Chlorophyll and carotenoids analysis spectrophotometer using method on microalgae

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Abstract. Microalgae is a very potential to be developed biota Because of its abundant amount on earth. Research on microalgae and its application as pigment analysis, provides important information about chemical compounds such as carotenoids and chlorophyll roomates that can be developed into drugs. Microalgae such as Chlorella vulgaris, Nannochloropsis sp., Porphyridium cruentum and Spirulina platensis has known had carotenoid pigments and chlorophyll content, but the optimization of the production of pigment in microalgae life phase has not been known in detail. Chlorophyll content in microalgae in the logarithmic phase of Chlorella vulgaris 200-1500 mg/L, Nannochloropsis sp. 100-500 g/L, Porphyridium cruentum 500-800 g/L and Spirulina platensis 1000-3500 mg/L and for stationary phases microalgae Chlorella vulgaris 100-1000 mg/L, Nannochloropsis sp. 200-500 g/L, Porphyridium cruentum 900-2000 mg/L and Spirulina platensis 2000-6000 mg/L. While the carotenoid content of microalgae in the logarithmic phase of Chlorella vulgaris 10-40 g/L, Nannochloropsis sp. 10-60 g/L, Porphyridium cruentum 10-60 ug/L and Spirulina platensis 20-40 ug/L and for stationary phases microalgae Chlorella vulgaris 10-50 g/L, Nannochloropsis sp. 10-70 g/L, Porphyridium cruentum 70-130 ug/L and Spirulina platensis 20-1100 mg/L.

1. Introduction
Microalgae have a wide distribution throughout the world, including Indonesia. In Indonesia, the microalgae are biological resources are abundant in number compared with other biological resources. Microalgae are microscopic aquatic organism that is both cellular and multicellular, living in all regions of freshwater and marine waters. Microalgae capable of transforming inorganic matter into organic matter through photosynthesis [1, 2, 3, 4, 5]. Microalgae have a high content of natural proteins that could potentially produce bioactive compounds such as pigments. There are four groups of photosynthetic pigments in microalgae, which is a green photosynthetic pigment (chlorophyll), brown (fikosantin), turquoise (fikobilin), and red (fikoeritrin). Other pigments contained in the microalgae is a carotenoid. Carotenoids are a group of natural pigments in red, orange or yellow and soluble in lipid.

Research on microalgae and its application to be used in various processes or economical and high-value products have been developed extensively over the past 50 years, including efforts in the utilization of secondary metabolites contained in them. Exploration microalgae addition to being used
as a business diversification, is also meant to empower the agricultural land that is not feasible. Biota land diminishing in number makes the study of replacement more and more, especially marine life. Marine life, particularly microalgae has a great potential to be explored because some of the advantages it has, such as easily cultivated, can grow in extreme conditions because it requires only sunlight and some nutrients that are simple and the cycles of rapid growth, lasting only a few days [6]. The main advantages of microalgae are the high biomass content, which can reach half of its weight.

Type of secondary metabolites most in microalgae are pigment chlorophyll and carotenoids. Microalgae pigments contained in the green of chlorophyll a, chlorophyll b aquamarine and carotene. Microalgae Phorphyridium cruentum pigments that participate directly in the light reaction, whilst carotene as photoprotection which absorbs and releases excessive light energy [7]. Almost all species of microalgae contain chlorophyll and carotenoid pigments, including Porphyridium cruentum species, Chlorella vulgaris, Spirulina platensis, and Nannochloropsis sp.

Porphyridium cruentum is a kind of unicellular red alga with selberbentuk like a ball. Porphyridium cruentum may contain sulfate polysaccharides and the protein red pigment fikoeritrin [8]. Moreover, it has three main components namely pigments: chlorophyll, fikobiliprotein, and carotenoids. Microalgae Phorphyridium cruentum and fikobiliprotein containing pigment chlorophyll, protein (28-39%), polysaccharides (40-57%) and lipids (9-14%) [9].

Chlorella vulgaris including in the division Chlorophycae (green algae). Chlorella vulgaris classified as low-level plants of 3-15 micron sized genetikyang properties without the mutation until now. Chlorella contains the green pigment (chlorophyll) and carotenoids.

Spirulina platensis belonging to the division Cyanophyta (blue-green algae) with morphologic characteristics are filaments composed of multicellular trichomes spiral merge into one, autotrof, and turquoise. The composition of the pigment in Spirulina platensis is a complex composition and common pigment found in blue-green algae. The composition of which is chlorophyll, xanthophylls, phycocyanin, and carotenoids consisting of myxoxanthophyll, beta carotene, and zeaxanthin [10].

Nannochloropsis sp. included in the phylum Chromophyta, have sel2 size - 4 micrometers, are green and have the two flagella (heterokontous) which is one of the thin-haired flagella [11]. Nannochloropsis sp. has a number of pigment content and nutrients such as protein (52.11%), carbohydrates (16%), fat (27.64%), vitamin C (0.85%), and chlorophyll-a (0.89%) [12].

