto, 2010) was:

Number of cells (cell / mL) = 13.4 x 10^6 x OD_{685}

The biomass productivity, lipid content and lipid productivity were calculated as follow:

Biomass productivity (g/L/d) = mass of dry alga (gram)/ (Culture volume (L) * day)

Lipid content (%) = (mass of extracted lipids/dry microalgae mass) x 100%

Lipid productivity (g/L/d) = (Biomass (g/L) x lipid content)/(Number of days of culturing (days)).

Then the oil results was analysed further by using TLC and GC-MS analysis.

3 Results and discussion

3.1. Growth of Microalgae Chlorella vulgaris In Sea Water Media

In this study, the growth of Chlorella vulgaris microalgae was affected by light intensity, CO_2 gas concentration, culturing period and nutrient concentration of walne. Sea water salinity was 24.7 ppt (2.47%). Based on the previous research, it was mentioned that Chlorella vulgaris was tolerant to the environment with salinity content of 0-70 ppt (Chalid et al).

The growth of Chlorella vulgaris was observed in this study by increasing the number of cells. Effect of light intensity, walne nutrient concentration and harvesting time at flow rate of 2mL/min (CO_2 gas concentration= 0.03%) on growth of Chlorella vulgaris microalgae can be seen in Fig 3.1 and 3.2.

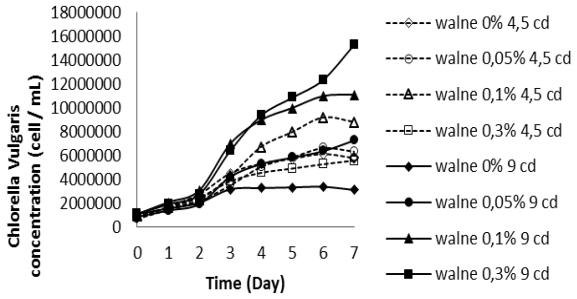

Fig 3.1. *Chlorella vulgaris* concentration in seawater media for 7 days of culturing period with flow rate of 2mL/ min (0.03% CO_2 gas concentration) for various nutrient addition of walne at 5000 lux and 10000 lux.

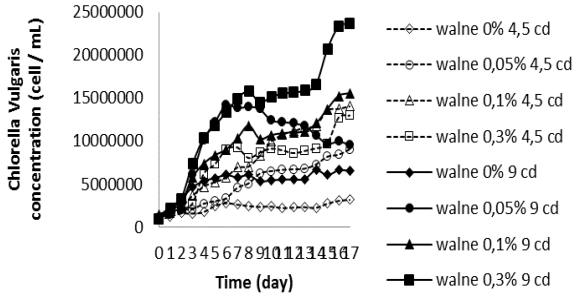

Fig 3.2. *Chlorella vulgaris* concentration in seawater media for 17 days of culturing period with flow rate of 2mL/ min (0.03% CO2 gas concentration) for various nutrient addition of walne at 5000 lux and 10000 lux.

CO_2 used in this study was from the air, it called CO_2 in ambient water. In both Figs, the highest growth was obtained in 0.1% walne nutrient concentration with a light intensity of 10000 lux. From the Fig was known that to increase cell concentration by increasing of walne nutrient concentration. For the trend line formed on

growth for 7 days showed a significant increase on the 3rd day to the 7th day. As for the trend line on growth for 17 days showed a tendency to rise until day 8, and then enter the stationary phase during its growth.

Effect of light intensity, walne nutrient concentration and harvesting time at flow rate of 62.5 mL/min (CO_2 gas concentration= 1 %) on growth of Chlorella vulgaris microalgae can be seen in Fig 3.3 and 3.4.

