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Fatty Acid Analysis of Microalgae *Chlorella* sp. Cultured Results in a Closed System

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***Abstract*— Research on the analysis of fatty acid from microalgae *Chlorella* sp. conducted to determine the oil content obtained from microalgae *Chlorella* sp. Microalgae oil from the extraction using methanol and n-hexane solvents was obtained 3.13%. The methyl ester function group uses FTIR: C-O, C_{sp}^3-H, C=O, and CH3. Analysis with GC-MS contained fatty acids.: methyl myristate (7.62%), methyl palmitoleate (36.37%), methyl palmitate (21.70%), and methyl oleate (11.56%), and tricyclo [8.6.0.0(2,9)] hexadeca-3,5-diane (22.75%).**

Keywords*— *Chlorella sp., Fatty acid, FTIR, GC-MS.

I. INTRODUCTION

Fatty acids consist of saturated and unsaturated fatty acids. Food quality and biodiesel are determined by the amount and components of the fatty acids they contain. Vegetable oil is composed of glycerol and fatty acids. Vegetable oil is a source of unsaturated fatty acids, including essential fatty acids such as oleic acid, linoleic acid, and arachidonic acid. These fatty acids are found in many vegetable oil-producing foods [1,2]. Vegetable oil-producing foods thrive in Indonesia such as palm oil, jatropha, sunflower, soybeans, and corn. However, these materials have several weaknesses that result in suboptimal oil production, such as time of harvest mass, require fertile and extensive land, disrupt food security, and have the potential to increase food prices due to competition with food needs. Therefore, it is necessary to look for vegetable oil-producing materials that can overcome these weaknesses [3,4].

The limited availability of petroleum encourages Indonesia to use biofuel as a substitute for fossil fuels. Palm oil is the primary source of biofuel has been developed in Indonesia. However, with the increasing need for biofuel in the future, as well as competition with the need for palm oil as food, it is necessary to look for sources of biofuel that do not compete with foodstuffs, and have high productivity so that they can produce more biofuel with fewer land requirements [5–9].

Microalgae is the most reliable biodiesel raw material compared to other biodiesel raw materials. Microalgae have a high oil content (70%) of dry weight, do not require fertile soil, and do not compete with food [10]. Microalgae are autotrophic organisms that grow through photosynthesis. Microalgae unicellular structure allows solar energy can convert into chemical energy. Microalgae can grow anywhere, both in aquatic ecosystems and in terrestrial ecosystems [11].

Another advantage of microalgae is fast growth and high productivity. Microalgae can produce 50 times more biomass than other higher plants. Microalgae can produce 200 times more oil compared to oil-producing plants, such as palm oil and jatropha [12]. Microalgae contain protein, carbohydrates, vegetable oils (lipids), minerals, and nucleic acids [13]. The oil content in microalgae varies greatly depending on the environmental conditions in which the microalgae grow [14]. Microalgae biomass *Chlorella* sp. can be used as an alternative energy source, producing bioactive components, pharmaceutical and medical materials, natural and artificial food sources, and also as a source of food supplements [15].

Microalgae cultivation can be done in a closed system with controlled environmental conditions or can be done in an open system. In a closed system, microalgae are cultivated in bioreactors that are connected to several light sources. The parameters needed to grow, such as CO_2, nutrient-rich water, temperature, and lighting must be entered into the system. Microalgae are cultivated for 12 to 15 days per cycle before being harvested. Microalgae obtained from the bioreactor system has a concentration of 1% solids, while to produce biodiesel is needed in the form of a paste with a concentration of 15% solids [16].

Conventional harvesting techniques are centrifugation, flocculation, and filtering with filter bags. After the microalgae paste is obtained, the extraction of microalgae oil is performed. Oil extraction from microalgae is the most decisive step, so it needs efforts to maximize the acquisition of oil in the

extraction process [13]. The microalgae oil obtained is transesterified to convert triglycerides into methyl esters (biodiesel) [10].

The oil content in microalgae is usually in the form of glycerol and fatty acids with a chain length of C_{14} to C_{22} [3]. Based on this background, this study was conducted the fatty Acid Analysis of Microalgae *Chlorella* sp. resulted by cultured in closed system.

