Research Article

Purkan Purkan*, Ersalina Nidianti, Abdulloh Abdulloh, Abdillah Safa, Wiwin Retnowati, Wiwie Soemarjati, Hamida Nurlaila, Seung Wook Kim

Biodiesel Production by Lipids From Indonesian strain of Microalgae *Chlorella vulgaris*

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Abstract: The fatty acid methyl ester (FAME) production from *Chlorella vulgaris* has been studied by sequential investigation such as microalgae culturing, lipid extraction, and lipid conversion to FAME. The *C. vulgaris* could grow well in the BG-11 medium and had a doubling time 3.7 days for its growth using inocula 16% (v/v). The optimum of dry cell biomass as 11.6 g/L was obtained after the microalgae culture harvested for 6 days. Lipid extraction from the biomass was carried out in various solvents and ultrasonication power, resulted lipid as 31% (w/w) when extracted with a mixed solvent of n-hexane-ethanol in ratio 1:1 and ultrasonication treatment at power 25 kHz/270W for 30 min. The lipid then converted to FAME through transesterification reaction with methanol using H_2SO_4 catalyst at 45ºC for 2 h, and resulted FAME with area 32.26% in GC-MS analysis. The area was corresponded to FAME output as 13.68% (w/w). Fatty acid profiles of FAME obtained from GC-MS analysis showed the major peaks of fatty acids found in *Chlorella vulgaris* were palmitic acid (C16:0), stearic acid (C18:0) and margaric acid (C17:0), and nonadecanoic acid (C19:0). Optimization of the transesterification reaction will be developed in future to improve the FAME product.

Keywords: microalgae; *Chlorella vulgaris;* FAME; ultrasonication

1 Introduction

Depletion of petroleum energy resources or fuel oil as a result of high fuel consumption becomes a major issue in many countries. To overcome this problem many countries develop biofuels, one of which is biodiesel as renewable energy. It is also classified as a safety energy, because it has no aromatic compounds, easily degraded, and free of SOx component. Biodiesel is more specifically defined as the monoalkyl esters of long-chain fatty acids derived from the chemical reaction (transesterification) of renewable feedstocks, such as vegetable oil or animal fats, and alcohol with or without a catalyst [1].

Biodiesel has a significant energy similar to petroleum-derived diesel oil, therefore it has more potential to substitute the diesel. Because the cost raw of material needs 75% of the total cost on biodiesel production [2], the choice of an appropriate resource is the most important thing to ensure a low production cost biodiesel. Microalgae has been suggested as a good candidate for fuel production because of their advantages of higher photosynthetic efficiency, higher biomass production and faster for growth than other energy crops [3]. Microalgal cells have a high oil content, so it is a suitable to be developed as a material source in the biodiesel production. The composition of various fatty acids in microalgae makes the biodiesel that has different characteristics [2]. In addition, the use of microalgae does not compete with food [3].

Indonesia has a high biodiversity of microalgae scattered in terrestrial and marine waters, however the potency of microalge has not yet explored optimally. Microalgae consist of various species such as diatom microalgae (Bacillariophyceae), green microalgae (Chlorophyceae), gold microalgae (Chrysophyceae), and blue microalgae (Cyanophyceae) [1]. Microalgal cultivation

***Corresponding author: Purkan Purkan,** Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya 60115- Indonesia, E-mail: purkan@fst.unair.ac.id
Ersalina Nidianti, Abdulloh Abdulloh, Abdillah Safa, Hamida Nurlaila, Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya 60115- Indonesia
Wiwin Retnowati, Microbiology Division, Faculty of Medicine, Airlangga University, Jl Prof Dr. Moestajab, Surabaya, Indonesia
Wiwie Soemarjati, Brachiswater Aquaculture Development Center Situbondo
Seung Wook Kim, Korea University, Department of Chemical and Biological Engineering; Adjunct Professor at Universitas Airlangga

is essential for the provision of sustainable feedstock sources in biodiesel production. Microalgal cultures can be performed in a bioreactor containing a liquid medium with additinal supply of air and irradiation. The cost of cultivation is also relatively cheap, since it does not require much fertilizer and nutrients.

