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Direct Extraction and Conversion of Microalgal Lipid from *Chlorella vulgaris* to Biodiesel Through In Situ Process Assisted by Sonication

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ABSTRACT. Direct extraction and conversion of microalgal lipid to biodiesel in one step process is developed to initiate an efficient method in biofuel production. The method that is called as in-situ process has been applied in the biodiesel production from microalgae *Chlorella vulgaris* lipid by involving sonication assisting. Various parameters that affect the in-situ transesterification were investigated to find the optimum conditions including power of sonication, co-solvent use, and biomass amount. The experiment was performed by acidic catalyst H_2SO_4. The use of sonication in 25kHz/270W yielded the highest biodiesel product. The apply of co-solvent *n*-hexane significantly increased the yield biodiesel rather than no co-solvent. The biodiesel yield of 10.39 % obtained when the process was accompanied by the *n*-hexane as co-solvent with increasing level as 47% rather than without co-solvent. The in situ process in the ratio biomass - methanol 3:50 that assisted by sonication for 60 mins produced the highest biodiesel, which was 20.31 % w/w. The yield was higher than assisting with reflux or a combination of reflux-sonication. The component of biodiesel yielded in the process consisting of 7,10,13-hexadecatrienoic methyl ester; pentadecanoic acid, 14-methyl-methyl ester; 9,12,15-octadecatrienoic acid-methyl ester; and eicosanoic acid-methyl ester. The combine of reflux-sonication exhibited a low biodiesel product. The disadvantage method from the combination may inhibit on lipid extraction or FAME transformation, the exact cause needs to be looked for in the future.

Keywords : Biodiesel, in-situ transesterification, microalgae *Chlorella vulgaris*, sonication.

INTRODUCTION

In the modern era, the need for energy is increasing from year to year, but its availability continues to decrease. Most of the energy consumed is from fossil fuels. However, burning fossil fuels cause air pollution, contrary for the biodiesel that its burning does not produce NOx and SO_2 gases in the air (Shoyeb et al., 2023). Therefore, alternative energy to meet the need for fuel and at the same time to reduce pollution is the use of biodiesel.

Biodiesel is a biofuel that comes from natural sources such as algae, soybeans, jatropha, corn, oil palm, and jojoba (Boocock et al., 1998). It constitutes as an efficient alternative fuel because it can reduce gas emissions and increase energy security (Mona et al 2020; Mostofa et al, 2021). In addition, it is also known as a friendly fuel (green fuel), non-toxic, no sulfure content, a biodegradable fuel, and having a higher viscosity (Alice et al., 2023; Shoyeb et al., 2023). One of the potential raw materials for producing biodiesel is microalgae (Teku et al., 2023; Thanigaivel et al., 2023; Imane et al., 2023). According to Chisti (2007), microalgae produce different complex lipids, hydrocarbons and oils depending on the species. Of the many types of microalgae, Chlorella vulgaris has a lipid content of 56% which has hight potential to be used for biodiesel production (Purkan et al., 2020; Purkan et al 2021, Saldivar et al., 2014).

There are two methods of biodiesel production from microalgae, conventional transesterification and direct transesterification (Milano et al., 2016). The direct transesterification method is also known as in-situ transesterification, which is an oil extraction process and the transesterification reaction is carried out simultaneously (Park et al., 2015, Purkan et al., 2022). Some papers have reported several methods to increase the efficiency of the in-situ transesterification process by using ultrasonication, microwave irradiation, supercritical conditions and the use of co-solvents to minimize mass transfer resistance and improve the biodiesel synthesis process (Kalsum et al., 2017; Lerin et al., 2011; Xu and Li., 2011). The transesterification reaction using the sonication method is a more efficient method because the biodiesel produced is 98-99% and can save energy because the reaction time is faster, which is approximately 5 minutes compared to the reaction time using the conventional method, which is 1 hour. The reflux method is generally used for in-situ transesterification reactions. In the in-situ transesterification reaction, microalgae biomass is reacted directly with a catalyst and solvent without lipid extraction (Purkan et el, 2022; Ningsih et al., 2022; Xu and Li., 2011). Thus, it takes high pressure and energy to break the cell walls so that the lipids can be extracted out and react to form fatty acid methyl ester (FAME). The reflux method is a mechanical stirring process assisted by heating which will help the transesterification reaction occur but it is slower when compared to using the ultrasonication method.