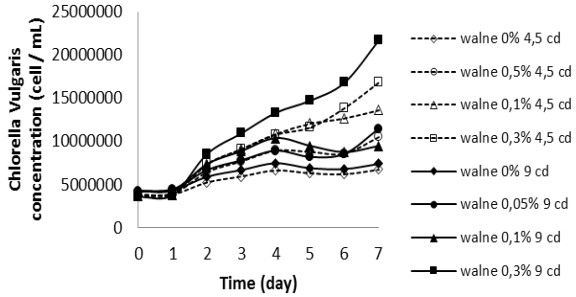

Fig 3.3. *Chlorella vulgaris* concentration in seawater media for 7 days of culturing period with flow rate of 62.5 mL/min (1% CO_2 gas concentration) for various nutrient addition of walne at 5000 lux and 10000 lux.

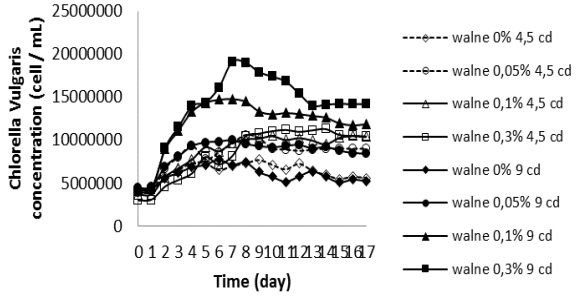

Fig 3.4. *Chlorella vulgaris* concentration in seawater media for 17 days of culturing period with flow rate of 62.5 mL/min (1% CO2 gas concentration) for various nutrient addition of walne at 5000 lux and 10000 lux.

Fig 3.3 and 3.4 shown that the highest cell concentration was obtained by giving walne nutrition concentration of 0.3% and light intensity of 10000 lux. From these can also be observed the tendency of increasing growth along with the increasing number of walne nutrient concentration. In Fig 3.3 it can be seen that the trend line formed on each chart increased on the third day and still continues to increase even though it has been the seventh day (not yet entered the stationary phase). The trend line on each graph is likely to have entered the stationary phase on the day of 5th to day of 17th.

Effect of light intensity, walne nutrient concentration and harvesting time at flow rate of 125 mL/min (CO2 gas concentration= 2 %) on growth of Chlorella vulgaris microalgae can be seen in Fig 3.3 and 3.4..

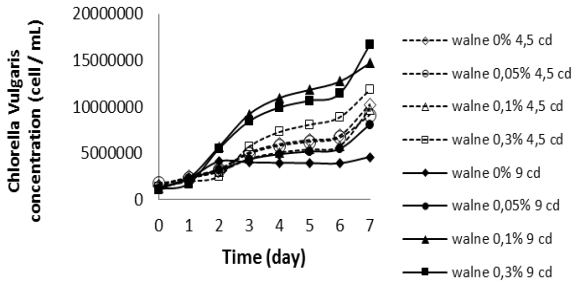

Fig 3.5. *Chlorella vulgaris* concentration in seawater media for 7 days of culturing period with flow rate of 125 mL/min (2% CO_2 gas concentration) for various nutrient addition of walne at 5000 lux and 10000 lux

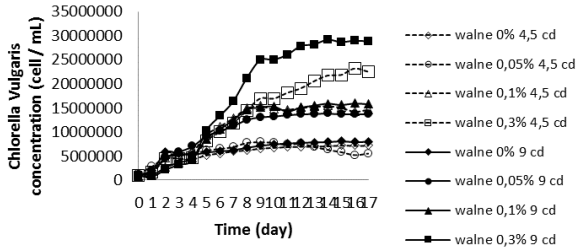

Fig 3.6. *Chlorella vulgaris* concentration in seawater media for 17 days of culturing period with flow rate of 125 mL/min (2% CO_2 gas concentration) for various nutrient addition of walne at 5000 lux and 10000 lux

In both Figs (Figs 3.5 and 3.6) it can be observed that the highest growth was obtained in the nutrient administration of 0.3% and the light intensity of 10000 lux. In Fig 3.5, it can be observed that the growth of microalgae at a light intensity of 10000 lux with the provision of nutrients by 0.1% higher than with the provision of nutrients by 0.3%. While in Fig 3.6, it can be observed also at the light intensity of 5000 lux with walne nutrition by 0.05% experienced a lower growth when compared with walne nutrition by 0%. This can be affected by the aeration in each of these conditions is not good. In addition, temperature and pH also affect each of these conditions.