The main components of triglycerides in microalgae can be converted to biodiesel or fatty acid methyl ester (FAME) through the transesterification reaction with methanol by using acid, base or enzyme catalysts. The reaction can be run in two ways, i.e., *ex-situ* and *in-situ* transesterification [4,5]. In the *ex-situ* method, the biodiesel is prepared through two stages, started by lipid extraction then followed by a transesterification reaction. While in the *in-situ* method, both lipid extraction and transesterification steps are performed in one process.

One of key parameters required for FAME production with *ex-situ* process is the high availability of lipid. Solvent extraction is used to obtain lipid from microalgae due to its simplicity and relatively inexpensive process which has almost no investment for equipment [6,7]. Various solvents such as hexane, methanol, chloroform, and combination of them are usually used in the extraction [8]. The efficiency on lipid extraction is highly dependent on the polarity of the solvent and the ease of solvent access to the lipid storage in the cell parts. In addition to the solvent extraction, some methods which facilitate the cell disruption are usually combined to enhance the lipid yield by some pre-treatments such as microwaves, sonication, bead-heating and supercritical extraction with CO_2 [9,10]. The most efficient method for extracting compounds from several species of microalgae including *C. vulgaris* has not yet been settled.

The superiority of the sonication method in lipid extraction has been widely reported in several references, which can reduce on the sample extraction times [23, 24, 25]. The sonication can disrupt microbial cells through a cavitation effect, because it produce high-energy microscopic bubbles along with mechanical pressure and shear [23]. Moreover, It has increased lipid extraction from vegetal tissue through the action of accelerating rehydration or swelling of plant cells accompanied by tissue matrix fragmentation, accompanied by mass transfer and penetration of the solvent into the cell and the release of cell contents into the solvent [23,24]. Based on this principle, then the ultrasonication is applied to assist the lipid extraction from Indonesia strain of *Chlorella vulgaris* microalgae as an effort to get a high yield of lipids. The comparison of lipid from the microalgae is also still unknown, so it is very interesting to be studied.

This paper reports the FAME production from microalgae *C. vulgaris* isolated from Indonesia which performed by ex-situ process including the cultivation and harvesting of the microalgae, lipid extraction and its conversion to biofuels.

2 Methods

2.1 Sample and chemicals

Chlorella vulgaris used in this research is obtained from Balai Perikanan Budidaya Air Payau (BPBAP), Situbondo, East Java, Indonesia. All chemicals for solvents and reagents were obtained from commercial sources and had a specification in analytical grade.

2.2 Cultivation of microalgae

C. vulgaris was cultured in the BG-11 medium [11] in the fotobioreactor system (1 L) equipped by a light source from three lamps (each 40 Watt). The culture was incubated under aerated CO_2 at room temperature. An inocula of 16% (v/v) was used in the cultivation. The cell density of culture was measured by spectrophotometry at 540 nm to determine the growth curve.

2.3 Lipid extraction

Lipid extraction was performed by using the Bligh and Dyer method [12]. The 5 g of dry biomass of *C. vulgaris* was dissolved respectively in 30 mL methanol, 30 mL mixed solvent of chloroform: methanol in ratio of 2:1, and 30 mL n-hexane. Every work was further subjected with an ultrasonic wave by using ultrasonicator (JY 92-IIDN) at 25kHz/270W for 30 min, then refluxed at room temperature for 2 h. After centrifuging at 6000 rpm for 10 min, the solvent phase was taken and evaporated in the rotary evaporator under vacuum at 60°C. This work was repeated for three times to get the entire lipid. The effects of solvents polarities on lipid extraction was also investigated in this study. Yield of lipid was calculated based on the equation:

$$\text{Lipid (\%)} = \frac{\text{mass of lipid (g)}}{\text{mass of microalgae (g)}} \text{x100}$$

2.4 Transesterification

The *ex-situ* transesterification was performed according to the Zhang method [13]. Lipids and methanol in molar ratio of 1:6 was mixed, then added 1% (w/w) concentrated sulfuric acid. The reaction was run at 45°C for 2 h. After cooling, the filtrate was evaporated in a rotary vacuum evaporator at 60 with 90 rpm. The filtrate was collected and added 10 mL n-hexane. The mixture was centrifuged at 8000 g for 20 min. Two layers which formed after centrifugation was shaken out for 20 min in the separation funnel. The botton layer containing hydrophilic phase was removed, whereas the top layer containing organic phase was taken and washed with 10 mL of hot water. After addition of anhydrous sodium sulfate, the organic phase evaporated in a rotary vacuum evaporator at 45 with a speed of 30 rpm. A solution containing FAME was collected and its weight was measured.