In recent years, the use of co-solvents is a method that can be used to increase the yield of direct (in-situ) transesterification reactions (Zhang et al., 2016). According to Cao et al. (2013), *n*-hexane solvent will help to easily extract lipids from microalgae. The *n*-hexane solvent in the reaction functions to remove (withdraw) biodiesel from the equilibrium system of the biodiesel synthesis reaction so that the equilibrium of the biodiesel synthesis reaction shifts to the right and the amount of biodiesel produced increases (Li et al., 2011). Another factor that affects the amount of biodiesel in the in-situ transesterification reaction is the weight of the biomass used. In the study of Aulakh et al. (2013), the weight of the biomass has an effect on the ethyl ester produced. If the percentage of biomass is higher, it will produce a lot of ethyl ester yield, but the yield will decrease if the percentage of biomass increases from the optimum amount. The effects of using co-solvents and biomass ratios in the in situ biodiesel production process have not been widely reported until now, even though it is important to reveal this.

This paper reports the synthesis of biodiesel through in-situ transesterification of lipids from the microalgae Chlorella vulgaris whose extract is assisted by sonication. Lipid extraction was carried out using the Bligh and Dryer solvent extraction method, namely the ratio of methanol to chloroform (2:1). The lipid content obtained was used to determine the biodiesel yield (%) in further studies. In the in-situ synthesis of biodiesel, various parameters were optimized including sonication power, use of co-solvents and the effect of total weight of biomass. A comparison of the in-situ transesterification reaction using three different methods, namely the sonication method, the reflux method, the combination of sonication and reflux methods on the effect of the total weight of biomass has been carried out. The biodiesel samples were analyzed using GC-MS to characterize the FAME composition obtained from the in-situ transesterification reaction and determine the biodiesel conversion (%) and biodiesel yield (%).

EXPERIMENTAL SECTION

Material and Instruments

The research materials consisted in Chlorella vulgaris microalgae from Balai Perikanan Budidaya Air Payau (BPBAP), Situbondo, East Java-Indonesia, methanol, *n*-hexane, distilled water, concentrated sulfuric acid, and methyl heptadecanoate solution. Then for the instruments included reflux, neck flask, hot plate, magnetic stirrer, thermometer, set of rotary vacuum evaporator (Buchi Rotavapor® R-300), separatory funnel, GC-MS instrument (Agilent 5977), stative, clamps, coarse filter paper, JY92-IIDN batch ultrasonicator.

Lipid extraction from the microalgae Chlorella vulgaris

A total of 1 g of microalgae biomass was put into a round bottom flask, added by 5 mL of methanol and 2.5 mL of chloroform with a ratio (2:1) and 1 mL of distilled water. The mixture was refluxed while stirring with a magnetic stirrer at 7 rpm for 2 hours at ± 60 °C. At t 40 minutes, 2.5 mL of chloroform was added. After the reaction completed, the mixture was allowed to stand for 30 minutes at at 30°C. After cooling, the mixture was centrifuged at 8000 x g for 20 minutes. The filtrate containing chloroform was taken, then evaporated using a rotary vacuum evaporator at 50 °C at 30 rpm. The solution containing lipid was put into a glass vial and then put into a desiccator and left overnight. The glass vial containing the pure lipid was weighed, and lipid weight was recorded. Lipid levels in Chlorella vulgaris microalgae (% lipid content) can be calculated by formula 1 (Purkan et al., 2019).

$$\text{Lipid yield (\%)} = \frac{\text{Lipid } weight \text{ (g)}}{\text{Biomass weight of microalgae (g)}} \text{ x } 100 \quad (1)$$

Optimization of sonication power in the in-situ biodiesel synthesis

The in-situ transesterification reaction by sonication was carried out by adding 1 g of dry microalgae biomass to a beaker glass. As much as 50 mL of methanol and 2.5 mL of concentrated sulfuric acid were added with a ratio of biomass to methanol volume of 1:50 (w/v) and the concentration of sulfuric acid used was 5% of methanol volume (v/v). The tip of the vibrating horn of the batch ultrasonicator is inserted into the solution. The beaker glass is covered with aluminum foil to minimize solvent loss due to the evaporation process. The sonication power used is a sonic power of 25kHz/90W; 25kHz/270W and 25kHz/450W. The maximum power of the sonicator is 900 W with a frequency of 20 - 25 kHz. The reaction time is 30 minutes with 1 pause (30 seconds) every 1 minute of sonication. Then, the mixture was refluxed with stirring at 5 rpm for 3 hours at 60 °C.

After cooling, the filtrate was separated from microalgal debris by centrifugation at speed 5000 g for 20 minutes The filtrate was then evaporated at 60℃ using roraty evaporator at 90 rpm. As much as 10 mL of *n*-hexane was added to the filtrate and centrifuged at speed 8000g for 20 minutes. The mixture containing the two phases was shaken for 20 minutes and then separated using a separatory funnel. The bottom layer was discarded while the top layer was washed with 10 mL of hot water. The organic phase is added with anhydrous sodium sulfate to minimize the water content in the organic phase. The organic phase was evaporated using a rotary vacuum evaporator at 40°C with a speed of 30 rpm. The solution containing FAME (biodiesel) was put into a glass vial, then analyzed by GC-MS.