Carotenoids (also known as carotene extract) are provide color pigments in a variety of plants, including fruits and vegetables. Carotenoids play an important role for the health and survival of human beings. Carotenoids can boost the immune system, protection against cancer and also serves as an antioxidant [13]. Chlorophyll is the green pigment color poster on plants, algae and photosynthetic bacteria. These compounds that play a role in the process of photosynthesis in plants to absorb and transform sunlight into energy chemistry. With the process of photosynthesis, there are 3 main function of chlorophyll is first who utilize solar energy, both triggered fixation of CO2 into carbohydrates and the third provides the energetic basis for the ecosystem as a whole. The resulting carbohydrate anabolism photosynthesis through the process is converted into proteins, fats, nucleic acids, and other organic molecules.

Analysis of levels chlorophyll on mikoalga been done on microalgae Porphyridium cruentum. The amount of carbohydrates in food products need to know, among other things for identity standardization of food, nutrition label, the detection adulterasi and for the development of a food product. Carbohydrate significant role especially in food products make an important total carbohydrate analysis [14].

2. Materials and methods

2.1. Preparation of culture medium microalgae Chlorella vulgaris, Nannochloropsis sp., Porphyridium cruentum, and Spirulina platensis

The culture medium used for microalgae cultivation process is different depending on the kind of microalgae to be cultured. Media culture is a material consisting of a mixture of nutrients or
substances - nutrients (nutrients) used to grow microorganisms on or in it. In addition, the culture media can be used also for the isolation, propagation, testing the properties - physiological properties and the calculation of the number of microorganisms [15].

The culture medium for microalgae *Chlorella vulgaris*, *Nannochloropsis* sp. and *Porphyridium cruentum* adalah Medium Johnson. While the culture medium for microalgae *Spirulina platensis* is Zarrouk Medium / Zarrouk Medium (ZM).

Stages of making microalgal culture media are as follows. At first, preparing the culture medium, and then weighed using the analytical balance according to the number listed. Selection as a means of weighing analytical balance analytical balance due has an accuracy of up to 0.0001 mg, so as to minimize the error [16]. Each ingredient is weighed and then put in culture bottles (glass bottles) 1L size had previously been washed clean. After all of the ingredients into the culture bottle, then add 500 ml of tap water. The solution is then homogenized using a magnetic stirrer until all the ingredients evenly mixed.

2.2. Cultivation of microalgae *Chlorella vulgaris*, *Nannochloropsis* sp., *Porphyridium cruentum*, and *Spirulina platensis*

Microalgae cultivation process is carried out in two periods, the first period and the second period. In each period of culture, there are four kinds of microalgae culture, namely *Chlorella vulgaris*, *Nannochloropsis* sp., *Porphyridium cruentum* and *Spirulina platensis*. Each culture of microalgae cultivated in duplicate, namely Deuteronomy I and II. Their repetition in the process of cultivation is because the chlorophyll and carotenoid content analysis was performed on two phases, i.e. when the microalgae reached log phase and stationary phase when microalgae experience. Analysis of chlorophyll and carotenoid content of microalgae in log phase of Deuteronomy I do use on each culture microalgae cultivated in duplicate, whereas for the stationary phase analysis using microalgae Deuteronomy II.

Microalgae cultivation process begins with the calculation of the volume of the culture to be added to the culture medium. Seedlings will be moved microalgae biomass density measured in advance using turbidimetry by measuring the Optical Density (OD) using a UV-Vis spectrophotometry, so that the volume of the culture to be added can be calculated using the formula:

\[ M_1 \times V_1 = M_2 \times V_2 \]

| Information : |
|---------------|
| **M1**        |
| = Inoculated cell density (g/L) |
| **V1**        |
| = The volume of the initial culture / to be added (mL) |
| **M2**        |
| = The desired cell density (g/L) |
| **V2**        |
| = Culture desired volume (mL) |

In this cultivation, the value of the expected initial Optical Density is 0.8 mg/L and the volume of microalgae culture is as much 1L for all types of microalgae. Therefore, the result of calculation microalgae seed volume should be added. Seed microalgae poured in a plastic measuring cup until it reaches the volume calculation, then added to the 1L culture bottles that had contained a culture medium. Culture bottle is then added again with tap water until the volume of 1L.

Glass bottles that have been filled with microalgae culture bottle is then sealed using a stopper. Bottle stopper is made of cotton which has been rolled with a tissue and with a straw inserted in it. There are two types of straws inserted, namely straw length and short sized straw. Straws length serves as the entry of air from the aeration process, while the short-sized straw serves as a discharge of residual air respiration microalgae. Then, use the cotton rolls wrapped in plastic wrap to paste and fixed to the mouth of the bottle culture. Culture bottle is equipped with a name tag for writing the name of culture microalgae culture along with the start date.

Culture bottle placed on racks and mounted aerator culture. Culture bottle rack placement on culture not just anywhere, but rather were separated according to type. It aims to avoid cross-contamination in cultures of microalgae. Microalgae cultivation is done at pH 7.0, by light using 40
watt fluorescent lamp with a light intensity of 2500 lux and carried out at a temperature of 25°C. Aeration was supplied continuously (continue - constantly) until the process of harvesting microalgae. Especially for microalgae Porphyridium cruentum, aeration given must be in large amounts because this microalgae sensitive to a given aeration. Small aeration causes tiny air bubble size, which can damage microalgae cells [17].