2.5 Gas Chromatography-Mass Spectrophotometry (GC-MS) Analysis

Sample was dissolved in n-hexane, then 1 mL of this injected into an Agilent GC-MS 5977 instrument using HP-5MS column with length 30 m, diameter 0.25 mm, and film thickness of 0.25 µm. Injection and detector temperature were maintained at 250°C. Initial column temperature was set at 100°C for 5 min, then increased to 300 °C with gradient of 20°C/min. The MS Source and Quard of the instrument were 230 °C and 150 °C, respectively, then set at low mass of 30 and high mass of 550 for sample measurement. Methyl heptadecanoate was used as standard for this analysis. The conversion of biodiesel resulting from the transesterification process is determined by the equation:

$$\text{FAME}(\%) = \frac{\sum A - As}{As} \text{x} \frac{Cs \text{ x } Vs}{m} \text{x } 100\%$$

Where, ΣA parameter constitutes as a total peak area of the methyl ester (C14:0-C24:1); As as a peak area of standard solution; Cs as a concentration of standard solution; Vs = volume of the standard solution and m as mass of samples [14].

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 The growth of *Chlorella vulgaris*

The *C. vulgaris* was cultured to observe its growth profile. The growth of microalgae in BG-11 medium with an inoculum showed typical pattern with 4 phases consisting of adaptation, logarithmic, stationary and death phases (Figure1). The adaptation phase was occured at day 0-1 marked by no significant growth, because the microalgae need initial adaptation to new environment. The logarithmic phase was occurred at day 1 – 5 that indicated by a significant increase in the growth of *C. vulgaris*. In this phase, the microalgal cells uptake the excessive nutrients in the medium to support their growth to obtain the energy, so the number of cells was increased up to (0,79x10%/ml) logarithmically. At days 5, the growth of *C. vulgaris* enterned to the stationary phase, characterized by stagnant growth. In this phase, the cell number of growth and death is balance. The *C. vulgaris* showed doubling time for it growth at 3.7 days, after determined based on the growth curve. Characteristics and morphological feature of the local strain of *C. vulgaris* have demonstrated its close similarity with genus *Chlorella vulgaris* . The individual cells of the strain are green colour, unicellular, spherical in shape its shows the Figure 2. Preparation of *C. vulgaris* biomass for lipid extraction was performed by using inoculum of 16% (v/v) followed by culturing for 5 days. A dry biomass of 11.6 g/L was obtained after draying overnight at 50ºC.

3.2 Lipid extraction from *C. vulgaris*

The lipid from *C. vulgaris* biomass was extracted by using several solvents both polar and non-polar as well as their mixtures. The effect of ultrasonication power and polarity of solvents on lipid extraction was investigated in this study. Lipid extraction without assisted by ultrasonication using solvents of methanol, n-hexane, and a mixture chloroform with methanol in ratio 1: 2 resulted in lipid yield as 15%, 24% and 19% (w/w) respectively, whereas those with additional ultrasonication resulted in 17%, 29% and 22% (w/w) (Figure 3). The work was performed by assisting of ultrasonication at power 25kHz/270W. The solvent of n-hexane extracted lipid higher than the methanol, and a mixture chloroform with methanol. It seems that n-hexane has a role to disrupt the existing hydrophobic interactions between non-polar and neutral lipid compounds. Hexane has a higher selectivity for

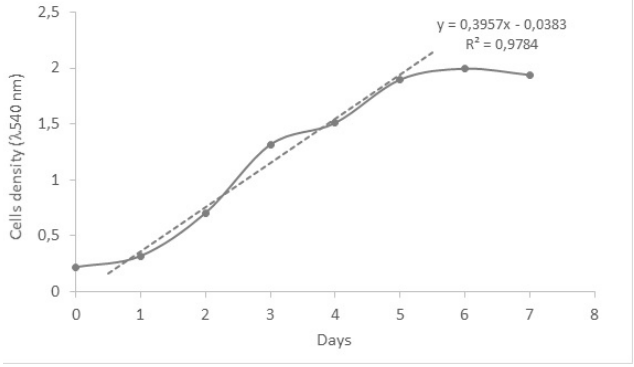

Figure 1: The growth curve of *Chlorella vulgaris* in BG-11 medium. The curve showed a doubling time for cell growth on 3.7 days.