Determination of co-solvent effect in the in-situ biodiesel synthesis

On the parameters of the using of co-solvent effect, the co-solvent solution is added with a ratio of methanol to co-solvent, namely methanol : *n*-hexane (3:1). On the same occasion, a control esterification reaction was carried out containing only methanol solvent. The biodiesel product formed was determined by GC-MS

Optimization of ratio biomass to methanol on biodiesel synthesis at various methods of sonication, reflux and both combinations.

The microalgae biomass was mixed with methanol at ratio 1:50; 2:50; 3:50 (w/v), then added by 2.5 mL of sulfuric acid respectively. The concentration of sulfuric acid used was 5% of methanol (v/v). The sonication was run for 60 minutes, while for reflux method was carried out for 3 hours. While for combinantions of sonication and reflux method, the process was set for 30 minutes for sonication, and then refluxed for 3 hours.

Characterization of biodiesel from Chlorella vulgaris microalgae by GC-MS

The biodiesel compounds (methyl esters) were analyzed by GC-MS. A total of 50 mg of standard methyl heptadecanoate was dissolved in 5 mL of *n*-hexane to make a standard solution of methyl heptadecanoate (10mg/mL). The synthesized biodiesel sample was dissolved in 5 mL of *n*-hexane. Then 100 µL of standard solution was added to 1 mL of biodiesel sample solution. From this analysis, it will be known the content of FAME compounds and the conversion of oil into biodiesel in the form of percentages. The conversion of biodiesel produced from the transesterification process can be determined by equation 2.

$$\text{Biodiesel conversion (\%)} = \frac{\sum A - A_{is}}{A_{is}} \times \frac{M_{standard}}{M_{sample}} \times 100\% \qquad (2)$$

$\sum A$ is the total area of the methyl ester peak ($C_{14:0} - C_{24:1}$), A_{is} is the peak area of the internal standard solution (methyl heptadecanoate), $M_{standart}$ is standard weight (g), M_{sampel} is the sample weight (g).

Determination of biodiesel yield from Chlorella vulgaris microalgae (%)

The percentage of biodiesel produced (% biodiesel yield) can be calculated by formula 3.

$$\text{Biodiesel } Yield\ (\%) = \frac{\text{FAME } weight\ \text{(g)}}{\text{Biomass weight of microalgae (g)}} \times 100\% \qquad (3)$$

RESULTS AND DISCUSSION

Lipid yield from microalgal biomass of Chlorella vulgaris

Lipid was extracted by reflux method using the mix solvents of methanol-chloroform in ratio 2:1. For the microalgal biomass of 1,0016 g could be obtained the lipid as 0.1925 g with a lipid content as 19.22 % w/w. The various percentage of lipid contents have reported between 17-50% according to the extraction method and microalgal strains used ((Araujo et al., 2013; Cao et al., 2013; Li et al., 2011). The chloroform that presented in the solvent has performance as a semi-polar solvent with in tends to be a non-polar character. It used to disrupt the hydrophobic interaction between non-polar and neutral lipids. In turn the lipid that wrapped as a single layer of phospholipids has performance as polar (Purkan et al., 2012). To break the phospholipid layer and release non-polar lipids, a high polarity solvent is needed (Zhang et al., 2016). Methanol as a polar solvent will help extract lipids more quickly. Methanol used to break the phospholipid layer while chloroform used to dissolve the lipids (Purkan et al., 2019).

Optimization of sonication power in the process of forming biodiesel in-situ

In this study, an acid catalyst was used due to the high content of FFA (free fatty acids) in microalgae lipids (Kim et al., 2014). The mixture of biomass, methanol and sulfuric acid was treated with an ultrasonicator for 30 minutes with 1 pause (30 seconds) every 1 minute of sonication. The sonication breaks down the cell wall so that it mixes perfectly with the solvent, the FAME that is formed, and the residue of the microalgal cells forms an emulsion (Zhang et al., 2016, Purkan et al., 2019). The mixture then was centrifuged and filtered to harvest the green filtrate that contain FAME. It was obtained by *n*-hexane and hot water. The water was used to prevent the precipitation of saturated fatty acid esters and slowed down the formation of emulsions (Demirbas & Kara., 2016). The biodiesel yield obtained was analyzed using GC-MS (Gas Chromatography-Mass Spectrophotometry).

The sonication power at 25kHz/270W produced the highest percentage in biodiesel conversion and biodiesel yield, which were 7.07% w/w and 1.32% w/w respectively. The power at 25kHz/90Wresulted as 6.89% w/w and 1.36% w/w respectively for biodiesel conversion and yield, whereas at power of 25kHz/450W obtained low rendemen of biodiesel conversion and yield, which were 0.92% w/w and 0.18% w/w respectively (**Figure 1** and **2**).