3. Results and discussion

3.1. First period culture
The first microalgae culture was conducted from January 19, 2016 to February 2, 2016. The Microalgae culture consists of Chlorella vulgaris, Nannochloropsis sp., Porphyridium cruentum and Spirulina platensis and culture is done in duplicate (Deuteronomy Deuteronomy I and II). The observations of growth of microalgae Chlorella vulgaris, Nannochloropsis sp., Porphyridium cruentum and Spirulina platensis was conducted by turbidimetry, ie by measuring the Optical Density (OD) using a spectrophotometer UV-Vis Spectrophotometer Hitachi U-3900H with a wavelength of 680 nm and with the microscopic method, ie using a haemocytometer cell density calculation is done every day. The observation of the growth of microalgae cultures can be seen in the following Figures 1, 2, 3, and 4.

![Figure 1. Optical density graph microalgae culture Deuteronomy I.](image)

The purpose uses two methods to observe the growth of microalgae culture is to mem-validate data obtained in order to determine more precisely the growth phase. Comparison chart between Optical Density calculations with cell density calculation shows that an increase in the density of microalgae cultures are aligned, although not all of the data in these days have the same increase. From the second graph above it can be concluded that the microalgae culture through a phase of growth is relatively normal, the same as microalgae growth in general. Microalgae growth phase consists of the lag phase, logarithmic phase, the phase of decline in growth rate, stationary phase and death phase [18].
The visual observations are also done by observing the color of microalgae cultures performed every day during the culture period. According to [19], the growth of plankton at the time of cultivation are visually marked by a water color changes from clear to colored initially (light green / brown and then green / brown and so on), this change is accompanied by a decrease in transparency. The results of the visual observations of microalgae culture first period is as follows.

Figure 2. Optical Density Graph Deuteronomy microalgae culture II.

Figure 3. Graph Deuteronomy culture microalgae cell density I.

In general, the visual appearance of microalgae culture seemed normal, except in microalgae Porphyridium cruentum I. Replicates normal visual appearance is an indicator of the success of culture
in microalgae culture. In Porphyridium cruentum I repeat, the resulting color is slightly different from microalgae Porphyridium cruentum general. This is due to the release of aeration hose on microalgae culture bottle, so the growth is slightly delayed.

Table 1. Visual appearance of microalgae culture.

| No. | Microalgae Culture   | Appearance                      |
|-----|----------------------|---------------------------------|
| 1.  | Chlorella vulgaris   | Light green soupy               |
| 2.  | Nannochloropsis sp.  | Dark green                      |
| 3.  | Porphyridium cruentum| Dark red-brown, dark red dark   |
| 4.  | Spirulina platensis  | Dense dark green, turbid        |

3.2. Second period culture
Microalgae culture both do the same as the culture of microalgae first period, commencing on February 4, 2016 until February 17, 2016.

The period for microalgae culture Deuteronomy I was starting on February 4, 2016 until the date of February 11, 2016 (7 days) until the harvesting stage, while microalgae Deuteronomy II was started on February 20 until the date of February 2, 2016 (13 days). Observations made by the microalgae growth turbidimetry method, by measuring the optical density (OD) and the microscopic method, i.e., using a haemocytometer cell density calculation is done every day.

Figure 5. Optical density graph microalgae culture.

Figure 6. Optical density graph microalgae culture.
Based on the second graph, the growth of microalgae in the culture of this second period is normal, due to the phase - the same phase with microalgae culture in general, the lag phase, logarithmic phase, the phase of decline in growth rate, stationary phase and death phase [18].

Figure 7. Graph microalgae cell density culture.

Figure 8. Graph microalgae cell density culture.

The visual observations are also done by observing the color of microalgae cultures performed every day during the culture period. The results of the visual observations of microalgae culture first period is as follows.

Table 2. The visual appearance of microalgae culture.

| No. | Microalgae Culture     | Appearance                      |
|-----|------------------------|---------------------------------|
| 1.  | *Chlorella vulgaris*   | Light green soupy               |
| 2.  | *Nannochloropsis sp.*  | Dark green                      |
| 3.  | *Porphyridium cruentum*| Concentrated dark red           |
| 4.  | *Spirulina platensis*  | Dense dark green, turbid        |

Based on the data describing both the culture period, mikralga *Chlorella vulgaris* grew more rapidly than the other microalgae culture. It can be influenced by several factors, including the availability of nutrients (macronutrients and micronutrients) and aeration. Elements - elements such as nitrogen (N) and phosphate (P) greatly affects the growth of microalgae culture. The addition of nitrogen can promote the growth of microalgae in accordance with the assumption that nitrogen is the limiting factor [20]. The benefits of the element phosphorus (P) which is in the media, is absolutely necessary because these elements are essential to the process of energy transformation in the process
of photosynthesis. Phosphorylation of adenosine produces adenosine monophosphate, diphosphate and triphosphate (AMP).

3.3. Harvesting of microalgae culture

Microalgae harvesting methods differ depending on the type of microalgae culture. Microalgae *Chlorella vulgaris*, *Nannochloropsis* sp. and *Porphyridium cruentum* harvested with a centrifuge method, whereas the microalgae *Spirulina platensis* harvested by means of filtration (filtration) using a filter cloth. Centrifuge method is done by using Hitachi Centrifuge CT6EL, by incorporating culture into Corning 50 mL bottle. Here is a method of harvesting microalgae culture.