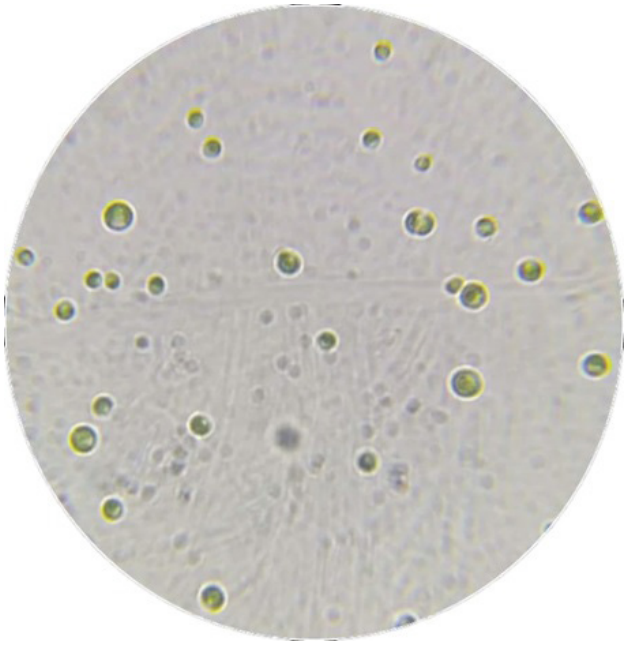

Figure 2: Microscopy picture of *C. vulgaris*.

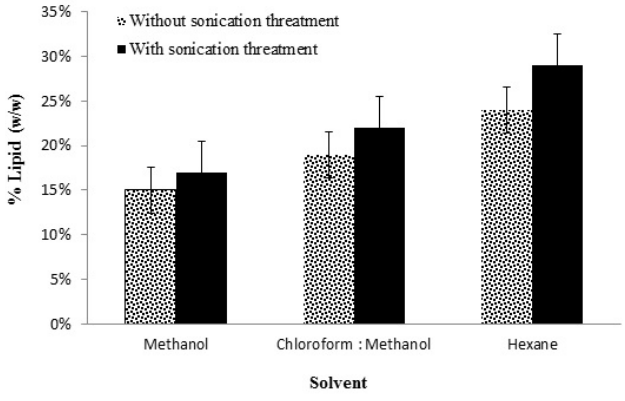

Figure 3: The yield of lipid extraction on various solvents with and no ultrasonication treatment.

non polar lipid than methanol and chloroform. It can partitions preferentially to the center of the lipid bilayer with a favorable entropy change, so this consistent with the hydrophobic effect [25, 27]. Similar result was obtained by Krishna et al. [28]. The applying of ultrasonication treatment which was initially setted at power 25kHz/270W also enhanced the lipid yield (Figure 3). The ultrasound-assisted extraction can increase the extraction efficiency through cavitation and some mechanical effects. Cavitation can disrupt microalgae cells then facilitate the lipid becoming easy to contact with organic solvent. Another mechanical effect caused by ultrasound may also be the agitation of the solvent used for extraction, thus increasing the contact surface area between the solvent and targeted compounds by permitting greater penetration of solvent into the cells [28]. Because of the cavitation role is affected by the solvent factor as solvent viscosity and surface tension [26], so the choice of precise solvent is needed. Compared to without ultrasonication, ultrasound-assisted extraction increased lipid yield as 2%, 5% and 3% in the solvent of methanol, n-hexane and a mixture chloroform with methanol respectively. The rise of lipid yield in n-hexane is higher than other two solvents. The n-hexane might support well the cavitation role of ultrasound so it could disrupt the microalgae cells optimally.