II. METHODOLOGY

A. Materials

The materials are microalgae *Chlorella* sp. N-hexane (Merck), anhydrous methanol (Merck), distilled water, filter paper Whatman no. 42, potassium hydroxide (Merck), sodium sulfate anhydrous, phenolphthalein, boron trifluoride methanol, urea (Merck), $NaH_2PO_4.2H_2O$ (Merck), $Na_2SiO_3.2H_2O$ (Merck), $FeCl_3$ (Merck), Na_2EDTA (Merck).

B. The equipment

The equipment used in this study: Soxhlet, a set of glassware (Pyrex), hotplate (Cimarec 2), thermometer, analytical scales (Ohaus), oven (Memert), separating funnel, a set of tools, reflux (pyrex), magnetic stirrer, rotavapor (Buchii), Gas Chromatography-mass Spectrometer (QP-2010 plus, Shimadzu), Fourier Transform Infrared Spectrometer (FTIR Prestige 21, Shimadzu).

III. PROCEDURE

A. Sterilization of the equipment and seawater

All equipment sterilized by spraying a 70 % alcohol solution. Seawater is filtered using filter paper then sterilized using 1.25 mL NaOCl. The sample is left standing for 24 hours. Sterile seawater neutralized by adding 1 mL of $Na_2S_2O_3$ and left for 12 hours.

B. Sterilization of the equipment and seawater

Microalgae growth media used is a modification medium for optimal conditions with the composition: Modified medium urea, $NaH_2PO_4.2H_2O$, $FeCl_3$, and Na_2EDTA (the first nutrient, while Na_2EDTA and $Na_2SiO_3.5H_2O$ (other nutrients) were sterilized using an autoclave at 121 °C for 15 minutes to prevent contamination.

C. Microalgae cultivation process

Chlorella sp. Microalgae cultivation performed at 27 ppt salinity and initial pH seven. The total volume of *Chlorella* sp., which used as much as 1000 mL. Cultivation is carried out by inserting 900 mL of sterilized seawater in a glass jar, then adding 100 mL of microalgae *Chlorella* sp. Inoculum. The jar tightly closed with a perforated cover for the inclusion of L tubes and pipes. Cultured aerated with air, aerated for 24 hours, room temperature (± 25-27 °C), photoperiod 12:12 (dark: light) using a 42 W spiral lighting lamp. The density of culture inoculum cells was measured using a hemocytometer under a microscope. *Chlorella* sp. Microalgae cultivation, the modification medium was carried out for 12 days.

D. Sample preparation stage

On the 12th day of *Chlorella* sp. harvested, then centrifuged for 20 minutes at a speed of 60 rpm to obtain biomass. Furthermore, it is dried in an oven at a temperature of 30 ^{o}C to obtain *Chlorella* sp. dry.

E. Analysis of water content

Porcelain dishes are heated in an oven at 105 ºC for 15 minutes, then cooled in a desiccator. The cup is weighed until a constant weight is obtained. A total of 5 g of microalgae powder *Chlorella* sp. dried in an oven at 105 ºC, then stored in a desiccator, then weighed until a constant weight is obtained.

$$\% \text{ water} = (c\text{-}b)/c \times 100\% \quad (1)$$

Where, c is Wet weight *Chlorella* sp., and b is Dry weight of Chlorella sp.

F. Determination of the Chlorella sp. oil content

A total of 64.38 g of dry biomass of *Chlorella* sp. extracted with n-hexane 200 mL and methanol 200 mL using soxhlet for 7 hours at a temperature of 80 ^{o}C. The extraction results are concentrated using a rotary evaporator, and then dried with a vacuum pump. A concentrated extract of microalgae oil *Chlorella* sp. then weighed.

G. Determination of free fatty acids

Chlorella sp. oil took as much as 0.97 g, then put into Erlenmeyer and added five drops of phenolphthalein indicator then titrated using 0.1 N KOH until the red color is calculated free fatty acid content.

H. Determination of free fatty acids

1.04 g of oil and 10 mL of BF_3-methanol were put into a round bottom flask and refluxed for 90 minutes. Then the mixture was put into a separating funnel, then added 100 mL of distilled water and 100 mL of n-hexane, then shaken and allowed to stand 2 layers (organic phase and water phase). The organic phase (top layer) taken, and 100 mL of distilled water and 15 mL of n-hexane were added, then the separation was carried out using a separating funnel. The obtained organic phase is concentrated using a rotary evaporator. Then the concentrated extract is dried using a vacuum pump. The layer then esterified with free fatty acids with methanol (1: 3). The mixture is refluxed at 75 ^{o}C for 3 hours. The result is formed two layers, namely a mixture of methanol and methyl esters (top layer) and triglycerides (bottom layer).