In general,it is assumed that the higher the sonication power input, the faster the transesterification reaction will result in a high biodiesel conversion (Araujo el al., 2013; Martinez-Guerra & Gude, 2015). However, in reactions using ultrasonic waves, this is true for a certain level of sonication power. This can be explained that the higher the amount of ultrasonic power given to the reaction mixture, the greater the number of ultrasonic cavitation bubbles formed in the solution which increases the surface area of the interface between the lipid and methanol. The cavitation bubbles that are formed also accelerate the process of cell wall lysis (disruption) of microalgae cells thereby increasing the extraction of lipids from cells (Park et al., 2015). This can directly accelerate the in-situ transesterification reaction of the Chlorella vulgaris microalgae. However, if the input sonication power is too high, this can trigger the formation of excess bubbles which will combine with each other to form larger and more stable cavitation bubbles which can become a barrier to the process of transferring acoustic energy. This phenomenon is known as the decoupling effect (Martinez-Guerra et al., 2015). Martinez-Guerra et al. (2015) reported that if the sonication power is given from 4.0 W/mL, the biodiesel yield is 90% and then increases to 94% if it is given a power of 5.0 W/mL. However, the biodiesel yield drops drastically to 83% if the sonication power intensity is increased to 11 W/mL (Martinez-Guerra et al., 2015).

Effect of co-solvent in in-situ biodiesel synthesis

The study was conducted using co-solvent *n*-hexane with a co-solvent ratio of methanol:*n*-hexane (3:1) and without using a co-solvent. The use of co-solvent *n*-hexane resulted in the highest percentage of biodiesel conversion and yield, which were 10.39% w/w and 2.00% w/w respectively Where as without co-solvent only produced as 7.07% w/w and 1.36% w/w of biodiesel conversion and biodiesel yield respectively.The results showed that there was an increase in the formation of biodiesel by 1.4 times when *n*-hexane was used as a co-solvent in the in-situ transesterification reaction. From **Figures 3** and **4**, there were shown that *n*-hexane which acts as a co-solvent produces high biodiesel yields.

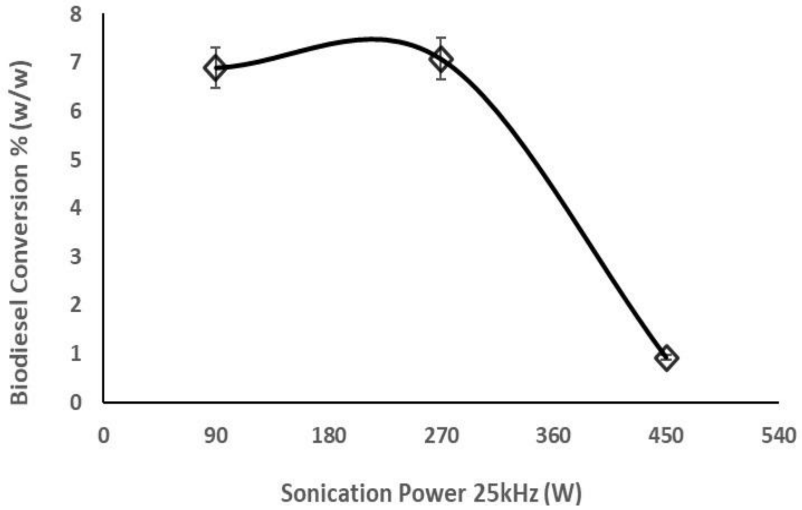

Figure 1. Effect of sonication power on biodiesel conversion (% w/w) from in-situ transesterification reaction

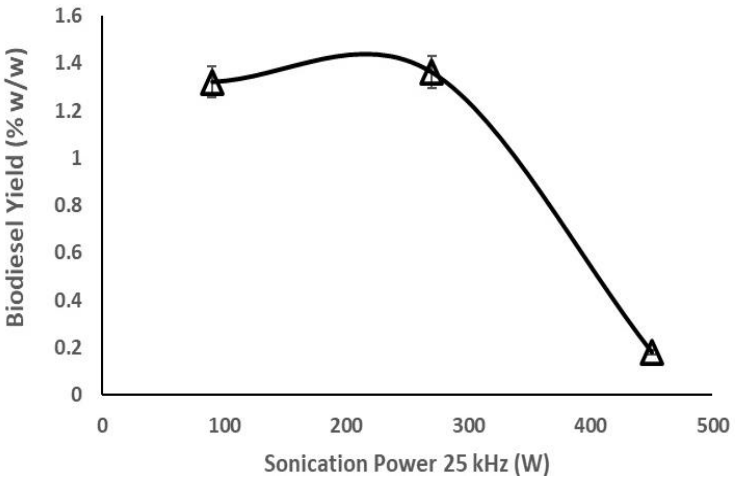

Figure 2. Effect of sonication power on biodiesel yield (% w/w) from the in-situ transesterification reaction