Since the presence of lipids in cells is enclosed by polar phospholipid layers of cell membrane, the splitting of the layer is required to release the non-polar lipids [13]. Based on this principle, the lipid extraction in the study was also conducted by using a mixed solvent of n-hexane - ethanol and n-hexane - methanol in various ultrasonication power. The highest lipid yield achieved when the ultrasonication-assisted cell disruption of microalgae was done at power 25kHz/270W (Figure 4). Although an increase in the power ultrasonicator can improve the cavitation process so the cell is easy to lysis, but if the power used is excessive, it can cause bubbles that actually reduce lipid yield.

Lipid extraction with a mixed solvent of n-hexane - methanol and n-hexane - ethanol in ratio of 1: 1 resulted in lipid yields of 25% and 31% (w/w), respectively (Figure 5). For this result showed that an increase in lipids extraction yields was obtained when non-polar and polar solvent mixtures were used. The use of polar and non-polar solvent combinations is enabled so that all lipids in both neutral and polar lipids can be extracted properly. The non-polar organic solvents is inadequate used to disrupt the membrane–lipid–protein associations, due to weak interactions to the complex. However, polar organic solvents can break the lipid–protein associations by forming hydrogen bonds with the polar lipids in the

Table 1: FAME composition of *Chlorella vulgaris*.

No	FAME	RT (min)	Formula	Area %
1	Butylated Hydroxytoluene	9.788	C15H24O	1.29
2	1,2,4-Triazol-4-amine, N-(2-thienylmethyl)-	11.082	$C_7H_8N_4S$	4.97
3	Pentadecanoic acid, methyl ester	11.67	C16H32O2	0.59
4	Hexadecanoic acid, methyl ester	12.204	C17H34O2	1.55
5	8-Octadecenal	12.596	C18H34O	1.36
6	Heptadecanoic acid, methyl ester*	12.768	C17H34O2	67.74
7	Heptadecanoic acid, methyl ester	12.791	C17H34O2	16.16
8	Methyl stearate	13.183	C19H38O2	1.37
9	Nonadecanoic acid, methyl ester	13.634	C20H40O2	3.96
10	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	14.548	C23H32O2	1.01
Total : FAME Sample				32.26
Total : FAME Sample + Standard				100

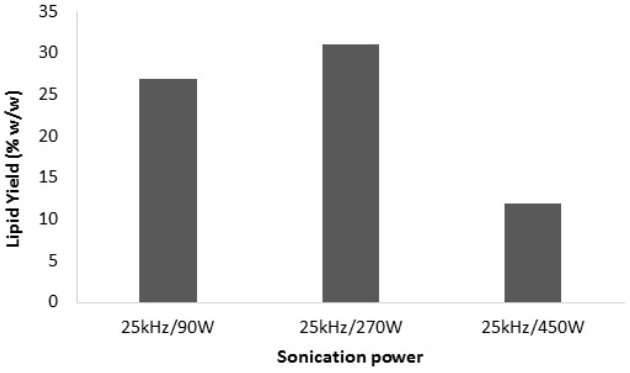

Figure 4: The yield of lipid extraction with binary solvent of n-hexane-ethanol in various ultrasonication power.

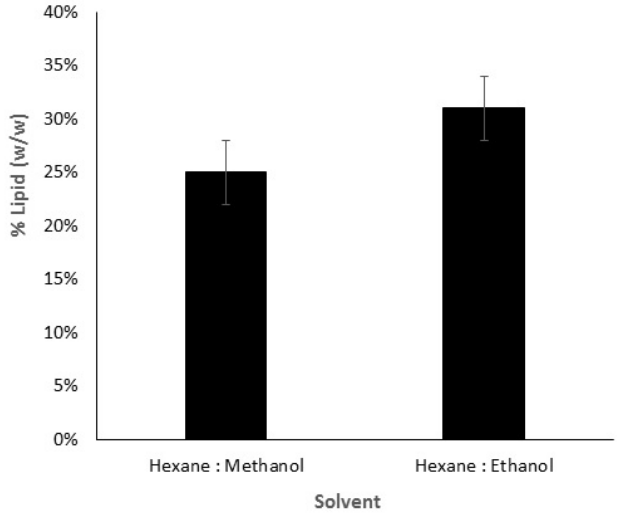

Figure 5: The yield of lipid extraction with binary solvents.