Then triglycerides are transesterified with methanol (1:12) and added with a BF_3-methanol acid catalyst weighing 2% by weight of the mixture. The mixture is refluxed again at 70 ^{o}C for 2 hours. The reaction mixture is cooled down, and two layers are formed, namely methyl ester (top layer) and glycerol (bottom layer). The methyl ester and glycerol layers are

separated by separating funnels. The methyl ester is evaporated to remove the remaining methanol. The methyl ester is then washed with distilled water in a separating funnel to dissolve the remaining glycerol. The final step is to add anhydrous Na_2SO_4 to bind the residual water, and then filter it with filter paper. Then biodiesel synthesis is carried out at the optimum conditions that have been obtained. The resulted methyl esters characterized by FTIR and GC-MS.

IV. Results and Discussion

A. Sterilization of Cultivation of Microalgae *Chlorella sp.*

Cultivation of *Chlorella* sp. carried out for 12 days using a light intensity of 3000 lux at room temperature ± 25-27 °C. Seawater used has a salinity of 27 ppt with a pH of 8. On the 12th day *Chlorella* sp. is in an exponential phase with stable growth and is the time of harvest. Cultivation media is a modification medium which is a culture medium with the ability to provide good growth for microalgae with nutrients in a small amount than other commercial media [13]. CO_2 used as a carbon source in the aeration process. The phytoplankton growth becomes faster in cultures that are given light and aeration with air containing CO_2 [11].

Microalgae growth is characterized by an increase in the number of cells in culture. Determination of the time to harvest microalgae Chlorella sp. performed based on phytoplankton growth curves during the cultivation process. During cultivation, the culture of *Chlorella* sp. experiencing a color change from light green to dark green. This result shows that an increase in the density of *Chlorella* sp. *Chlorella* sp. during the cultivation process shown in Fig. 1.

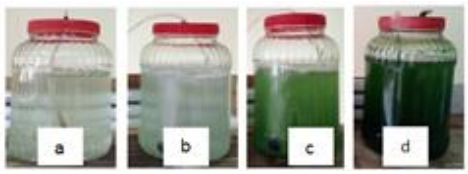

Figure 1. Cultivation results of *Chlorella* sp. on day (a) 0, (b) day 4, (c) 6th day, and (d) 12th day.

Fig. 1 shows the color of the culture of *Chlorella* sp. always changing from day to day, from colorless (a and b), then light green (c) to dark green (d). Changes in the color of this culture indicate that the growth of microalgae. *Chlorella* sp. the better by increasing the number of cell densities.

Harvesting of *Chlorella* sp. performed when the microalgae cells reach optimum growth on the 12th day. This process is done so that the condition of microalgae is still good, and not many cells have died. Harvesting is done by centrifugation techniques to obtain biomass *Chlorella* sp. This technique was chosen for harvesting because of *Chlorella* sp. has a tiny cell size. From the research results obtained by biomass *Chlorella* sp. dried 64.38 g.

B. Results of water content determination

Water content is the percentage of the water content of a material that can be expressed based on wet weight or dry weight. Determination of the water content of the microalgae *Chlorella* sp. gravimetric method used direct heating (oven). The principle of the drying oven method is that the water contained in material will evaporate if the material heated at 105 ^oC for a specific time. The difference between the weight before and after heating is calculated as water content.

The presence of water affects the deterioration of the quality of chemical material, so it is necessary to determine the water content so that water molecules cannot inhibit the process of absorption of solvents. Water content that is too high in the sample can cause the solvent to become saturated with water. The content of fatty acids *Chlorella* sp. was 74.036%.

C. Determination of the oil content of *Chlorella sp.*

Extraction is a method of separating a substance based on differences in solubility of two insoluble liquids [15]. *Chlorella* sp. Microalgae oil extraction performed using the soxhlet extraction method. The main principle in soxhlet is the taking of a particular component using a solvent that is always new, so that continuous extraction occurs with a constant amount of solvent in the presence of a reverse cooler or condenser. The advantages of extracting oil from microalgae *Chlorella* sp. compared to other natural ingredients, the dry powder can be directly extracted with non-polar solvents without the need for a grinding process [10].

Figure 2. The results of the extraction of the *Chlorella* sp.