Each volume culture of microalgae, *Chlorella vulgaris*, *Nannochloropsis* sp., *Porphyridium cruentum* and *Spirulina platensis* separated 100 mL for the analysis of chlorophyll, while the remaining volume for carotenoid analysis. The harvested volume for each - each microalgae culture first period and the second period is enclosed in 9 and 10. The biomass obtained is then weighed using the analytical balance Precisa 40SM-200A. Weight biomass is the amount of weight of a population at a given time period and is expressed in units of weight. Furthermore, the biomass for carotenoid analysis is placed on a petri dish that has been coated with plastic and labeled with the name of microalgae culturing and put in the oven to remove the water content (drying) to obtain dry biomass. While biomass for chlorophyll analysis is not dried using an oven, wet biomass is dried in a cool room for 1 day (± 24 hours) until the moisture content is reduced. It aims to avoid damage to the pigment chlorophyll in the biomass, because the chlorophyll will be damaged if exposed to high temperatures. Chlorophyll is located in the chloroplasts are composed of protein, so that when heated in the temperature exceeds 500°C, a protein in the chloroplast would denatured causing the release of chlorophyll of chloroplasts [22].

3.3.1. Chlorophyll and carotenoids extraction process

Extraction is the process of withdrawal of the desired constituents are selected using a solvent in which the desired substances can be dissolved. The solvents used are selected based on its ability to dissolve the maximum amount of the active substance and the minimum for undesirable elements [23]. The material to be analyzed is chlorophyll and carotenoids of microalgae. The type of extraction used is maceration, the method of extraction by means of curing arrive at a certain time until equilibrium is reached between the concentration of the compound concentration in the solvent with a concentration in microalgae cells. The process of extraction of chlorophyll and carotenoid pigments in culture microalgae *Chlorella vulgaris*, *Nannochloropsis* sp., *Porphyridium cruentum*, and *Spirulina platensis* will be described as follows [24, 25, 26].

A. Chlorophyll extraction

Chlorophyll extraction process performed by using the method of Becker (1982). Extraction is a process of separation of the material from the mixture by using a suitable solvent [27]. Samples for analysis of chlorophyll that had been taken earlier, as many as 100 mL separated again 33 ml three times as replicates (triplo), namely A, B and C. Each sample (33 mL) is inserted into the bottle for the next Corning 50 mL centrifuge and filtrated to obtain biomass. Furthermore, this biomass is weighed using the analytical balance Precisa 40SM-200A.

The solvent used for the extraction process chlorophyll is acetone. Acetone (CH3COCH3) is a semi-polar solvent with a dielectric constant of 21. According to [28], chlorophyll is a pigment that is non-polar and had to be extracted using organic solvents such as acetone with a certain polarity (polarity index 5.2). Chlorophyll a is more polar than the chlorophyll b [29]. Therefore, acetone is used as solvent for the extraction of chlorophyll.

Step extraction of chlorophyll is as follows. Mula - first adding ± 5 ml acetone into Corning 50 mL bottle that had contained the biomass. After that, homogenized (divortex) strong during ± 1 minute. Each - each sample was then transferred to a test tube and heated over a water bath with a temperature of 400°C for 5 minutes. Then, the sample was cooled to room temperature, as the heat will affect the
next process. The entire sample was then centrifuged (6000 rpm) for 5 minutes and separated into two layers, the filtrate and precipitate. The filtrate is then separated using a pipette and put into Corning 15 mL bottle for spectrophotometric process. The filtrate is green, which is an extract of chlorophyll in acetone. Polar and non-polar pigments in a pure state and the single has a different color. Pigment chlorophyll a has a bluish-green color, orange carotenoids, phycocyanin blue, turquoise and allofikosianin red fikoeritrin [30]. The remaining precipitate was then added acetone again and extracted to obtain the extract pale green chlorophyll. Extracts were obtained and then added to the Corning 15 mL bottle that had contained the extract chlorophyll from the previous process. Chlorophyll extracts were then analyzed using a spectrum UV - Vis with wavelengths between 300-700 nm wavelength and absorbance at 645 and 663 nm. Korofil extraction process is done in duplicate for each phase of the growth of microalgae (logarithmic phase and stationary phase).

B. Extraction of carotenoids

The process of extracting carotenoids is done using two different methods, the method for culturing Tanticharoen first period and Bin Li Hua methods for culturing the second period. Each method using different solvents, Tanticharoen method using a solvent diethyl ether, Hua Bin Li sedangkan method using dichloromethane solvent. Carotenoids are pigments soluble in nonpolar organic solvents with a certain polarity (polarity index 5.2) [28]. Good extraction procedure is to release the carotenoids from the microalgae sample into the solvent without resulting in a change of its own [31]. Diethyl ether and dichloromethane is a type of organic solvent with a semi-polar nature, but diethyl ether is more polar than the dichloromethane. Extraction of carotenoids from biological samples such as microalgae that contains a lot of water is done with dried samples (dry biomass) in order to run the extraction process more efficient [32]. The extraction process is done in triplo carotenoids (three repetitions) for each microalgae.