From Fig 3.1 to 3.6 above can be observed that the growth of the microalgae has increased along with the increasing amount of CO_2 given. In the Figs 3.1 to 3.6 it is also found that the highest growth absorbance was obtained in the 10000 lux, 0.3% walne and 17-day cultures at 125 mL / min CO_2 concentration (2% CO_2).

Microalgae require a light / dark regime for doing photosynthesis, needing light for the photochemical phase to produce Adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH). And also need a dark phase for biochemical phase synthesize molecules essential for growth (Al Qasmi et al, 2012). Brautovic learned that Chlorella sp. Which was cultured on 900 lux then changed its exposure to 12,000 lux on the 23rd day of the

experiment. The result is that the algae can grow well at higher light intensity stimulate growth until the stable stage is achieved (Brautovic). This is consistent with the results of this experiment shown in Figs 3.1 to 3.6 where at higher light intensities of 10,000 lux (9 cd), the majority of *Chlorella vulgaris* growth is higher than 5,000 lux (4.5 cd). In general, the inhibition of photosynthesis (CO_2 fixation and growth) increases with increasing oxygen concentration and with increasing intensity of light (Salih, 2011). On the other hand, a further increase in light intensity (above 10,000 lux) can make the growth lower. In his research Brautovic also reported that the use of 12,000 lux light from day 0 experiments resulted in a lower growth graph of the use of 6,000 lux intensity.

In his research Brautovic cultured Chlorella sp. With the light intensity of 900, 2000, 4000, 6000, and 12000 lux until stationary phase of growth was achieved, then obtained the result that the shortest period of recorded log phase for algae exposed at 6000 and 12000 lux (on day 18), and longest 2000 lux (On day 26). So from Brautovic's research it can be seen that growth on day 17 will be much higher than day 7 because in that range the linear growth curve is in the log phase. This is in accordance with the experiments shown in Figs 3.1 to 3.6 where the 17-day talk time has a higher growth majority than 7 days.

Basic nutrient growth should be available to maintain proper physiological integration of culture (Salih, 2011). According to BBAP Situbondo, Cultivation of *Chlorella vulgaris* done with the addition of nutrients 1 mL of walne nutrition per 1 liter of culture. In this experiment, however, we tried to use various walne concentrations to find out which variables gave the best growth effect. From the experiments shown in Figs 3.1 to 3.6 it was found that the majority of the best growth trends were obtained when the use of 3 mL walne (0.3%) nutrients.

The effect of CO_2 concentration on growth of *Chlorella vulgaris* can be seen from graph 3.1 - 3.6 above where *Chlorella vulgaris* growth is best found in variable CO_2 with flow rate 125 mL / min (2% CO_2). Chlorella sp microalgae were reported to have a maximum tolerance of CO_2 to 40% (Salih, 2011). On the other hand, in the previous study it was reported that the use of CO2 with a flow rate of 200mL / min (3.33% CO_2) resulted in a lower absorbance graph of CO_2 with a flow rate of 50mL / min (1.67% CO_2) (Widjaja, 2008) . While in another study it is estimated that the rate of CO_2 absorption and ammonia removal of *Chlorella vulgaris* in wastewater is 260 g CO_2 m^{-3} h^{-1} and 0.92 g NH3 m^{-3} h^{-1} (Yun et al., 1997). With the rate of CO_2 absorption it could be one of the reasons that affect the growth of *Chlorella vulgaris* is higher at 125 mL / min.