complex [29]. Ethanol has a polarity index lower that ethanol [25], so the mixing of ethanol into n-hexane produce a mixed solvent with a lower polarity index than if n-hexane is mixed with methanol. However the polarity of mixed solvent of n-hexane – ethanol that formed might facilitate an optimal cavitation role for *C. vulgaris* cells disruptio, in turn it could extract lipid higher than n-hexane - methanol mixed solvent.

The lipid product for the extraction was analyzed by GC-MS to search the fatty acids component, and resulted pentadecylic acid, palmitic acid, heptadecanoic acid, stearic acid, margaric acid and nonadecylic acid as component of the lipid (Table 1).

3.3 Lipid conversion to fatty acid methyl ester (FAME)

The production of fatty acid methyl ester (FAME) is conducted by mixing lipid with methanol in transesterification reaction using H_2SO_4 catalyst. The glyceride that presented in the lipid is transformed to glycerol and methyl esters. Because of the transesterification is included in the reversible reaction, so the excessive of methanol is needed to shift the reaction toward the FAME product [16]. Methanol was chosen as a reactant in the study because it is classifed as a cheap material, having a low boiling point and its excess in the glycerol phase easily to be separated [15].

Fatty acid profiles analyzed by GC-MS showed 32.26% FAMEs yield (Table 1). The area is corresponded to FAME

rendement as 13.68% (w/w). GC-MS chromatogram of FAMEs produced from ex-situ transesterification of *Chlorella vulgaris* . The major peaks of fatty acids found in *Chlorella vulgaris* were palmitic acid (C16:0), stearic acid (C18:0) and margaric acid (C17:0), and nonadecanoic acid (C19:0). For fuel properties, the length of carbon chain and the number of double bonds are important, in which C16:1 and C18:1 are the ideal biofuel feedstock [17, 18]. Five common feedstocks includes C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid) which were suitable for biodiesel production [19,20]. Fatty acids conversion to fatty acid methyl esters (FAMEs) can be economically applied at remote biomass production facilities for servicing production site and community energy and transport fuel needs [21].

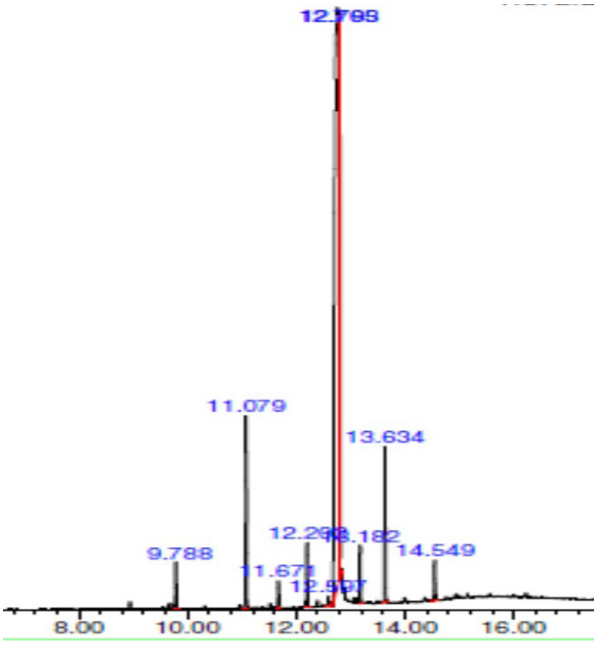

Figure 6: GC-MS chromatogram of FAMEs produced from ex-situ transesterification of *Chlorella vulgaris*. FAME products represented on retention time of 9.788; 11.082; 11.671; 12.204; 12.596; 12.791; 13.183; 13.634 and 14.548 min.