Step extraction karotenid Tanticharoen method is as follows. Initially, a total of 0.3 grams of dry biomass of microalgae are weighed and put into a test tube three times (triplo) for each type of microalgae. In the test tube was added with 5 ml of 0.9% NaCl, 1 ml of 1N KOH and 5 mL of ethanol. Preparation of 0.9% NaCl solution is prepared by dissolving 0.9 grams of NaCl in 100 mL of distilled water. While the manufacture of 1N KOH solution is prepared by dissolving 2.8 grams of KOH in 50 mL of distilled water. This process is the saponification, the reaction that occurs between fat / oils with lye. Saponification aims to eliminate chlorophyll and fats are not expected in microalgae, so it does not affect the analysis of carotenoids [32]. It runs an exothermic process, indicated by the heat in the tube when the solution is added. After that, the samples were sonicated using a Branson Sonifier 250 with a frequency of 40 kHz for 4 minutes. Sonication aims to break down the cell walls of microalgae using ultrasonic waves, especially Chlorella vulgaris which has three layers of the cell wall so that the carotenoids extracted perfectly. Ultrasonic waves can create cavitation bubbles in a solvent. When the bubble burst near the cell walls, creating wrinkles and waves cause rupture the cell walls so that the release of oil present in the cell [33]. The samples were then divortex strong until homogeneous. Next, the sample was heated above water bath with a temperature of 40-500C for five minutes. Heating in this water bath aims to accelerate the reaction, because the heat will increase the kinetic energy of the molecules, so it will be faster carotenoids extracted through heating. The samples were then cooled to room temperature and centrifuged menggunakan Hitachi CT6900 GEL (6000 rpm) for 5 minutes. There are two phases after centrifugation process, the filtrate and precipitate. The filtrate was added with 10 mL of diethyl ether for further separation process to form two layers, the top layer of yellow and green colored undercoat. The top layer (yellow) This is an extract carotenoids, while the bottom layer (green) is participating chlorophyll extracted with a solvent. Deposition rest of centrifuge then added with 10 mL of diethyl ether to form a pale yellow color on the top layer of the filtrate. Furthermore,

Carotenoids extraction procedure using the Hua Bin Li is as follows. A total of 0.3 grams of dry biomass of microalgae are weighed and put into a test tube three repetitions (triplo) for each - each type of microalgae. This method is used for the extraction of carotenoids stationary phase, because the
extraction process using Tanticharoen, carotenoids are not extracted completely. Then, into a test tube was added to 4 mL of 10N KOH. This solution is prepared by dissolving 28 grams of KOH in 50 mL of distilled water. The addition of KOH aims to saponification, namely the removal of chlorophyll and fats are not expected so do not interfere with the extraction of carotenoids [32]. The samples were homogenized using a stirrer for 5 minutes. After that, the sample was heated to 600°C in a water bath with a temperature of selama 10 minutes. Heating above this water bath aims to accelerate the reaction, so that the process of extracting carotenoids run faster. The samples were then cooled to room temperature and added with 2.6 mL of dichloromethane. Each - each sample divortex strong and centrifuged at 6000 rpm power for 5 minutes. There are two phases after centrifugation process, the filtrate and precipitate. The yellow filtrate is an extract carotenoids in dichloromethane solvent. The filtrate is separated using a pipette and put into Corning 15 mL bottle. Deposition centrifuge remainder is then added back in with 2.6 mL of dichloromethane to obtain a pale yellow filtrate and added to the Corning 15 mL bottle for the analysis process. Heating above this water bath aims to accelerate the reaction, so that the process of extracting carotenoids run faster. The samples were then cooled to room temperature and added with 2.6 mL of dichloromethane. Each - each sample divortex strong and centrifuged at 6000 rpm power for 5 minutes. There are two phases after centrifugation process, the filtrate and precipitate. The yellow filtrate is an extract carotenoids in dichloromethane solvent. The filtrate is separated using a pipette and put into Corning 15 mL bottle. Deposition centrifuge remainder is then added back in with 2.6 mL of dichloromethane to obtain a pale yellow filtrate and added to the Corning 15 mL bottle for the analysis process. 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Figure 9. Carotenoid extract using method (a) Tanticharoen and (b) Hua Bin Li.

UV-Vis spectrophotometry is an analytical method based on the interaction between electromagnetic radiation near ultraviolet (190-380 nm) and visible light (380-780 nm) using a spectrophotometer instrument with a material (compound) [34]. This method is based on the absorption of ultraviolet rays or visible light that causes the transition of electrons (electron transfer from a low energy level to the level of higher energy) [34]. UV spectrophotometric analysis - Vis uses Biochrom Libra instruments UV-Vis Spectrophotometers. Spectrophotometric analysis was done by determining the highest absorbance at a specific wavelength spectrum and calculate the concentration of substance. UV spectrophotometric analysis procedures -Vis for chlorophyll and carotenoids are described as follows.

C. Analysis spectro UV-vis extract chlorophyll

Each sample (triplo) of each  algae Chlorella vulgaris, Nannochloropsis sp., Porphyrydium cruentum and Spirulina platensis then measured absorbance at wavelengths of 645 and 663 nm. This wavelength is the maximum wavelength that can be absorbed by chlorophyll [35]. Then, the characterization of the highest absorbance spectrum of chlorophyll with a specific wavelength range, which is between 300-700 nm. Results obtained in the form of chlorophyll absorption spectrum. The calculation result chlorophyll content in microalgae are described in the following table.

a. Culture First Period

The results of the analysis of chlorophyll in microalgae culture of the first period when the logarithmic phase and stationary phase are as follows.

