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Effect of Pre-treatment for Lipid Extraction from Tisochrysis Lutea (T-iso)

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These days, the environmental issues from the usage of fossil fuels have been widely highlighted since it is high in demand to come up with the mitigation actions. These environmental issues may be derived from the extensive pollution and release of greenhouse gas (GHG) emissions especially from the industry and transportation sector. GHG will trap the heat in the atmosphere which leads to global warming. Therefore, the usage of microalgae as biomass for the feedstock can help in tackling these issues. As a feedstock that is common in marine environment, microalgae can be converted into high-quality biomass with its accelerated metabolism. In addition, microalgae can rapidly achieve optimal growth and harvesting conditions for further usage on generating renewable energy in the form of liquid or gaseous fuels. Besides, the extraction of lipid from microalgae biomass was widely studied. Specifically, lipid extraction is currently being innovated and modified in order to meet the sustainability aspects. Prior to lipid extraction, pre-treatment method can be considered as an essential step to boost the amount of lipid produced. Thus, the effect of pre-treatment method called ultrasonication before lipid extraction from dry biomass *(Tisochyris Lutea, T-iso)* has been discussed. Dry *T-iso* did undergoes ultrasonication water treatment and followed by lipid extraction. Methanol: chloroform (2:1) was used as the solvent system for control during extraction. The amount of lipid produced while adding the pre-treatment method before extraction has also been determined and compared with the conventional method. 2-Methyltetrahydrofuran (2-MeTHF) was used as the solvent system in this due to its green properties. Research. An addition parameter of isoamyl alcohol was also used to compare the effect of this solvent with 2-MeTHFFrom this research, the lipid yield without any pre-treatment method for chloroform:methanol (2:1 v/v), 2-MeTHF and 2-MeTHF:isoamyl alcohol (2:1 v/v) were 20.33%, 20.67% and 30.00%. Meanwhile, the lipid yield with ultrasonication pre-treatment method for chloroform:methanol (2:1 v/v), 2-MeTHF and 2-MeTHF:isoamyl alcohol (2:1 v/v) were 22.67%, 31.00% and 36.67%. In a nutshell, the addition of pre-treatment method in this study will help to boost the lipid efficiencies and as well as tackling the environmental concern by achieving a more sustainable process on producing biodiesel.

1. Introduction

Recently, microalgae have grown in popularity as a third-generation biodiesel feedstock. Microalgae has the advantage on the generation of energy from renewable sector in the form of fuels (either liquid or gaseous) as it can rapidly achieve growth at optimal state with a great condition or harvesting (Gielen et al., 2019). The major benefits of using microalgae biomass are due to the properties of microalgae which has the efficiency that is high photosynthetically, high content of lipid and high production yields. Other than that, In this research, the microalgae strain used was *Tisochrysis Lutea (T-iso)*. *T-iso* has been classified under haptophytes and has a high lipid oil content (approximately 20% and more) while comparing with other types of microalgae species,which made it very compatible for the extraction of lipid. Haptophytes are unicellular algae that are important members of the marine phytoplankton involved in many important biochemical cycles and considered as a red alga. Haptophytes, like other chromalveolates (such as diatoms), have secondary plastids made from

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red algal endosymbionts that mostly contain chlorophyll c.In addition, several techniques for pre-treatment on the extraction of lipid have been widely explored either with physical, chemical or biological pathways such as microwave, bead-mills, thermal, enzymatic and ultrasonication. Based on a previous study by Ren et al. (2021), the effect of pre-treatment method for vortex, microwave and ultrasonic were discussed but by using different microalgae strains and solvent systems. The results show that ultrasonication can achieved the best results of lipid yield with haptophytes microalgae. Another study by Liu et al. (2022) shows that ultrasonic cell disruption is a sustainable "green" technique, but additional study and work are required to scale up this process without compromising performance or using more energy. Compared with another methods, ultrasonication has lower operating temperature, with little solvents used for valorization and as well as shorter time. Thus, in this paper, ultrasonication has been chosen to be used for further investigation when being paired with *T-iso*. Besides, the organic solvent used during the extraction of lipids from microalgae oil might not be environmentally friendly. Green solvents or also known as bio solvents are solvents that has been labelled environmentally friendly which are derived from crops of agriculture. The necessity of using solvents with a petrochemical base did contribute fairly to chemical operations, but it has serious environmental implications. Comparing with petrochemical derived solvent, lesser harms has been created with the implementation of green solvents. With the recent research of interest, many researchers are shifting on implementing the green solvents like ethyl lactate and 2-MeTHF instead of the conventional petroleum solvent like hexane or chloroform/methanol. To sum it up, this paper discussed the effect of ultrasonic pre-treatment method with 2-MeTHF in order to achieve a higher lipid yield and fatty acid production.

