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Synthesis of Methyl Ester from Microalgae *Chlorella* sp. TAD Using the In-Situ Transesterification Method

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ABSTRACT

Synthesis of methyl ester from *Chlorella* sp. TAD microalgae was carried out using the in-situ transesterification method. This study aims to determine the methyl ester composition of *Chlorella* sp. TAD microalgae using the in-situ transesterification method. The in-situ transesterification method is a modified method that allows extraction and transesterification into methyl ester products in one process simultaneously. The in-situ transesterification process lasted 8 hours, followed by a distillation process to remove the n-hexane content and an oven for 2 hours to evaporate the remaining water. The results of the analysis using GC-MS to determine the chemical content of the methyl ester compound from *Chlorella* sp. TAD, showed the methyl ester composition of 7,10-hexadecanoic methyl ester, 8,11,14-docosatrienoic methyl ester, 9-hexadecanoic methyl ester, hexadecanoic methyl ester, heptadecanoic methyl ester, 10-octadecenoic methyl ester, octadecenoic methyl ester, 9,12-octadecadienoic methyl ester, 10-octadecenoic methyl ester, 2-hexyl cyclopropaneoctanoic methyl ester, eicosanoic methyl ester, 10-heptadecen-8-ynoic acid methyl ester, nonahexacontanoic methyl ester, and tetracosanoic methyl ester.

***Keywords*:** *Chlorella* sp. TAD, in-situ transesterification, methyl ester, microalgae, synthesis.

1. INTRODUCTION

As a tropical country through which the equator passes, Indonesia has two seasons, making plants thrive because they get enough sunlight, an average of 12 hours per day. Approximately 67% of Indonesia's territory is sea, one of which is the Province of Maluku. Geographically, most of the area of Maluku is the sea. Only 10% is land (islands totaling around 1700 islands), especially Ambon City, which is located at position: 3^{o}–4^{o} South Latitude and 128^{o}–129^{o} East Longitude, which is divided into Inner Ambon Bay (TAD) and Outer Ambon Bay (TAL) [1]. Inner Ambon Bay waters can be found in various types of microalgae, such as microalgae in the class Bacilariophyceae, Flagilariophyceae, Coscinodiscophyceae, Dinophyceae, and Chlorophyceae [2].

Microalgae are photosynthetic microorganisms that do not have roots, stems or leaves that are capable of producing bioactive compounds such as astaxanthin, fucoxanthin, carbohydrates and lipids as an ingredient production of biofuels such as biodiesel and bioethanol [3-5]. Microalgae have a simple cell structure, high photosynthetic ability, and can survive in extreme environmental conditions [6].

The Chlorophyceae class belongs to a potential type of microalgae and is very intensively developed, one of which is *Chlorella* sp. The advantages of *Chlorella* sp. include multiplying quickly and easily in cultivation [4]. The adaptation period is relatively short and resistant to ideal environmental conditions for the growth of *Chlorella* sp., making it easy to culture. *Chlorella* sp. contains many lipids of 28–32% of its dry weight. A previous study by Saadudin et al. reported that *Chlorella* sp. had the highest lipid content of 9.8% compared to other species such as *Nannochloropsis* sp., *Spirulina* sp., *Dunaliella* sp., *Tetraselmis* sp.

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which has a lipid content of about 7% [7]. Widianingsih et al. reported that there were two main fatty acid contents in *Chlorella* sp., dominated by palmitic acid (C16:0) which reached 31%, and palmitoleic acid (C16:1) at 28%. [8] The fatty acid content of *Chlorella* sp. is very well converted as methyl ester [9,10]. In comparison, research conducted by Bandjar et al. reported that *Chlorella* sp. produced biodiesel containing methyl myristate (7.62%), methyl palmitoleate (36.37%), methyl palmitate (21.70%), and methyl oleate (11, 56%) [11].