Table 3. Levels of chlorophyll logarithmic phase of the first period.

| Name Culture          | Chlo-a | Chlorophyll levels (ug/L) | Chlo-a + b |
|-----------------------|--------|---------------------------|------------|
| Spirulina platensis   | 782.2483 | 2576.7652                | 3354.1371  |
| Porphyrydium cruentum| 131.7393 | 409.5173                 | 540.4559   |
| Chlorella vulgaris    | 302.8225 | 1149.9998                | 1450.806   |
| Nannochloropsis sp.   | 92.3287  | 355.9949                 | 447.7044   |

Table 4. Levels of chlorophyll stationary phase first period.

| Name culture            | Chlo-a | Chlorophyll levels (ug/l) | Chlo-a + b |
|-------------------------|--------|----------------------------|------------|
| Spirulina platensis     | 1431.3618 | 4999.1931              | 6421.3925  |
| Porphyrydium cruentum  | 635.2764  | 1187.9142               | 1819.9929  |
| chlorella vulgaris      | 346.5353  | 561.9425               | 906 806    |
b. Culture Second Period

The results of the analysis of chlorophyll in microalgae culture of the first period when the logarithmic phase and stationary phase are as follows.

| Name Culture | Logarithmic phase | Chlorophyll levels (μg/L) | Chlo-a | Chlo-b | Chlo-a + b |
|--------------|-------------------|---------------------------|--------|--------|-----------|
| Spirulina platensis | 638.1866 | 950.8846 | 1586.0633 |
| Porphyridium cruentum | 601.6441 | 123.7023 | 723.1619 |
| Chlorella vulgaris | 219.4495 | 46.0529 | 264.7049 |
| Nannochloropsis sp. | 81.229 | 24.7127 | 105.64 |

In the culture of the first period, the highest chlorophyll content when the logarithmic phase and stationary phase contained in microalgae *Spirulina platensis*, with a total chlorophyll content 782.2483 μg/L, chlorophyll b 2576.7652 μg/L and chlorophyll a + b 3354.1371 μg/L for the logarithmic phase and concentration of chlorophyll a much 1431.3618 μg/L, chlorophyll b 4999.1931 μg/L and chlorophyll a + b 6421.3925 μg/L for the phase stationary.

In the culture of the second period also obtained the same results, ie the highest chlorophyll content found in *Spirulina platensis* microalgae with high levels of chlorophyll a much 638.1866 mg/L, chlorophyll b 950.8846 mg/L and chlorophyll a + b 1586.0633 μg/L for a logarithmic phase and chlorophyll content a total 1344.3362 μg/L, chlorophyll b 509.0843 mg/L and chlorophyll a + b 2273.9527 μg/L for stationernya phase. The highest chlorophyll content of microalgae *Chlorella vulgaris* is actually owned by, instead of *Spirulina platensis*. *Spirulina platensis* contains phycocyanin (blue-green pigment) which is more than the chlorophyll content. The solvents used in the extraction process is acetone, which are semi-polar acetone with a dielectric constant value of 21.

The larger the dielectric constant, the greater the solubility of the solvent in water, so the more polar. At the microalgae *Chlorella vulgaris*, a cell wall composed of three layers whose structure is composed of microfibrils and pyrenoid. This Pyrenoid separated by two thylakoid membrane [36]. The existence of this layered cell wall that led to the extraction process is slow due to chlorophyll hard out through the thick cell walls, making it difficult extractable. Therefore, it takes a longer maceration for extraction that can be extracted perfectly lorofil. Additionally, acetone is more polar than the other semi-polar solvents, therefore pigment in microalgae *Spirulina platensis* fikobiliprotein more easily extractable at this solvent causing the higher the absorbance value. Fikobiliprotein on microalgae *Spirulina platensis* consists of consists of pigment phycocyanin and allofikosian. *Spirulina platensis* more dominant pigment phycocyanin thus classified as blue-green algae (Cyanophyta) [37]. While microalgae *Nannochloropsis* sp. contains chlorophyll a little, because *Nannochloropsis* sp. have a smaller cell size compared with other microalgae cell sizes with diameters ranging from 4-6 μm [38].

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Table 5. Chlorophyll content logarithmic phase of the second period

| Name Culture | Chlorophyll levels (μg/L) | Chlo-a | Chlo-b | Chlo-a + b |
|--------------|---------------------------|--------|--------|-----------|
| Spirulina platensis | 1344.3362 | 509.0843 | 2273.9527 |
| Porphyridium cruentum | 614.754 | 277.4365 | 911.727 |
| Chlorella vulgaris | 142.7612 | 38.6042 | 180.8393 |
| Nannochloropsis sp. | 26.1199 | 207.7286 | 499.625 |

Table 6. Chlorophyll content of the stationary phase in the second period.

