Fatty Acid Analysis of Marine Microalgae *Chlorella vulgaris* in Modified Medium Used GC-FID

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**Abstract.** Microalgae oil can replace the role of fish and other vegetable oils. One of them is lipid in microalgae *Chlorella vulgaris*. This study aims to determine the composition of fatty acids in marine microalgae *Chlorella vulgaris* grown in fertilizer medium. Cultivation was carried out at room temperature, photoperiod 12:12 (dark : light); light intensity 10,000 lux and aerated for 24 hour. The optimum medium used was medium GMF fertilizer with concentration 10% (w/v). Lipid extraction used Blight-Dryer method with methanol, chloroform and aquadest as solvent. The lipid obtained was esterified by NaOH in methanol and H\(_2\)SO\(_4\) as a catalyst for 12 hours and analyzed used GC-FID with capric acid as a standard. The analysis result shows that was 18 fatty acid methyl ester compounds. The highest known fatty acid methyl esters compositions was 61.52% methyl stearate; 15.52% methyl palmitate; 4.69% methyl eicosanoate and 3.34% methyl heptadecanoate from total fatty acids. The unsaturated fatty acids identified were 0.17% EPA (eicosapentaenoic acid) also 0.23% omega 3 and omega 6. Microalgae grown in a fertilizer medium produce quite high lipids. Although the levels of unsaturated fatty acids are still low, it does not rule out the possibility of being increased.

1. Introduction
Indonesia is one of the countries that has a vast sea area of 5.8 million km\(^2\) or three quarters of the entire territory of Indonesia. There are about 17,508 islands and surrounded by a coastline of 81,000 km, which is the second longest coastline after Canada [1]. This fact makes Indonesia have a sea that is rich in diverse natural resources. Indonesia's marine natural resources consist of recoverable resources (such as fisheries, coral reefs, mangroves, seaweed, and biotechnology products) and natural resources cannot be recovered (such as oil and natural gas, gold, silver, lead, hematite and other minerals). Marine resources also have the potential to be used as medicinal raw materials considering the number of secondary metabolites contained in marine species. The high biodiversity in the ocean can reflect the economic potential in the area of the coastal waters, in the sense that the higher the biodiversity contained, the greater the potential that can be developed [2]. Indonesia is a tropical country through the equator so that Indonesia has a large and stable sunlight intensity throughout the year. This condition makes photosynthetic plants, including microalgae, able to thrive [3].
Microalgae are microorganisms that resemble the most primitive plants with miniscule sizes. Microalgae can live in fresh, brackish, sea water and wet areas. Generally microalgae are known as phytoplankton. Phytoplankton are the primary producers in the waters and have photosynthetic ability like most high-level plants. Microalgae that live in the sea are known as marine microalgae. Microalgae are classified as organisms that are autotrophic and have the ability to convert carbon dioxide into potential biofuel, high-value foods and bioactives with the help of sunlight. Microalgae contain protein, fat, unsaturated fatty acids, pigments and vitamins. Lipids content and fatty acid within microalgae is a good source of energy [4].

The total of microalgae oil and fat content ranges from 1% to 70% of the dry weight. Microalgae oil can play a role in replacing fish oil and other vegetable oils. One of the microalgae containing lipids or fatty acids is *Chlorella vulgaris*. The oil content in *Chlorella vulgaris* is around 28 to 32% [5]. Fatty acids are carboxylic acids which can be obtained from the hydrolysis process of a triglyceride compound within fat or oil. Generally, these compounds have a long range of carbon as much as C2-C80 [6].

Based on the presence of double bonds, these molecules are classified into saturated fatty acids (there are no double bonds in carbon saturated fatty acids) and unsaturated (there are double bonds in the carbon unsaturated fatty acids chain). Furthermore, unsaturated fatty acids are divided into monounsaturated fatty acids (there are 1 double bonds of mono unsaturated fatty acids) and plural (there are more than 1 double bonds of polyunsaturated fatty acids) [7].

Free fatty acids are used by several food industries as emulsifiers or stabilizers in ice cream products, especially the multiple unsaturated fatty acids that are widely used by the health industry in some of their dairy products because its can help the development of brain cells. Therefore, this study aims to determine the composition of the lipid-forming fatty acids from *Chlorella vulgaris* microalgae by using GC-FID (Gas Chromatography-Flame Ionization Detector).

2. Materials and Methods

2.1 *Chlorella vulgaris* Biomass Harvesting

Heat-resistant glass sterilization is carried out using an autoclave at 121 °C for 15 minutes, while the sterilization of non-heat-resistant equipment can be carried out by spraying 70% alcohol solution evenly. Sterilized tools can be stored at 70 °C when not in use [8].