Chlorella sp. Oil extraction performed using n-hexane and methanol (1:1) solvent. The soxhlet method for extracting oil in *Chlorella* sp. selected based on the nature of the oil which is resistant to the heating process. *Chlorella* sp. obtained blackish-green oil (Fig. 2) with a yield of 3.12%. According to Saadudin, et al (2011) *Chlorella* sp. has a lipid content of about 9.8% of the dry weight of biomass.

D. Determination of free fatty acid content

The content of free fatty acids in microalgae *Chlorella* sp. is 19.65%. Lipids extracted by microalgae biomass usually contain neutral lipids and polar lipids. Neutral lipids (triglycerides) can be used as raw material for biodiesel, in which the molecular composition consists of three long-chain fatty acids that are bound to one glycerol. The type of fatty acid bound to glycerol varies greatly with carbon chain

lengths from 4 to 30, in the form of saturated chains (no carbon double bonds), or in the form of unsaturated chains (there are one or more carbon double bonds).

The higher free fatty acids, the lower the quality of biodiesel. High levels of free fatty acids cause sediment formation, which can cause a decrease in quality in the combustion system. In addition, high free fatty acids can also reduce the age of pumps and filters [2].

E. Esterification reaction results

Chlorella sp. oil is a triglyceride that will be hydrolyzed into free fatty acids and glycerol. The principle of hydrolysis is the decomposition of a compound by breaking the glycoside bond using water and a base or acid catalyst. Hydrolysis of microalgae oil *Chlorella* sp. performed using an acid catalyst (BF_3-methanol). The hydrolysis of *Chlorella* sp. black green viscous extract was obtained as much as 1.04 g.

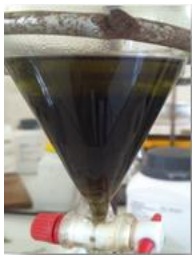

Figure 3. The results of the esterification of the *Chlorella* sp.

Esterification is an ester formation reaction by refluxing carboxylic acid with alcohol using an acid or base catalyst. The esterification process is carried out by reacting methanol with *Chlorella* sp. (1: 3). The reflux process is carried out for 3 hours at a temperature of 75 ^oC, i.e. the temperature approaches the boiling point of methanol. The result is formed two layers, namely a mixture of methanol and methyl esters in the upper layer and triglycerides in the lower part (Fig. 3). The two layers are separated and taken the bottom for further transesterification, while the top will be combined with the methyl ester resulting from the transesterification process.

The esterification process is carried out to reduce the levels of free fatty acids. The smaller the level of fatty acids, the higher the methyl ester obtained. The esterification process aims to convert the carboxylic acid into an ester using an acid catalyst with water as a by-product of the reaction. The byproduct in the form of water can be overcome with excess methanol, where the water formed will dissolve in methanol and not inhibit the reaction process. Methanol also inhibits the rate of hydrolysis because methanol in the form of methoxide ions reacts quickly with triglycerides to produce methyl esters [7].

F. Transesterification reaction

In the transesterification process, the catalyst used is the acid catalyst from the amount of oil and methanol. The ratio of methanol to oil in the transesterification process is higher than the esterification process, which is 1:12 because the amount of triglycerides that will be converted into methyl esters is higher than free fatty acids that are converted into methyl esters.

Figure 4. Results of transesterification of the *Chlorella* sp. Oil.

The transesterification process uses the reflux method at temperatures close to the boiling point of methanol, which is 70 ^oC for 2 hours. The results obtained are in two layers, namely methyl ester in the upper layer and glycerol in the lower layer (Fig. 3). The methyl ester is evaporated at 70 ^oC according to the boiling point of methanol. The methyl ester is then washed with distilled water in a separating funnel to dissolve the remaining glycerol. The anhydrous methyl ester in the upper layer is added Na_2SO_4 to bind the remaining water, then filtered until methyl ester is obtained.

G. Fatty acid analysis with FTIR spectrophotometer

Analysis by FTIR was carried out to prove the presence of esters in the transesterification product seen from the typical absorption of C=O and C-O. FTIR analysis of fatty acid microalgae *Chlorella* sp. is presented in Table 1.

TABLE I. FTIR FATTY ACID OF MICROALGAE *CHLORELLA* SP.