| Name Culture | Chlorophyll levels (μg/L) | Chlo-a | Chlo-b | Chlo-a + b |
|--------------|---------------------------|--------|--------|-----------|
| Spirulina platensis | 1344.3362 | 509.0843 | 2273.9527 |
| Porphyridium cruentum | 614.754 | 277.4365 | 911.727 |
| Chlorella vulgaris | 142.7612 | 38.6042 | 180.8393 |
| Nannochloropsis sp. | 26.1199 | 207.7286 | 499.625 |
The small cell size that causes the chlorophyll content in the cell is less than the content of chlorophyll in microalgae cells larger. Fikobiliprotein on microalgae *Spirulina platensis* consists of pigment phycocyanin and allofikosianin. *Spirulina platensis* more dominant pigment phycocyanin thus classified as blue-green algae (Cyanophyta) [37]. While microalgae *Nannochloropsis* sp. contains chlorophyll a little, because *Nannochloropsis* sp. have a smaller cell size compared with other microalgae cell sizes with diameters ranging from 4-6 μm [38]. The small cell size that causes the chlorophyll content in the cell is less than the content of chlorophyll in microalgae cells larger. Fikobiliprotein on microalgae *Spirulina platensis* consists of pigment phycocyanin and allofikosianin. *Spirulina platensis* more dominant pigment phycocyanin thus classified as blue-green algae (Cyanophyta) [37]. While microalgae *Nannochloropsis* sp. contains chlorophyll a little, because *Nannochloropsis* sp. have a smaller cell size compared with other microalgae cell sizes with diameters ranging from 4-6 μm [38].

The small cell size that causes the chlorophyll content in the cell is less than the content of chlorophyll in microalgae cells larger. *Spirulina platensis* more dominant pigment phycocyanin thus classified as blue-green algae (Cyanophyta) [37]. While microalgae *Nannochloropsis* sp. contains chlorophyll a little, because *Nannochloropsis* sp. have a smaller cell size compared with other microalgae cell sizes with diameters ranging from 4-6 μm [38]. The small cell size that causes the chlorophyll content in the cell is less than the content of chlorophyll in microalgae cells larger. *Spirulina platensis* more dominant pigment phycocyanin thus classified as blue-green algae (Cyanophyta) [37]. While microalgae *Nannochloropsis* sp. contains chlorophyll a little, because *Nannochloropsis* sp. have a smaller cell size compared with other microalgae cell sizes with diameters ranging from 4-6 μm [38]. The small cell size that causes the chlorophyll content in the cell is less than the content of chlorophyll in microalgae cells larger.

Based on the analysis above, the pigment chlorophyll content of microalgae in the stationary phase more than the content of chlorophyll pigments in the logarithmic phase. In the logarithmic phase, cell division occurs at a growth rate increased intensively followed with an active metabolic processes [39]. In this phase, the primary metabolite is a product that generated a lot due to the metabolism of microalgae. While in the stationary phase, the rate of reproduction and mortality rates are relatively equal. Addition and subtraction number microalgae balanced so that its density is relatively fixed (stationary) [39]. In this phase, the dominant product produced by the microalgae are secondary metabolites, such as chlorophyll because in this phase mainly anabolism metabolism has been inactive happen, microalgae cells tend to produce the active compound to survive in its environment. The figure below shows the characterization of the absorbance spectrum of chlorophyll.

**Kultur First Period - Phase Logarithmic**

![Image A](image1.png)

![Image B](image2.png)
Figure 10. Absorbance spectrum of chlorophyll a logarithmic phase of the first period on microalgae (a) Chlorella vulgaris, (B) Nannochloropsis sp., (C) Porphyridium cruentum and (d) Spirulina platensis.

Kultur First Period - Stationary Phase

Figure 11. Absorbance spectrum of chlorophyll a stationary phase first period in microalgae (a)Chlorella vulgaris, (B) Nannochloropsis sp., (C) Porphyridium cruentum and (d) Spirulina platensis.

Kultur Second Period - Phase Logarithmic
Figure 12. Spektrum chlorophyll absorbance logarithmic phase of the second period on microalgae (a) *Chlorella vulgaris*, (B) *Nannochloropsis* sp., (C) *Porphyridium cruentum* and (d) of *Spirulina platensis*.

Kultur Second Period - Stationary Phase

Based on the results of the absorption spectrum of chlorophyll, chlorophyll absorption peak highs on microalgae showed almost the same results. on microalgae *Chlorella vulgaris*, the highest chlorophyll absorption peak occurs at a wavelength of 663 nm, *Nannochloropsis* sp. at 664 nm, *Porphyridium cruentum* at 665 nm and *Spirulina platensis* is located at a wavelength of 666 nm. based on[35], characteristics of the highest chlorophyll absorption peak is at a wavelength of 645 and 663 nm, which means that the extract chlorophyll analysis results have approached the pure extract chlorophyll. The maximum absorption wavelength of chlorophyll pigment extracts of different - different, because it is influenced by the type of solvent and type of spectrophotometer used [35].

Analysis Spectro UV - Vis Extracts Carotenoids
Each sample (triplo) of each - each ie microalgae *Chlorella vulgaris*, *Nannochloropsis* sp., *Porphyridium cruentum* and *Spirulina platensis* then measured absorbance at a wavelength of 455 nm. This wavelength is the maximum wavelength that can be absorbed by carotenoids [35]. Then, the characterization of the highest absorbance spectrum of carotenoids with a specific wavelength range, which is between 400-700 nm. Results obtained in the form of the carotenoid absorption spectrum. The results of calculations levels of carotenoids in microalgae are described in the following table.