Marine microalgae culture of *Chlorella vulgaris* was obtained from Biochemistry research group, Chemistry Department Institute of Technology Bandung in Walne medium with a salinity of 25 ppt. Activation of microalgae *Chlorella vulgaris* was carried out in the Bandung School of Pharmacy research laboratory in fertilizer medium with a total volume of 900 mL culture, microalgae culture inoculum 10% (v/v) of total culture volume, and medium 0.1% (v/v). The salinity of seawater medium is regulated at a concentration of 25 ppt, aeration for 24 hours, room temperature (± 25–27 °C), photoperiod 12:12 (dark:light) with 10,000 lux light intensity. The activation process is carried out for 4 days [9].

*Chlorella vulgaris* growth activity was carried out for 15 days in a modified medium using NPK Grow More fertilizer 10-55-10 (GMF) with a concentration of 5, 10 and 15% in sea water with 25 ppt salinity, aeration for 24 hours, room temperature (± 25–27 °C), photoperiod 12:12 (dark: light) with 10,000 lux light intensity [10]. Cultivation was then carried out using a GMF medium with a concentration that gave the best growth rate activity of the *Chlorella vulgaris* microalgae.

Harvesting of microalgae *Chlorella vulgaris* was carried out using Beckman J2-HS centrifuge at a speed of 8,000 × g for 20 minutes. The supernatant is then separated and wet biomass are collected. Then weighed with an analytical scale. After that the microalgae samples were dried by Freeze-Dry method, so that dry biomass was obtained [11].
2.2 Extraction and esterification of lipids Chlorella vulgaris

2 grams of dry biomass from Chlorella vulgaris was extracted by the Bligh-Dyer method. The dried microalgae is crushed, then added with 10 mL of methanol and 5 mL of chloroform then mixed, then authenticated for 10 minutes and stirred using a stirrer for 24 hours then re-added with 5 mL of chloroform and 5 mL of distilled water then evaporated so that the lipids are tolerated [12]. A total of 40 mg of extracted lipids were put into a beaker and then added with 2 mmol NaOH in methanol then stirred for 12 hours at 55-60 °C. Then after 12 hours added with concentrated H₂SO₄ and incubated for 2 hours at a temperature of 55-60 °C followed by stirring with the stirrer [13].

2.3 Chlorella vulgaris Fatty Acid Analysis with CG-FID

Analysis of Chlorella vulgaris fatty acids using GC-FID. The samples used were esterified fatty acids. The concentrated methyl ester Chlorella vulgaris was injected as much as 2 µL in the GC-FID instrument. The column used is DB-5 with helium carrier gas. Measurement parameters used are column temperature 104 °C; injection temperature 200 °C; detector temperature 300 °C with a flow rate of 40mL/min. The results of the analysis were compared with the capric acid standard GCMS chromatogram which was treated the same as the sample [13].

3. Results and discussion

Microalgae are the most primitive cellular-sized plant organisms commonly known as phytoplankton. Microalgae is classified based on its pigment content, namely Chlorophyta (green algae), Chrysophyta (golden algae), Pyrrophyta (fire algae), Euglenophyta and Cyanophyta (blue-green algae). Chlorophyta is the most diverse group of algae because there are single-celled and colonized ones [4]. Chlorophyceae is a type of class of green algae that comes from the phylum Chlorophyta. This class is considered a pioneer of high-level plants [14]. Chlorella vulgaris belongs to the class Chlorophyceae, a group of single-cell green algae that can live in fresh water, marine and wet. Generally, Chlorella vulgaris is round like a ball, with a cell diameter of about 2-10 µm with cup-shaped chloroplasts.

![Figure 1. Chlorella vulgaris cells under a microscope with 100 × magnification](image)

Chlorella vulgaris breeds in a vegetative way by dividing. Vegetative breeding begins with forming spores. Each stem cell will release zoospores called aplanospores which will then become new individuals [15]. Microalgae growth is influenced by environmental conditions such as temperature, pH, light intensity, salinity, nutrition, and CO₂ concentration. In this study, activation was carried out for 4 days because it was estimated that during this time microalgae could grow and adapt to new mediums and environments. This can be observed from the color and number of cells of the microalgae Chlorella vulgaris. Changes in growing conditions can be a cause of disruption in the growth of microalgae such as slowing growth can even cause contamination and death in cells if the environmental conditions are not suitable. Activation is carried out using GMF medium to adapt microalgae to grow first. GMF medium is chosen as a modification medium because it is easy to dissolve in water so it does not need chelating agents such as Na-EDTA. In addition, the composition of the constituent minerals in the GMF medium composition resembles the rich medium such as...
Walne, although there are differences in levels. The price is cheap, easy to get and the preparation of the medium is simple [11].