Methyl ester is a raw material in making biodiesel or as an emollient in cosmetic products. At the same time, glycerol can be used as a raw material in various industrial applications such as cosmetics, soap, and pharmaceuticals. Methyl ester compounds can be used as additives in cosmetic formulations; one example is caprylic or caprylic triglyceride, which has been used in cosmetic formulations as an emollient. Therefore, it does not rule out the possibility that other methyl ester compounds can also be used as additional substances, either emollients or for different functions.

Most of the reported studies about methyl esters synthesis were using 2 main steps, starting from the lipids extraction and continuing with methyl esters synthesis by using the lipids esterification and transesterification.

In this study, the synthesis of methyl esters from *Chlorella* sp. TAD was carried out using the in-situ transesterification method. The in-situ transesterification method is a modified method that allows extraction and transesterification to produce methyl ester products to be processed simultaneously. This aims to shorten the time of the methyl ester production process and reduce synthesis costs [11].

2. RESEARCH METHODS

2.1. TOOLS AND MATERIALS

All the chemicals used in this research are pro-analysis grade (Merck, Germany): methanol, sulfuric acid, potassium hydroxide, and n-hexane. The equipment used includes glassware, analytical balance (Ohaus Adventurer^{TM} Pro), hot plate (Cimarec 2), autoclave (TOMY ES-215), refractometer, falcon tube, Eppendorf micropipette size 10-100 and 100-1000 µL, hemocytometer, Thermo Scientific S16 centrifuge, water bath, light microscope (Nikon YS-100), *Scanning Electron Microscopy* (SEM), *Rotary Vaccum Evaporator*, and UV-Vis spectrophotometer (Shimadzu UV-2450).

2.2. CULTIVATION OF *Chlorella* sp. TAD

Chlorella sp. TAD cell was grown in a modified medium [3]. The modification medium is a simple growth medium containing KNO_3, $NaH_2PO_4.2H_2O$, $FeCl_3$, Na_2EDTA, and $Na_2SiO_3.5H_2O$. Cultivation was carried out with an initial cell density of 5×10^5 cells mL^{-1} in a simple photobioreactor at room temperature under a light intensity of 67.5 mmol m^{-2} s^{-1} with photoperiod 12:12 hours (dark:light), salinity 28 ppt, pH 8.2–8.5 and aerated with free air bubbles. The simple photobioreactor used in this study was made of a transparent jar with a volume of 12 L. Cell growth in culture was measured by counting the number of cells (in units of cells mL^{-1}) using a Neubauer Haemocytometer under a light microscope.

2.3. HARVESTING OF BIOMASS *Chlorella* sp. TAD

Chlorella sp. TAD cell cultivated was harvested using sedimentation and filtration techniques using Masini cotton cloth (Industrial grades). Wet biomass *Chlorella* sp. TAD was weighed using an analytical balance to get the wet biomass weight.

2.4. CELL BREAKDOWN PROCESS OF *Chlorella* sp. TAD

Wet biomass was frozen in the refrigerator for 72 hours, then heated in boiling water for 20 minutes and cooled in the refrigerator for 20 minutes. Then, it was dried using an oven at 60°C for 48 hours to obtain dry biomass.

The dry biomass of *Chlorella* sp. TAD cells was pulverized for 30 minutes to become powdered microalgae.

2.5. SYNTHESIS OF METHYL ESTERS FROM *Chlorella* sp. TAD BY IN-SITU TRANSESTERIFICATION METHOD

15 g of biomass powder was weighed and put into a three-neck flask, then 97.8 mL of methanol solution and 2.2 mL of H_2SO_4 were added. The reaction mixture was then refluxed at 65°C for 8 hours. The mixture from in-situ transesterification was filtered to separate the residue from the filtrate. The residue was then washed with 50 mL of n-hexane solvent to recover the remaining oil in the residue. Then, the separation was did in a flask with 50 mL of n-hexane solvent to separate the oil from the glycerol. The separation process in the separating flask was formed two layers, namely the upper layer (a mixture of FAME, hexane, remaining triglycerides, and free fatty acids (FFA)) and the lower layer (a combination of methanol, water, and glycerol). The top layer resulting from the transesterification process was then washed with distilled water to repeatedly dissolve the glycerol and methanol residues until the bottom layer is clear. FAME layers were collected and evaporated at 45°C until constant weight for analysis. The results of the in-situ transesterification were then carried out to determine the fatty acid content using GC-MS.