**Kultur First Period**

**Table 7. Carotenoid levels logarithmic phase of the first period.**

| Name culture           | Levels of carotenoids (ug/L) |
|------------------------|------------------------------|
| *Spirulina platensis*  | 21.1737                      |
| *Porphyridium cruentum*| 16.4058                      |
| *Chlorella vulgaris*   | 11.6512                      |
| *Nannochloropsis* sp.  | 16.0612                      |

**Table 8. Levels of carotenoids stationary phase first period**

| Name culture           | Levels of carotenoids (ug/L) |
|------------------------|------------------------------|
| *Spirulina platensis*  | 28.8663                      |
| *Porphyridium cruentum*| 71.3309                      |
| *Chlorella vulgaris*   | 11.7375                      |
| *Nannochloropsis* sp.  | 17.132                       |

**Kultur Second Period**

In the culture of the first period obtained the highest carotenoid levels found in microalgae *Spirulina platensis* to logarithmic phase and *Porphyridium cruentum* on the stationary phase with high levels of 21.1737 g/L and 71.3309 g/L. Differences in results is due to the different culture conditions. In the logarithmic phase culture (culture replicates I), microalgae *Porphyridium cruentum* not grow well, indicated by a greenish brown color that tends of conditions that should be dark red-brown.

**Table 9. Carotenoid levels logarithmic phase of the second period.**

| Name Culture           | Levels of carotenoids (ug/L) |
|------------------------|------------------------------|
| *Spirulina platensis*  | 40.4929                      |
| *Porphyridium cruentum*| 44.5922                      |
| *Chlorella vulgaris*   | 39.8702                      |
| *Nannochloropsis* sp.  | 55.1197                      |

**Table 10. Carotenoid levels logarithmic phase of the second period.**

| Name culture           | Levels of carotenoids (ug/L) |
|------------------------|------------------------------|
| *Spirulina platensis*  | 108.0638                     |
| *Porphyridium cruentum*| 127.5917                     |
| *Chlorella vulgaris*   | 50.7389                      |
| *Nannochloropsis* sp.  | 71.0021                      |

It is caused by factors that are less good aeration, the aeration hose copotnya on microalgae culture flask, resulting in the growth of microalgae are less normal, and even tended to stress because
Porphyridium cruentum requires a considerable supply of aeration. In the culture of the second period, the highest levels of carotenoids produced by the microalgae.

Kultur First Period - Phase Logarithmic

![Carotenoid absorbance spectrum logarithmic phase of the first period on microalgae](image)

**Figure 14.** Carotenoid absorbance spectrum logarithmic phase of the first period on microalgae (a) Chlorella vulgaris, (B) Nannochloropsis sp., (C) Porphyridium cruentum and (d) Spirulina platensis.

Porphyridium cruentum, both during logarithmic phase and stationary phase. Levels of carotenoids produced was 40.4929 g/L for a logarithmic phase and 127.5917 mcg/L for the stationary phase. Porphyridium cruentum have included high carotenoid content, after Dunnnaliella sp., Which is 1020 ± 140 pg per dry weight [40], so that in this extraction carotenoid levels highest in Porphyridium cruentum microalgae. Carotenoids are the fraction of the fatty acids do not tersaponifikasi and includes classes of compounds terpenoids and a photosynthetic pigments. Microalgae Porphyridium cruentum have a low lipid content, but has a fatty acid content is very significant, so the highest carotenoid content contained in these microalgae [40]. Most types of carotenoids in microalgae Porphyridium cruentum is zeaxantin, which covers 97.4% of total carotenoids and β-carotene, reaching only 2.6% in total [41].

Kultur First Period - Stationary Phase
Figure 15. Carotenoid absorbance spectrum stationary phase of the first period on microalgae (a) Chlorella vulgaris, (B) Nannochloropsis sp., (C) Porphyridium cruentum and (d) Spirulina platensis.

In the culture of the first period, the levels of carotenoids in microalgae are obtained fewer compared with the levels of carotenoids in the extraction of microalgae culture in the second period. This is due to differences in the solvent used. When the extraction of carotenoids culture first period, used solvents diethyl ether and dichloromethane as solvent extraction of the culture of the second period. Dichloromethane solvent to extract the carotenoids more because of dichloromethane are more non-polar than the solvent diethyl ether. Carotenoids are compounds that dissolve in lipids, so that a suitable solvent is a solvent that is lipid soluble or non-polar solvents. Therefore, with the solvent dichloromethane extract showed a much more concentrated than solvent extract with diethyl ether, so that the levels of carotenoids in microalgae culture in the second period showed a higher yield. The figure below shows the maximum absorbance spectrum characterization of the extract carotenoids.

Kultur Second Period - Phase Logarithmic
Based on the results of the absorption spectrum of carotenoids, the highest carotenoid absorption peak in microalgae showed different results - different, between 411-460 nm. However, in general, carotenoids maximum absorbance peak occurs at a wavelength of 455 nm. This difference is due to the absorbance greatly influenced by the type of solvent and type of spectrophotometer used [35].
In carotenoid extract with dichloromethane solvent, absorbance peak is highest at a wavelength of 455 nm which is nearing. In accordance with the characteristics of a carotenoid that has a peak absorbance at a wavelength of 455 nm, then the dichloromethane extract nearing a pure extract of carotenoids. Carotenoid extract with diethyl ether solvent showed absorbance peak occurs at a wavelength which is quite far from 455 nm, this means in diethyl extracts are mixtures of compounds extracted in addition to the carotenoid.

4. Conclusion

Based on the results has been done in the Laboratory of Freshwater Microalgae Biotechnology Research Center on the analysis of chlorophyll and carotenoids in microalgae spectrophotometric method can be concluded the extraction of chlorophyll in microalgae using the method with acetone.
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