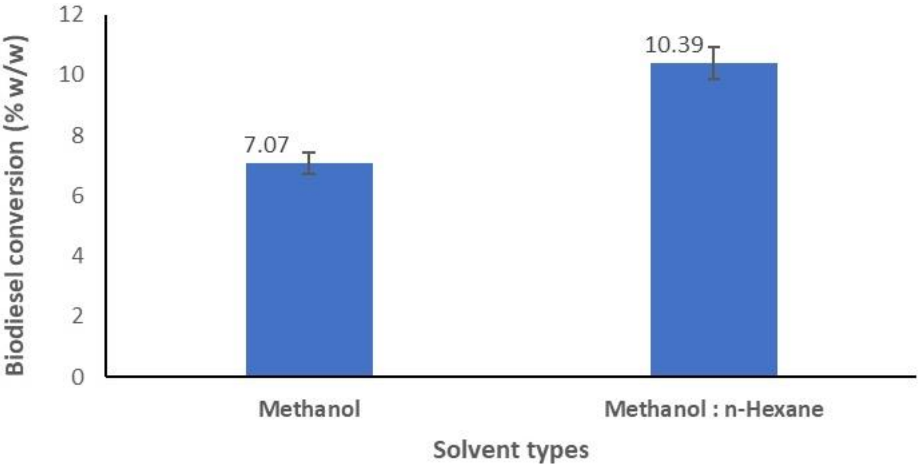

Figure 3. Effect of solvent type on biodiesel conversion (% w/w) from in-situ transesterification reaction

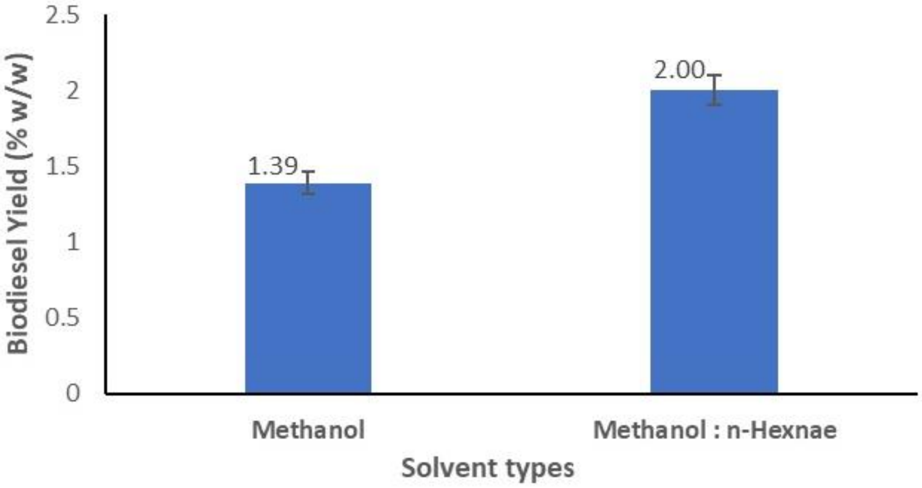

Figure 4. Effect of solvent type on biodiesel yield (% w/w) from in-situ transesterification reaction

The *n*-hexane solvent produced the percentage of biodiesel conversion and biodiesel yield higher than the mixture of methanol:*n*-hexane. This is because the polarity of the *n*-hexane solvent, which tends to be non-polar, facilitates contact between methanol and lipids, which are generally non-polar. Thus, the lipid extraction process will occur more quickly and easily (Cao et al., 2013). *n*-hexane is not only capable of dissolving long chain triglycerides but also miscible with methanol in a homogeneous catalyst system (Zhang et al., 2016). From the research results of Zhang et al., (2016) was reported that if *n*-hexane is used as a co-solvent with a ratio of 1:3 (v/v) to ethanol, it yielded of 87.75% compared to other co-solvents such as petroleum ether, acetone, and diethyl ether.

Effect of total weight of biomass using the sonication method, the reflux method and the combination of sonication and reflux methods

The research was carried out by varying the weight of biomass to volume of methanol in ratio w/v (1:50; 2:50; 3:50) with a constant volume of methanol, namely 50 mL. The in situ trans-esterification process assisted by sonication using a mixture of 3:50 (w/v) between biomass and methanol produced the largest percent conversion and yield of biodiesel compared to the method assisted by reflux or a combination of reflux and sonication. The process assisted by sonication produces 20.31% w/w and 3.87% w/w for biodiesel conversion value and yield value. A mixture of biomass and methanol 3:50 (w/v) gave the highest biodiesel yield compared to the other two mixtures, 1:50 and 2:50 (w/v) (**Figure 5** and **6**).