3. RESULTS AND DISCUSSION

Chlorella sp. TAD cells were cultivated using a modified medium [3], with the initial cells being 5×10^5 cells mL^{-1} using a light intensity of 5000 lux. The growth of microalgae is characterized by an increase in the number of cells in the culture (Figure 1a). The change in culture color shows that the density of *Chlorella* sp. TAD cells has increased, and the growth is going well. Figure 1b shows the image of *Chlorella* sp. TAD cells which are round and green in color, under a light microscope with a magnification of 400 times. The green color in the *Chlorella* sp. TAD culture indicates the dominance of the chlorophyll pigment in the cell cytoplasm.

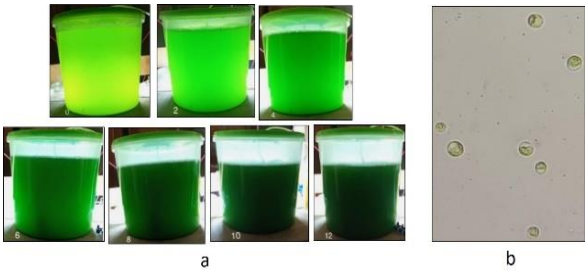

Figure 1. Cultivation of *Chlorella* sp. TAD on a laboratory scale: (a) change in color of culture (days 2, 4, 6, 8, 10, and 12), (b) the observation of cells under a light microscope.

The harvesting of *Chlorella* sp. TAD is carried out on the 12th day, which is included in the exponential phase with growth that tends to be stable [4,12]. The culture was harvested using sedimentation and filtration techniques (Figure 2a-b). The sedimentation technique was carried out for 24 hours, and then the biomass was separated by filtration for ±15 minutes. In this method, the biomass obtained is a thick green paste containing water. The wet biomass weight obtained was 121.815 g, with a biomass productivity of 0.920 g L^{-1} h^{-1} (Figure 2c).

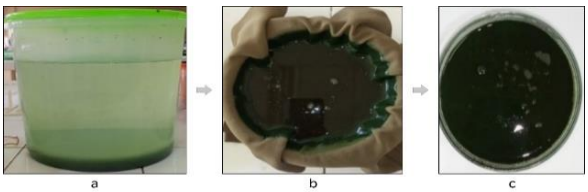

Figure 2. The process of harvesting *Chlorella* sp. TAD biomass: (a) Sedimentation of biomass, (b) Filtration of Biomass with a cotton cloth, (c) Wet biomass.

Chlorella sp. TAD has a cell wall composed of thick glucosamine [13-15], so it is expected to obtain more significant amount of lipid by breaking down the cell wall. In this study, two methods of breaking the cell wall

were used. The first method is the cold-hot method. This method aims to make the *Chlorella* sp. TAD cell wall stiff. The second method is a mechanical method that aims to break down the cell wall of *Chlorella* sp. TAD. The cold-hot method involves temperature, which is an important limiting factor for the life of organisms because each organism has a limited ability to tolerate temperature changes that occur. The cells observation from the hot-cold method under a light microscope showed that the surface of *Chlorella* sp. TAD cells looked different compared to normal cells (Figure 3a); this indicated that the cell walls became stiff due to temperature changes (Figure 3b). Then, the process of splitting cells with a second method through a mechanical method. Cell division results at this stage showed cell morphology with irregular shapes (Figure 3c) compared to normal cells (Figure 3a). The morphology of *Chlorella* sp. TAD cells from the breakdown results show that the cell wall was successfully broken.