The GC-MS chromatogram gave an unique retention time and ion fragmentation profile for every FAME. A chromatogram data corresponded to the retention time of 12.2 min showed fragmentation profile with the highest of molecular ions m/z at 270 (Figure 6 and 7). The fragment of m/z 270 might represent to the $C_{17}H_{34}O^{2+}$ ion for methyl hexadecanoic. Abdulloh *et al* [16]. described the m/z 74 fragment is derived from $C_3H_6O^{2+}$ ion yielded by the β-breakdown from $C_{17}H_{34}O^{2+}$ ion through McLafferty rearrangement [16]. The m/z 239 fragment represents the $C_{16}H_{31}O^{+}$ which a methoxy group was lost, whereas the m/z 43 fragment was produced by the release of a radical from $C_{12}H_{24}COOCH_3$ molecule. The molecular ion with m/z 87, 101, 115, 129, 143, 157, 171, 185, 199, 213 and 227 emerged due to the CH_2CH_2 fragmentation respectively, known as ion fragmentation pattern of the series $CnH_{2n-1}O^{2+}$. This similiar mechanism was also reported for methyl palmitate [22]. The result showed the FAME could be produced well by ex-situ transesterification of lipid extracted from *C. vulgaris*. Optimizing the condition for the reaction will be perfected in future to improve the biodiesel yield.

4 Conclusion

The *C. vulgaris* could grow well in the BG-11 medium and showed the doubling time at 3.7 days for its growth using inocula 16% (v/v). The microalgae growth resulted

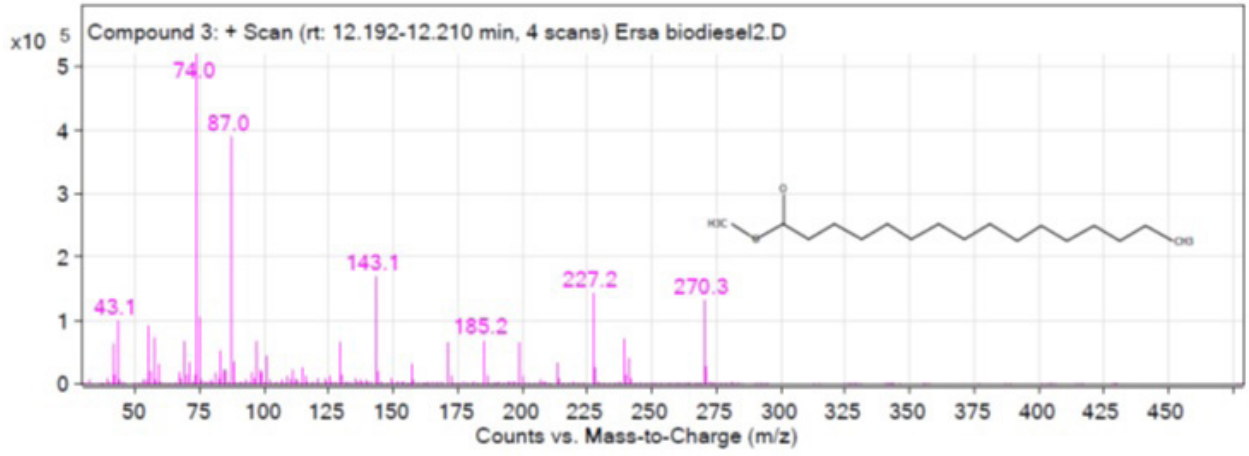

Figure 7: The MS spectrum of hexadecanoid methyl ester fragmentation.

dry cell biomass as 11,6 g/L after cultured for 6 days. The lipid as 31% (w/w) could be resulted after the biomass extracted by a mixed solvent of n-hexane-ethanol in ratio 1:1 and ultrasonication treatment at power 25 kHz/270W for 30 min. The lipid could be converted to FAME in the transesterification reaction with methanol using H_2SO_4 catalyst at 45ºC for 2 h, and appeared FAME with area 32.26% in GC-MS analysis that corresponded to FAME rendement as 13.68% (w/w). Fatty acid profiles analyzed by GC-MS showed the major peaks of fatty acids found in *Chlorella vulgaris* were palmitic acid (C16:0), stearic acid (C18:0) and margaric acid (C17:0), and nonadecanoic acid (C19:0). Optimization of the transesterification reaction will be developed in future to improve the FAME product.

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Conflict of interest: Authors state no conflict of interest.

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