3.2. Biomass Productivity from *Chlorella vulgaris*

Another quantity observed in this study was the production of biomass from the *Chlorella vulgaris* microalgae. Some of the conditions that influence this research are the light intensity, the amount of nutrients given, the CO_2 that flows and the different harvesting times. The following data show the relationship between the intensity of light, the amount of nutrients, the CO_2 that flows and the time of harvest that is different from the biomass productivity obtained by each of these conditions.

Table 1. Biomass Productivity from *Chlorella Vulgaris*

	CO_2 concentration		
Cultivation Condition	0%	1%	2%
0% 5000 lux 7 days (mg/L/d)	77	91	95
0,05% 5000 lux 7 days (mg/L/d)	80	99	101
0,1% 5000 lux 7 days (mg/L/d)	97	116	119
0,3% 5000 lux 7 days (mg/L/d)	96	119	123
0% 10000 lux 7 days (mg/L/d)	90	101	106
0,05% 10000 lux 7 days (mg/L/d)	107	117	121
0,1% 10000 lux 7 days (mg/L/d)	120	127	134
0,3% 10000 lux 7 days (mg/L/d)	126	134	139
0% 5000 lux 17 days (mg/L/d)	45	52	55
0,05% 5000 lux 17 days (mg/L/d)	56	58	66
0,1% 5000 lux 17 days (mg/L/d)	54	68	74
0,3% 5000 lux 17 days (mg/L/d)	59	78	82
0% 10000 lux 17 days	74	84	89
0,05% 10000 lux 17 days	78	90	95
0,1% 10000 lux 17 days	89	116	120
0,3% 10000 lux 17 days	99	126	133

From the above data can be seen the highest biomass productivity obtained in conditions with a light intensity of 10000 lux, the amount of nutrients given 0.3%, 2% CO_2 and 7 days culturing period.

The effects of light and CO_2 on biomass production are related to photosynthesis, where the reaction is as follows:

$$6\,CO_2 + 6\,H_2O \xrightarrow[\text{chlorophyl}]{\text{light}} C_6H_{12}O_6 + 6\,O_2$$

The more CO_2 and light obtained, the greater the efficiency of photosynthesis so that the biomass production will also increase. The variables exposed to the 10000 lux light intensity resulted in more biomass production than those exposed to a light intensity of 5000 lux. This is in accordance with the literature which states that increasing the intensity of light will also affect the increase in the number of cultivation of existing microalgae. (Al-Qasmi, et al, 2012)

As for the parameters of the amount of nutrients given, biomass productivity is more generated in the provision of nutrients by 0.3%. Biomass productivity is more when compared with the provision of nutrients by 0, 0.05% and 0.1%. As mentioned earlier, Nitrogen is the most needed nutrient for growth as an essential element in the formation of chlorophyll and protein (Kaplan, et al., 1986). Therefore, the more walne is added the more nitrogen and $FeCl_3$ are added, so the growth of the microalgae becomes higher. When growth is higher, then the biomass productivity obtained by the microalgae will also increase.

Another parameter given is the amount of CO_2 that flows. From the data obtained, at 2% CO_2 condition the microalgae have higher production compared with 0% and 1% CO_2. This is consistent with the literature which states that an increase in the amount of CO_2 will cause a significant increase in the growth of the algae (Widjaja, 2008). From the data can be seen also the tendency of significant increase from the provision of CO_2 0% to 1% CO_2. But at 2% CO_2, there was no significant increase in 1% CO_2 to 2% CO_2.

In the literature it is said that giving 50 mL / min CO_2 can increase growth significantly. But on giving 100 and 150 mL / min CO_2 does not provide better growth (Widjaja et al.). In this research, 0% CO_2 is equal to 2 mL / min (ambient air) CO_2 flow while CO_2 of 1% and 2% is equal to CO_2 flow of 62,5 mL / min and 125 mL / min. It can be concluded that in literature that 1% CO_2 (62.5 mL / min) will provide significant increase in the amount of growth and biomass productivity compared to 0% (ambient air) CO_2, but did not provide a significant improvement when compared with 2% CO_2 (125 mL / min).