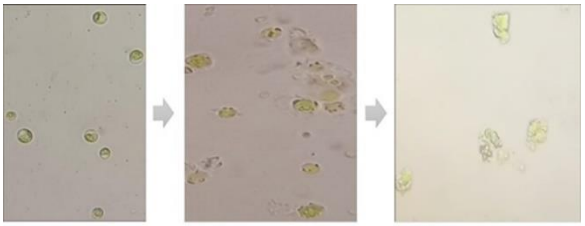

Figure 3. Cell morphology of *Chlorella* sp. TAD as a result of a breakdown: (a) normal cells, (b) cells after the cold-hot method process, (c). cells after the mechanical method process.

Cell splitting using both methods was declared successful based on the results obtained from the lipid extraction process. Extraction of lipids from *Chlorella* sp. TAD was carried out using the Bligh Dryer method with methanol:chloroform (1:1 v/v) solvent. The extraction results obtained a lipid content of 45%. Lipid yields obtained through cell breakdown using the maceration method carried out by Widyastuti and Dewi amounted to 15.775% [9], Bandjar et al. obtained 3.12% [11] and Kurnia et al. obtained 23.5% [16]. These results indicate that cell breaking using the two cell-breaking methods in this study has a high potential to increase the yield of the lipid extract obtained.

The synthesis of methyl esters in this study used the in-situ transesterification method. In this in-situ transesterification process, biomass (obtained from the previous cell breakdown process), methanol and acid catalyst (H_2SO_4) were used. The in-situ transesterification process used the reflux method at 65°C for 82 hours.

The mixture from in-situ transesterification was filtered to separate the residue from the filtrate. The residue was then washed with n-hexane solvent to recover the remaining oil. The separation process using the separating flask formed two layers, namely the upper layer (a mixture of FAME, hexane, remaining triglycerides, and free fatty acids (FFA)) and the lower layer (a combination of methanol, water, and glycerol) (Figure 4). The top layer resulting from the transesterification process was then washed with distilled water to repeatedly dissolve the glycerol and methanol residues until the bottom layer was clear. FAME layers were collected and evaporated at 45°C until constant weight for analysis.

Figure 4. Results of transesterification in-situ of the *Chlorella* sp. TAD.

The results of in-situ transesterification were analyzed using GC-MS to determine the chemical content of the methyl ester

compound from *Chlorella* sp. TAD. GC-MS analysis showed that 97 peaks stated 97 compounds in the methyl ester microalgae *Chlorella* sp. TAD, But only 23 peaks were detected as fatty acid methyl esters (Figure 5). The twenty-three fatty acid methyl ester compounds analyzed from the oil of the microalgae *Chlorella* sp. TAD were presented in Table 1. The dominant methyl ester in *Chlorella* sp. TAD are hexadecanoic acid methyl ester (palmitic acid), 7,10-hexadecanoic acid methyl ester, 8,11,14-docosatrienoic acid methyl ester, and 9-hexadecanoic acid methyl ester (palmitoleic acid).

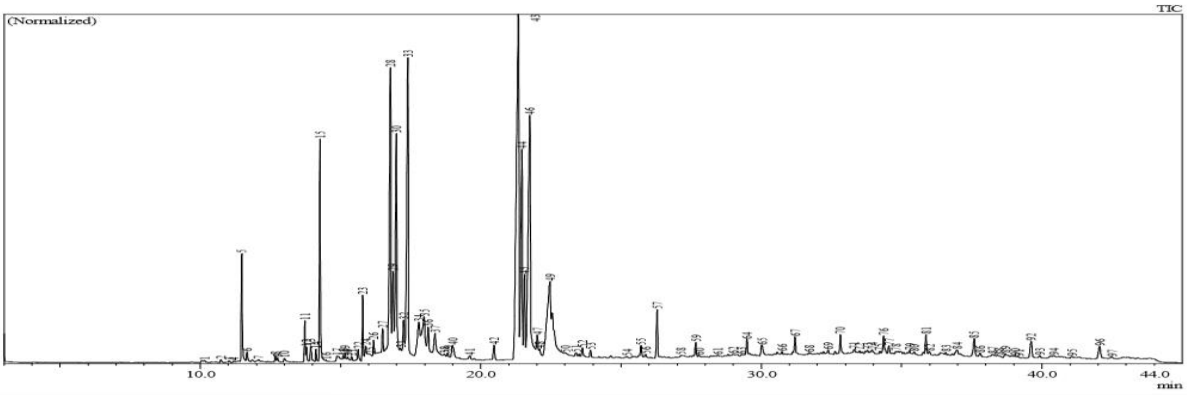

Figure 5. Methyl ester chromatogram from the in-situ transesterification process of *Chlorella* sp. TAD.