Data for the growth curve is carried out for 15 days because after that microalgae is estimated to have entered the phase of death. Microalgae is grown in 5%, 10% and 15% GMF medium with cell density starting from 1 x 10^6 cells / mL. For 15 days, the number of cells in the bioreactor was calculated daily using a haemocytometer. Table 1 shows data on cell density and table 2 shows the growth rate of Chlorella vulgaris microalgae cells.

Table 1. Cells density of microalgae Chlorella vulgaris in GMF medium

| Day | 5% (x10^6) | 10% (x10^6) | 15% (x10^6) |
|-----|------------|-------------|-------------|
| 0   | 1          | 1           | 1           |
| 1   | 2          | 2.2         | 2.8         |
| 2   | 3          | 4           | 4           |
| 3   | 4          | 5           | 8           |
| 4   | 5          | 6           | 12.85       |
| 5   | 6          | 5.5         | 9.35        |
| 6   | 4.65       | 6           | 11          |
| 7   | 5          | 6           | 11.9        |
| 8   | 5          | 6           | 10          |
| 9   | 6          | 6           | 11          |
| 10  | 6          | 8           | 9           |
| 11  | 5          | 10          | 10          |
| 12  | 6          | 13          | 13          |
| 13  | 8          | 13          | 17          |
| 14  | 9          | 13          | 18          |
| 15  | 11         | 15          | 19          |

Table 2. Growth rate microalgae Chlorella vulgaris in GMF medium

| Day | GMF concentration variation |
|-----|-----------------------------|
|     | 5%  | 10% | 15%  |
| 1   | 0.693 | 0.788 | 1.029 |
| 2   | 0.405 | 0.598 | 0.357 |
| 3   | 0.288 | 0.223 | 0.693 |
| 4   | 0.223 | 0.182 | 0.474 |
| 5   | 0.182 | -0.087 | -0.318 |
| 6   | -0.255 | 0.087 | 0.162 |
| 7   | 0.072 | 0 | 0.079 |
| 8   | 0 | 0 | -0.174 |
| 9   | 0.182 | 0 | 0.095 |
| 10  | **0** | **0.288** | **-0.201** |
| 11  | -0.182 | 0.223 | 0.105 |
| 12  | 0.182 | 0.262 | 0.262 |
| 13  | 0.288 | 0 | 0.268 |
| 14  | 0.118 | 0 | 0.057 |
| 15  | 0.201 | 0.143 | 0.054 |
The data above shows that GMF with a concentration of 10% gives better growth results of \textit{Chlorella vulgaris} compared to other concentrations. In addition, GMF medium with a 10% concentration has the composition and concentration of nutrient content that is close to the Walne medium compared to of 5% and 15%. From these data, the cultivation medium used is 10% GML with harvest time on the 10th day.

![Growth curve of \textit{Chlorella vulgaris} in GMF medium](image)

\textbf{Figure 2.} \textit{Chlorella vulgaris} growth curve in GMF medium

Cultivation and cell harvesting were carried out to obtain microalgae culture wet biomass. The wet biomass obtained is concentrated green and shaped like a paste. The harvesting technique used is using centrifugation so that the cells will collect at the bottom of the centrifugation tube. Other harvesting techniques besides centrifugation techniques are coagulation techniques. Centrifugation techniques are more effective and efficient to do when harvesting small cells such as \textit{Chlorella vulgaris}. In addition the cells obtained from centrifugation are intact cells, there has been no change in cell composition compared to the harvesting of coagulation methods by the addition of coagulant compounds. Biomass obtained in green indicates that the cells obtained are still in good condition. But the wet biomass still contains a lot of water. The water content must be removed because it can interfere with the non-polar lipid extraction process. Drying with freeze-dried techniques is done to eliminate water content in microalgae. Water content removal functions so that the water contained in biomass does not interfere with the extraction process and microalgae biomass samples last a long time and not covered by the fungus. In previous studies, harvesting was done using coagulation with NaOH. The color of the biomass obtained is light green. The results of the analysis of fatty acid compositions also detected only a few fatty acids. It is suspected that there has been a breakdown of the fatty acid composition in the harvesting process with the coagulation [16].

Lipid extraction was carried out using the Bligh-Dyer method. This method is a modification of extraction with maceration techniques combined with sonication and liquid extraction processes. The solvents used in this extraction are methanol, chloroform and water. First, dry biomass was extracted by adding methanol and chloroform, and sonication for 10 minutes. Methanol functions as a polar solvent that can dissolve non-lipid compounds and also to prevent bacteria from breaking down fatty acids. Chloroform functions as a nonpolar solvent that will dissolve lipids. The purpose of the sonication process is to break down the cell wall of \textit{Chlorella vulgaris}. The maceration process continued with stirring without heat for 24 hours [12]. Addition of chloroform and water is part of liquid extraction. Chloroform will attract lipids which are still emulsified in methanol during the maceration process while water will dissolve the methanol phase. Lipid extraction is obtained after
chloroform and water phase separated. After chloroform solvent was evaporated, a dark green lipid extract was obtained with a yield of 23.5% (w/w).