The ratio of the total weight of biomass to the volume of methanol using the sonication method of 3:50 resulting highest percentage of biodiesel conversion and biodiesel yield. The same trend can be seen using the reflux method and the combination of sonication and reflux methods, namely the ratio of biomass weight to methanol volume of 3:50 (w/v) produces the highest percentage of biodiesel conversion and biodiesel yield. From the experimental results it can be seen that the total weight of the biomass can affect the biodiesel conversion and biodiesel yield. If more biomass (substrate) reacts with methanol (reactant) in a certain amount, it will produce more FAME. In this experiment, a constant volume of methanol solvent was used, namely 50 mL.

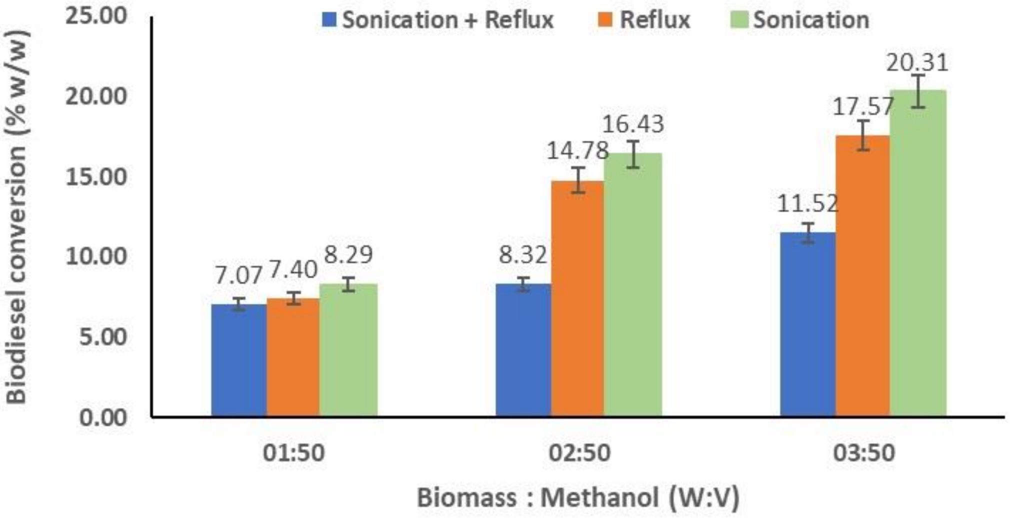

Figure 5. Effect of total weight of biomass depending on the type of method used on biodiesel conversion (%) from the in-situ transesterification reaction

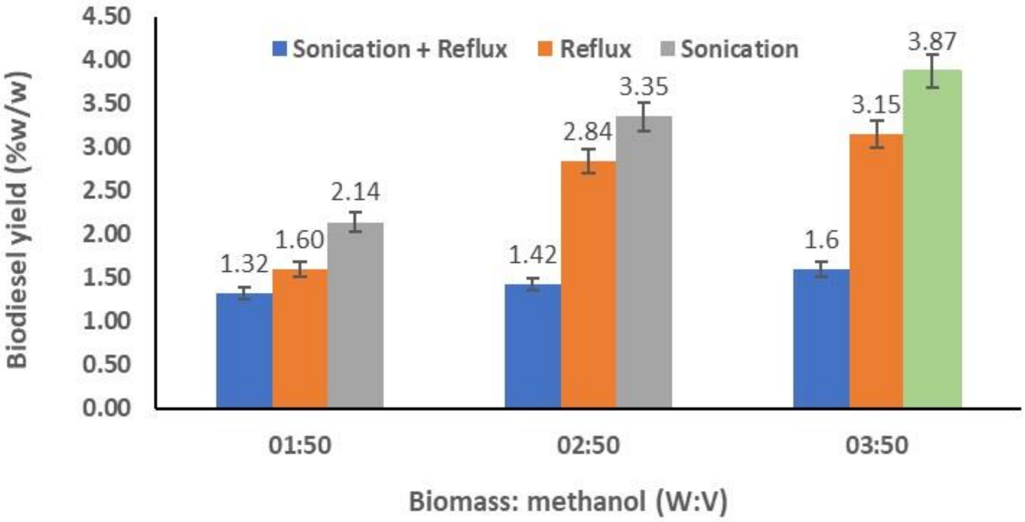

Figure 6. Effect of total weight of biomass depending on the type of method used on biodiesel yield (% w/w) from the in-situ transesterification reaction

At this volume of methanol, a lot of biodiesel conversion can be produced because excess alcohol is needed so that lipids are fully converted to FAME (Tang et al., 2016). The transesterification process occurs when lipids come into contact with methanol. Lipids are products of intercellular microorganisms, so the process of extracting them is more difficult in in-situ transesterification reactions where there is a cell wall that separates the lipids from the methanol. Thus, it is necessary to add more methanol as a solvent to weaken or disrupt it and then penetrate the cell wall and as a reactant to produce FAME (Zhang et al., 2016).