Table 1. Fatty acid methyl ester components from *Chlorella* sp. TAD.

Peak No.	Retention time (minutes)	Concentration (%)	Molecular Formula	Compound Name
18	15.092	0.11	$C_{16}H_{32}O_2$	Pentadecanoic acid methyl ester
21	15.393	0.11	$C_{19}H_{36}O_2$	9-Octadenoic acid methyl ester
22	15.626	0.30	$C_{16}H_{32}O_2$	Pentadecanoic acid methyl ester
28	16.779	7.89	$C_{17}H_{30}O_2$	7,10-Hexadecadienoic acid methyl ester
29	16.868	2.18	$C_{23}H_{40}O_2$	8,11,14-docosatrienoic acid methyl ester
30	16.987	5.52	$C_{17}H_{32}O_2$	9-hexadenoic acid methyl ester
31	17.137	0.16	$C_{17}H_{32}O_2$	9-hexadenoic acid methyl ester
32	17.247	0.91	$C_{19}H_{36}O_2$	9-Octadenoic acid methyl ester
33	17.399	8.21	$C_{17}H_{32}O_2$	Hexadecanoic acid methyl ester
34	17.791	1.57	$C_{17}H_{32}O_2$	7,10-Hexadecadienoic acid methyl ester
40	18.991	0.51	$C_{17}H_{30}O_2$	Hexadecadienoic acid methyl ester
41	19.608	0.11	$C_{18}H_{36}O_2$	Heptadecanoic acid methyl ester
44	21.463	5.86	$C_{23}H_{40}O_2$	8,11,14-docosatrienoic acid methyl ester
45	21.557	1.90	$C_{19}H_{36}O_2$	10-Octadenoic acid methyl ester
47	22.012	0.72	$C_{19}H_{38}O_2$	Octadecanoic acid methyl ester
51	23.396	0.05	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z) methyl ester
53	23.912	0.21	$C_{20}H_{38}O_2$	10-nonadecenoic acid methyl ester
56	25.875	0.11	$C_{18}H_{34}O_2$	2-hexyl cyclopropaneoctanoic acid methyl ester
57	26.286	1.06	$C_{21}H_{42}O_2$	Eicosanoic acid methyl ester
61	28.445	0.16	$C_{18}H_{30}O_2$	10-heptadecen-8-ynoic acid methyl ester
65	30.019	0.38	$C_{31}H_{62}O_2$	Triacontanoic acid methyl ester
68	31.704	0.08	$C_{70}H_{140}O_2$	Nonahexacontanoic acid methyl ester
71	33.313	0.28	$C_{25}H_{50}O_2$	Tetracosanoic acid methyl ester

4. CONCLUSION

Methyl ester synthesis using the in-situ transesterification method of *Chlorella* sp. TAD can be produced through 2 cell breakdown methods, increasing the lipid extract yield by 48%. The methyl ester composition obtained consisted of 7,10-hexadecanoic methyl ester, 8,11,14-docosatrienoic methyl ester, 9-hexadecanoic methyl ester, hexadecanoic methyl ester, heptadecanoic methyl ester, 10-octadecenoic methyl ester, octadecenoic methyl ester, 9,12-octadecadienoic methyl ester, 10-octadecenoic methyl ester, 2-hexyl cyclopropaneoctanoic methyl ester, eicosanoic methyl ester, 10-heptadecen-8-ynoic acid methyl ester, nonahexacontanoic methyl ester, and tetracosanoic methyl ester.

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