Number of waves (cm^{-1})	Functional groups
2924.09-2854.65	C_{sp3}-H (aliphatic)
1735.93	C=O (ester)
1097.5	C-O
1375.25	-CH_3

The analysis data in Table 1 shows the presence of esters in the microalgae oil *Chlorella* sp. with strong absorption characteristics of carbonyl (C = O ester) around 1735.93-1718.58 cm^{-1} and C-O at 1097.5 cm^{-1}. CH_3 from the fatty acid chain appears at 1375.25 cm^{-1}, whereas in the area of 2924.09-3369.64 cm^{-1} is absorption for the Csp^3-H (aliphatic) group.

H. Analysis of fatty acids with GC-MS

Analysis using GC-MS aims to determine the type of methyl esters contained in fatty acids from microalgae *Chlorella* sp. esterification and transesterification results. Identification of GC-MS

methyl esters of microalgae *Chlorella* sp. presented in Table 2.

In GC-MS analysis, the peak that comes out first is the ester with a short carbon chain, then followed by a longer carbon chain. The column (stationary phase) used is non-polar, whereas in general, the ester is polar. Short-chain esters are more polar than long-chain esters. According to the law like dissolve like, the ester with a longer chain will be held in the column, while the short-chain ester will escape with the mobile phase-out of the column (Suntoro, 2012).

TABLE II. COMPOUNDS IN THE EXTRACT OF *CHLORELLA* SP. GC-MS ANALYSIS RESULTS

Peak No.	Compound	Molecular formula	Concen. (%)	Category
1	Methyl myristate	$C_{15}H_{30}O_2$	7.62	Saturated fatty acids
2	Methyl palmitoleate	$C_{17}H_{32}O_2$	36.37	Unsaturated fatty acids
3	Methyl palmitate	$C_{17}H_{34}O_2$	21.70	Saturated fatty acids
4	Methyl oleate	$C_{19}H_{36}O_2$	11.56	Unsaturated fatty acids
5	Tricyclo [8.6.0.0(2,9)] hexadene-3,15-diene	$C_{16}H_{24}$	22.75	-

GC-MS analysis showed that 5 peaks stated 5 compounds contained in the methyl ester microalgae *Chlorella* sp., But only 4 peaks were detected as fatty acid methyl esters. The second peak with a retention time of 19.91 minutes and m/z = 268 has the most significant area (36.37%), namely methyl palmitoleate ($C_{17}H_{32}O_2$); the third peak with a retention time of 20.23 minutes and m/z = 270, namely methyl palmitate ($C_{17}H_{34}O_2$) of 21.70%; the fourth peak with a retention time of 22.41 minutes and m / z = 296, namely methyl oleate ($C_{19}H_{36}O_2$) of 11.56%, while the first peak with a retention time of 15.88 minutes and m/z = 242, namely methyl myristate ($C_{15}H_{30}O_2$) of 7.62%.

Triglycerides are transesterified using alcohol to produce glycerol and fatty acid methyl esters (biodiesel). The resulting methyl ester profile determines the quality of biodiesel. Methyl esters with 16 or more C atoms have cetane numbers (Suntoro, 2012). The research results obtained methyl esters, namely methyl palmitoleate, methyl palmitate, and methyl oleate, that meet the requirements as biodiesel because it has a C>16 atoms. Methyl esters of microalgae *Chlorella* sp. contain saturated methyl esters (29.33%), namely methyl myristate and methyl palmitate, while unsaturated (47.93%), namely methyl palmitoleate and methyl oleate.

Biodiesel contains predominantly unsaturated methyl esters, then the stability of biodiesel is low. This is due to the oxidation of fatty acid chains with oxygen molecules to form hydroperoxides. Its condition turn will encourage the formation of polymerization to form gum (sap). If biodiesel has a predominantly saturated methyl ester, then the performance of biodiesel will be worse at low temperatures because the melting point becomes increasing. The length of the carbon chain also affects the quality of biodiesel especially in the cetane number, where usually biodiesel with a dominant methyl ester profile with a shorter chain will have a lower cetane number.

V. CONCLUSIONS

Based on research that has been done, it can be concluded (1) microalgae *Chlorella* sp. contains 3.12% oil in 100 mL of inoculum and 900 mL of seawater. (2) identification of components with GC-MS shows *Chlorella* sp. containing fatty acids: methyl myristate (7.62%), methyl palmitoleate (36.37%), methyl palmitate (21.70%), and methyl oleate (11.56%).

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