The sonication method can produce biodiesel conversion and high yield biodiesel compared to using conventional methods, the reflux and the combination of sonication and reflux methods. The sonication method produces a high FAME conversion in a short time due to the principle of the sonication method, namely the formation of many cavitation bubbles and microscopic emulsion particles in the reaction system. This causes an increase in the interfacial area which will accelerate the reaction rate (Guo et al., 2017). By using sonication, cells can be completely lysed so that lipids can be extracted and then react with solvents and catalysts to form FAME. The reaction time is also short so this can have a positive impact on saving reaction time and energy required. The conventional method, namely reflux, produces lower biodiesel conversion than using the sonication method. This is because mechanical stirring produces lower energy so that cells cannot be completely lysed and only part of it is extracted. The process of lipid diffusion out of the cell membrane becomes slower (Araujo et al., 2012, Purkan et al., 2017). This causes the transesterification reaction to produce FAME cannot run more optimally and efficiently resulting in lower biodiesel conversion and biodiesel yield. By using the reflux method, the reaction time is longer and the required reaction temperature must be higher to produce maximum biodiesel conversion and biodiesel yield. The combination method of sonication and reflux results in a lower biodiesel conversion than using the sonication or reflux method. In this study, the in-situ transesterification reaction was initiated with the sonication stage as a pretreatment which aims to completely disrupt or lyse the cells so that the lipids are fully extracted and will react with the acid catalyst and methanol solvent. So, the transesterification reaction has occurred at this stage with a reaction time of 30 minutes. Then proceed with the reflux stage for 3 hours at 60 °C. During the reflux process, the transesterification reaction continues from the sonication stage. However, the biodiesel conversion is lower. It can be assumed that the longer reaction time and reaction temperature have an effect on the FAME formation process. The transesterification reaction is a reversible reaction (Teku et al., 2023), so if there has been maximum formation for the FAME and glycerol in non-optimum condition, it is possible to shift the reaction equilibrium to the left so that less on the FAME and glycerol products. Therefore in this study, the combination of sonication and reflux methods resulted in a lower biodiesel conversion.

The Component of biodiesel product

The FAME yielded in several method of transesterification process via sonication, reflux and a combination of both methods were detected by GC-MS chromatography. The chromatogram display of FAME is shown in Figure 7. The FAME obtained from sonication-assisted esterification was higher than those assisted by reflux or a combination of reflux-sonication. The dominant biodiesel products consisted of 7,10,13-hexadecatrienoic methyl ester; Pentadecanoic acid, 14-methyl-methyl ester; 9,12,15-Octadecatrienoic acid-methyl ester; and Eicosanoic acid-methyl ester (Table 1). Joining of reflux-sonication exhibited a low biodiesel product. Disadvantage method from the combination may inhibit on lipid extraction or FAME transformation, the exact cause needs to be looked for in the future.