In this research, culture time is also used as research parameter. It can be seen from the growth chart of each variable (Fig 3.1 - 3.6), in each picture has a tendency to increase on day 6 to day 14. So on the 7th day of the culturing period, the microalgae are still not in the phase of stationer or in the sense still can grow. Therefore, at culturing period, the number of microalgae will be more obtained by 17 days harvesting variable. This applies also to the production of biomass obtained by each parameter. The culturing period of 17 days will result in more biomass production than the 7 days culturing period. However, biomass production is inversely proportional to biomass productivity. This is because biomass productivity has a divisor of the number of cultivation days.

3.3. Lipid Productivity from Chlorella vulgaris

Below is the data of lipid productivity of *Chlorella vulgaris* with several variations of the conditions of light intensity, the amount of nutrients given, the amount of CO_2 that flows and the time of harvesting the microalgae.

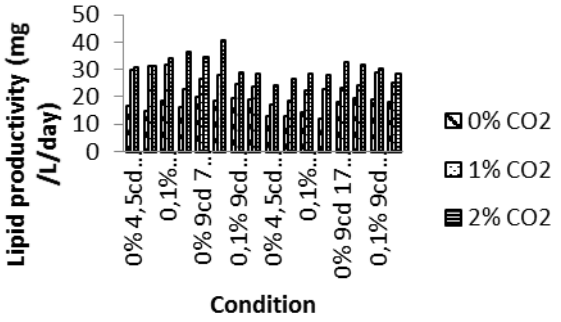

Fig 3.7 Relationship between light intensity, amount of nutrients, CO_2 flowed and culturing period different from lipid productivity from microalgae *Chlorella vulgaris*

From Fig 3.7 can be observed highest lipid productivity obtained at 10000 lux light intensity, walne nutrition 0,05% with 2% CO_2 concentration and 7 day culturing period. In order to enlarge oil productivity microalgae, then the microalgae can be conditioned in a state of stress. Ie by making the microalga nutrient deficient. In principle, nutrients are a source of nitrogen and phosphorus that have a role in influencing the productivity of lipids. This is related to the process of lipid biosynthesis. Lipid biosynthesis in microalgae requires acetil-CoA as the starting point of lipid binding. Acetil CoA carboxylase and several lipid liposuction or pathological enzymes have been used as targets for increased lipid production Schenk et al., (2008). The limitation of nutrients (nitrate and phosphate) in microalgae culture preservation media will increase the activity of ACCase enzyme which is the precursor for lipid formation in microalgae. The lower the concentration of nitrate derived from NaNO3 and the phosphate of NaH2PO4, the total lipid content in microalgae higher (Widianingsih, 2011).

Lipid productivity at culturing period 7 days is higher when compared with lipid production with 17 days culturing period. The longer the microalgae suffer from nutritional deficiencies, the more lipid produced (Widjaja et al, 2008). However, in the production of this lipid has a function of time where if divided per time culture will result in greater value will be available at smaller divisors.

CO_2 concentration also affects the biomass production obtained in this study. The more CO_2 that flows, the higher the production of lipid. This is in accordance with the literature which states that under normal conditions, oil production will increase in line with increased flowrate of CO_2 provided (Widjaja et al., 2008).

In the table below obtained data of biomass production, lipid content and lipid productivity from *Chlorella vulgaris* microalgae. In the table below is given data on the concentration of CO_2 given by 2%. This is because biomass production, lipid content and lipid productivity are highest in this condition.