Lipid esterification is carried out to convert fatty acids into fatty acid methyl esters, so that they can be analyzed using GC-FID (Gas Chromatography-Flame Ionization Detector). Esterification is performed by reacting the lipid extract with alcohol under acidic or alkaline. Lipids obtained from extraction were added with NaOH in methanol, incubated at 55-60 °C and stirrer for 12 hours. At the time of esterification, the lipids will react with methanol. H₂SO₄ is added as a catalyst to accelerate the esterification reaction.

![Figure 3. Chromatogram of fatty acid methyl esters Chlorella vulgaris with GC-FID](image)

| No | Peak | Compound                      | Area     | (% fatty acid) |
|----|------|-------------------------------|----------|---------------|
| 1  | 3    | C8:0 (methyl caprilate)       | 519113   | 0.28          |
| 2  | 6    | C14:0 (methyl miristate)      | 1754033  | 0.95          |
| 3  | 7    | C15:0 (methyl pentadecanoat)  | 530425   | 0.29          |
| 4  | 13   | C16:0 (methyl palmitate)      | 28689897 | 15.58         |
| 5  | 14   | C16:0 (methyl palmitate)      | 19290511 | 10.48         |
| 6  | 17   | C17:1 (methyl heptadecenoate) | 1580824  | 0.86          |
| 7  | 18   | C17:0 (methyl heptadecanoate) | 6152842  | 3.34          |
| 8  | 21   | C18:2 (Omega 6,methyl linoleate) | 281115  | 0.15          |
| 9  | 22   | C18:3 (omega 6)               | 429189   | 0.23          |
| 10 | 23   | C18:0 (methyl stearate)       | 113284519| 61.52         |
| 11 | 38   | C20:4 (methyl arakidonat)     | 311137   | 0.17          |
| 12 | 39   | C20:5 (EPA, omega 3)          | 316907   | 0.17          |
| No | Peak | Compound                      | Area   | (%) fatty acid |
|----|------|-------------------------------|--------|----------------|
| 13 | 40   | C20:3 (omega 3, omega 6)      | 279993 | 0,15           |
| 14 | 41   | C20:2 (methyl docosanoate)   | 220758 | 0,12           |
| 15 | 42   | C20:1 (methyl heneikosanoate)| 118122 | 0,64           |
| 16 | 43   | C20:0 (methyl eicosanoa)     | 8632814| 4,69           |
| 17 | 44   | C22:0 (methyl docosanoate)   | 352520 | 0,19           |
| 18 | 45   | C24:0 (omega 6)              | 322526 | 0,17           |

The results of the analysis of the composition of fatty acid methyl esters with GC-FID can be seen in figure 3. The chromatogram is compared with capric acid which is a standard mixture of C4-C24 fatty acids [13]. From the results of GC analysis, there were 46 peaks which showed 46 compounds. There were 18 compounds identified as fatty acid methyl esters consisting of 10 saturated fatty acid compounds and 8 unsaturated fatty acid compounds (table 3). Another unidentified peak is thought to be a byproduct of the esterification process or impurity from the solvent. High levels of fatty acid methyl esters are known as methyl palmitate ±15%, methyl heptadecanoate ±3.34%, methyl stearate ±61.52% and methyl eicosanoate ±4.69%. In addition, there have been identified high economic value of unsaturated fatty acids, EPA (eicosapentaenoic acid), omega 3 and omega 6. These fatty acids are contained in lipid microalgae *Chlorella vulgaris* where EPA concentrations are 0.17% while omega 3 and omega 6 are ± 0.23%. Although the levels of unsaturated fatty acids are still low, it does not rule out the possibility of being increased. One of them is by looking for another alternative medium. The composition of the medium will affect the biomass and lipid content that will be produced by microalgae. At low nitrogen concentrations, microalgae will form lipids which are used as food reserves, whereas if the nitrogen concentration and high light intensity, the growth of microalgae and biomass is high but the lipid content is low [17].

4. Conclusion

Plant fertilizer can be used as an alternative medium to replace rich medium to grow microalgae *Chlorella vulgaris*. Microalgae grown in a fertilizer medium produce quite high lipids. The result of GC FID analysis shows that there are 18 fatty acid methyl esters compounds, 8 of the compounds were unsaturated fatty acid methyl ester. The highest known fatty acid methyl esters composition is methyl stearate 61.52%, methyl palmitate 15:52%, methyl eicosanoate 4.69% and methyl heptadecanoate 3.34% from total fatty acids.

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