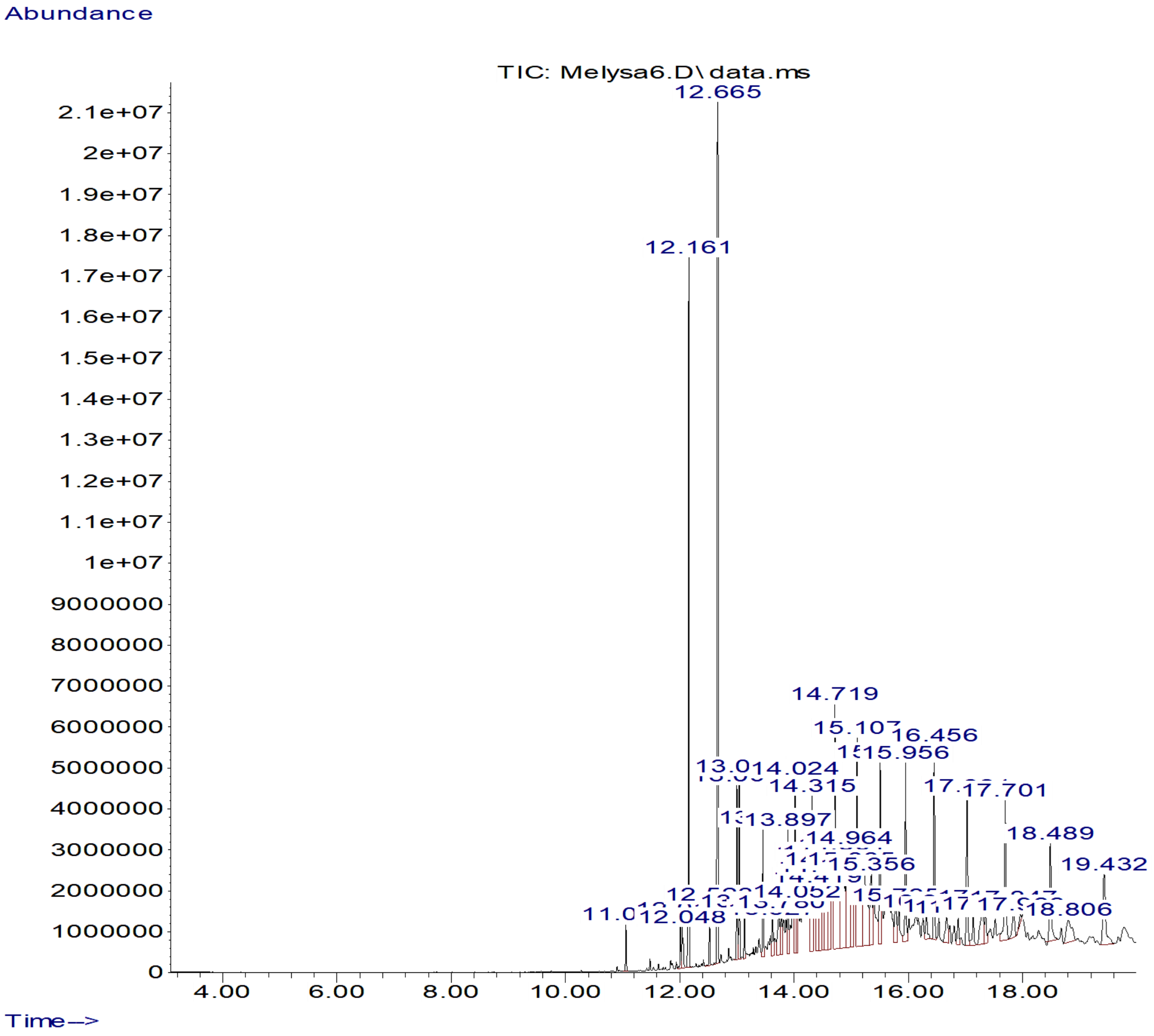

Figure 7. Chromatogram of biodiesel product detected by GC-MS

Table 1. The FAME composition obtained from in situ transesterification process

No.	Methyl Ester Name	FAME Area (%)			RT (min)
		Sonication	Reflux	Combination of sonication -reflux	
1	Methyl tetradecanoate	0.66	0.93	0.51	11.046
2	7,10-Hexadecadienoic acid, methyl ester	9.80	ND	ND	11.996
3	7,10,13-Hexadecatrienoic acid, methyl ester	4.31	3.64	0.74	12.049
4	Pentadecanoic acid, 14-methyl-, methyl ester	12.00	14.37	7.72	12.144
5	Valeric acid, tridec-2-ynyl ester	0.91	ND	ND	12.37
6	Methyl 10-trans,12-cis-octadecadienoate	5.64	ND	ND	12.506
7	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	10.53	6.51	2.10	12.649
8	Methyl stearate	0.80	8.19	0.54	12.714
9	2-Ethylbutyric acid, octadecyl ester	0.58	ND	ND	12.851
10	Eicosanoic acid, methyl ester	3.98	1.12	2.53	12.993
11	10,13-Octadecadienoic acid, methyl ester	ND	0.96	ND	13.035
12	2-Ethylbutyric acid, nonadecyl ester	ND	2.93	ND	13.118
13	Fumaric acid, 2-chloroethyl pentadecyl ester	ND	ND	3.13	13.444

*ND = Not Detected

CONCLUSIONS

Optimization of biodiesel production from *Chlorella vulgaris* through in-situ process assisted by sonication resulted the highest percentage of biodiesel took place at a sonication power of 25kHz/270W. The yield had more increased when combined with *n*-hexane as co-solvent rather than without co-solvent. In-situ transesterification with *n*-hexane co-solvent produced 10.39% biodiesel, an increase of 47% compared to without co-solvent. The use of biomass: methanol in ratio 3:50 (w/v) significantly produced the biodiesel yield at of 20.31% (w/w). The methyl ester compound that was produced consisted of 7,10,13-hexadecatrienoic methyl ester; pentadecanoic acid, 14-methyl-methyl ester; 9,12,15-octadecatrienoic acid-methyl ester; and eicosanoic acid-methyl ester. Joining of reflux-sonication exhibited a low biodiesel product. Disadvantage method from the combination may inhibit on lipid extraction or FAME transformation, the exact cause needs to be studied further.

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