Table 2. Production Data of Biomass, Lipid Content and Lipid Productivity from *Chlorella vulgaris* at CO_2 concentration of 2%

Condition	Biomass (g/L)	Lipid Content (%)	Lipid Productivity (g/L/day)
0% 5000 lux 7 days	0,67	32,10	30,62
0,05% 5000 lux 7 days	0,71	30,90	31,34
0,1% 5000 lux 7 days	0,83	28,70	34,03
0,3% 5000 lux 7 days	0,86	29,50	36,24
0% 10000 lux 7 days	0,74	32,70	34,57
0,05% 10000 lux 7 days	0,85	33,50	40,68
0,1% 10000 lux 7 days	0,94	21,70	29,14
0,3% 10000 lux 7 days	0,97	20,50	28,41
0% 5000 lux 17 days	0,93	44,20	24,18
0,05% 5000 lux 17 days	1,12	40,10	26,49
0,1% 5000 lux 17 days	1,25	38,40	28,24
0,3% 5000 lux 17 days	1,40	33,90	27,92
0% 10000 lux 17 days	1,52	36,30	32,46
0,05% 10000 lux 17 days	1,61	33,60	31,82
0,1% 10000 lux 17 days	2,04	25,10	30,12
0,3% 10000 lux 17 days	2,26	21,40	28,41

From the above data, it can be known that the highest biomass production is obtained at 10000 lux light intensity condition, walne nutrition given by 0.3% and culturing period of 17 days. The largest lipid content was obtained under conditions of 5000 lux light intensity, without the addition of walne nutrition, and culturing period for 17 days. While the largest lipid productivity obtained at 10000 lux light intensity conditions, the addition of nutrients walne 0.05%, and the cultivation time for 7 days.

From these data it can be concluded that the longer harvesting will be the greater the amount of biomass and lipid content obtained. This is because the longer the harvesting the more the number of microalga contained in the culture so that the production of biomass, lipid content and lipid productivity also more and more. Meanwhile, to increase the amount of lipid production, the microalgae must be conditioned on stress condition. In this study it was done with the restriction of nutrients on the microalgae so that the microalgae suffered from Nitrogen (Nitrogen starvation) deficiency. The limitation of nutrients (nitrate and phosphate) in microalgae culture preservation media will increase the activity of ACCase enzyme which is the precursor for lipid formation in microalgae. The lower the concentration of nitrate derived from NaNO3 and the phosphate of NaH2PO4 then the total lipid content in microalgae is greater (Widianingsih, 2011).

After doing the calculation, then analyzed to one sample in this research that is at the condition of light intensity of 5000 lux, without addition of nutrients (0% walne), without addition of CO_2 (0% CO_2) and 7 days culturing time. In this research, GC-MS (Gas Chromatography Mass Spectrometry) and TLC (Thin Layer Chromatography) were analyzed. Based on the results of GC-MS analysis, it is found that in the microalgae lipid there are characteristics of Hexadecanoid Acid and Octadecanoid Acid. And based on the results of TLC analysis, data obtained that the microalgae oil contains Triglycerides characteristic in it. This may serve as a reference for further research leading to the making of biodiesel from these microalgae oils.

4 Conclusion

The highest biomass productivity was obtained 139 mg/L/d in conditions with 10000 lux light intensity, 0.3% walne concentration, 2% CO_2 concentration and 7 days cultivation days. The highest lipid productivity was obtained at 10000 lux light intensity, walne addition of 0,05%, and 7 days time of 40,68 gr / L / day.

References

1. M. Al-Qasmi. *Proceedings of the World Congress on Engineering 2012 Vol I WCE*, (2012).

2. A. Bajguz, Journal of Plant Physiology, **166** (8), 882 – 886 (2009).
3. E. W. Becker, *Microalgae: Biotechnology and Microbiology*, Baddiley et al. (Eds.). New York: Cambridge University Press, (1994).
4. E. G. Bligh, and W. J. Dyer, Can. J. Biochem. Physiol., **37**, 911, (1959).
5. I. Brautovic, *The influence of light intensity on growth of the marine planktonic alga Chlorella sp. under laboratory conditions*. Institute of Oceanography and fisheries, Dubrovnik, Croatia
6. K. O. Cassidy, *Evaluating Algal Growth that Different Temperature* Theses an Dissertations--Biosystems and Agricultural Engineering. University of Kentucky, (2011).
7. S. Y. Chalid, *Kultivasi Chlorella Sp Pada Media Tumbuh Yang Diperkaya Dengan Pupuk Anorganik dan Soil.* (Program studi Kimia, Fakultas Sains dan Teknologi UIN Syarif Hidayatullah Jakarta).
8. S. Chinnasamy, B. Ramakrishnan, A. Bhatnagar, and K. C. Das, Int. J. Mol. Sci. **10** (2), 518-532. (2009).
9. A. Converti, A. A. Casazza, E. Y. Ortiz, P. Perego, and M. Del Borghi. Chem. Eng. Process. **48** (6), 1146-1151, (2009).
10. V. Endar, Sarjito, J. Hutabarat, B. Prayitno, Journal of Coastal Development, **16** (1), (2012).
11. E. P. Feofilova, Y. E. Sergeeva, and A. A. Ivashechkin. Appl. Biochem. Microbiol. **46** (4), 369 – 378, (2010).
12. Hadiyanto. I. Samidjan, A. C. Kumoro, and Silviana. *Produksi Mikroalga Berbiomasa Tinggi dalam Bioreaktor Open Pond. Pengembangan Teknologi Kimia untuk Pengolahan Sumber Daya Alam Indonesia*. 2010.
13. N. A. Handayani, D. Ariyanti, *Potensi Mikroalga Sebagai Sumber Biomasa dan Pengembangan Produk Turunannya*. **33 (**2), ISSN 0852 1697.2012 (2012).
14. S. Hiremath, P. Mathad, Journal of algal biomass utilization. Phycospectrum Inc. 2010.
15. http://toddcaldecott.com/herbs/chlorella/
16. http://www.chlorella-vulgaris.eu/
17. http://www.onr.navy.mil/focus/ocean/motion/default.htm
18. A. Isnansetyo, and Kurniastuty. *Teknik Kultur Phytoplankton dan Zooplankton*. Kanisius. Yogjakarta (1995).
19. R. P. John, G. S. Anisha, K. M. Nampoothiri, and A. Pandey, Bioresour. Technol., **102**, 186-193 (1995).
20. J. H. Lee, J. S. Lee, C. S. Shin, S. C. Park, and S. W. Kim. J. Microbiol. Biotechnol. **10** (3), 338-343 (2000).
21. Y. Liang, N. Sarkany, Y. Cui, Springer Science, Business Media B.V. Biotechnol Lett 31:1043–1049 DOI 10.1007/s10529-009-9975-7 (2009).
22. A. W. Mayo, Water Environ. Res. **69** (1), 64-72 (1997).
23. X. Miao, and Q. Y. Wu. Bioresour. Technol., **97**, 841 (2006).
24. J. C. Ogbonna, H. Masui, and H. Tanaka. J. Appl. Phycol. **9** (4), 359-366 (1997).
25. F. M. Salih, J. Environ. Prot., **2**, 648-654 (2011).
26. Soeroso, *Kilang Pengolahan BBM dioptimalkan*, Harian Pagi Jawa Pos, 11 Maret 2005.
27. M. Tsuzuki, E. Ohnuma, N. Sato, T. Takaku, A. Kawaguchi, Plant Physiol., **93**, 851-856 (1990).
28. A. Widjaja, C-C. Chien, Y-H. Ju, J. Taiwan Inst. Chem. Eng., (2008).
29. S. Wirosaputro, *Chlorella Untuk Kesehatan*, Global Teknik Budidaya dan Pengolahan Buku II. Gajah Mada University Press. Yogyakarta (2002).
30. Y. S. Yun, and J. M. Park. Appl. Microbiol. Biotechnol. **55** (6), 765-770 (2001).
31. M. Zhu, P. P. Zhou, and L. J. Yu, Bioresour. Technol., **84,** 93 (2002).