2. Materials and methods

2.1 Ultrasonic pre-treatment method

In this research, the dry biomass of 100 mg was used as the initial weight before undergoes sonication. 5 mL of distilled water was added to the dried biomass in the 15 mL microcentrifuge tube. Then, it was sonicated for 5 min in ice bath by using (Diogenode-Bioruptor, Iwaki Magnet Pump-MD series) at a medium frequency of 50 kHz and 150W. The interval for the cooling down of the machine was set to 30 s.

2.2 Lipid extraction

The lipid extraction was with three sets of parameters with different solvent system. The first solvent system consists of the control which was the extraction by chloroform/methanol. Then, the second set of solvent system was the extraction by 2-MeTHF and the last parameter was the combination of 2-MeTHF with isoamyl alcohol. After the pre-treatment, the biomass was with mixture of 6 mL chloroform:methanol (2:1 v/v). Next, with the concentration of 500µg/mL, 50 µL of C17 fatty acid (internal standard) was added to the mixture of the cell to calculate the concentration of endogenous fatty acids. The cell mixture was then being vortex for 30 s. Afterwards, the cells mixture was centrifuged at 25 °C for 10 min at 2500 rpm. The supernatant was then transferred to a new 15 mL centrifuge tube. Moreover, supernatant was also treated with 1.25 mL of 0.1 M KCl. Cells mixture was vortex again for 30 seconds and as well as centrifugation at 25°C for 5 minat 1500 rpm. Meanwhile, empty 15 mL buchi glass tube was weighted and labelled according to the three different sets of samples. After centrifugation, double layer had formed. Bottom layer of the mixture was extracted as it contains the lipid into the previously weighted buchi glass tube. All of these steps were repeated by using 2-MeTHF and mixture of 2-MeTHF and isoamyl alcohol (2:1 v/v). As a reminder, for these two sets of solvents system, the upper layer should be extracted into the buchi glass tube after centrifugation as the lipid oil is less dense than the KCL. Finally, all of the mixture were dried by using the rotary evaporator (Buchi Multivapor P-12) for 1 h and 30 min or until it dried up. The dry lipids obtained from evaporation were weighted by using the electronic scale and expressed as the percentage of dry cell weight (%) as mentioned in Eq(1).

$$Yield\ (\%) = \frac{Weight\ of\ lipid}{Weight\ of\ dry\ biomass}\ x\ 100\ \% \quad (1)$$

2.3 Fatty acid methyl ester (FAME) analysis

The dried lipid samples were treated with 4 mL of 0.1 N methanolic: hydrochloric acid (MeOH: HCl). The solution was then transferred to new 15 mL glass extraction tube. The mixture was left at 100 °C for 1 h. After that, the mixture needs to be cooled down at room temperature. 4 mL of hexane was added to the cooled FAMEs. The mixture was shaked vigorously for the formation of double layer. The upper layer of the mixture was then being transferred to a new 15 mL glass tube. 2 mL distilled water and 2 mL hexane were added to the remaining lower layer and double layer will form again. The newly formed upper layer was transferred to the glass tube in the previous steps (combined). The mixture was then dried again by using centrifugal evaporator (CC-105, TOMY)

for 30 min to the constant weight. After drying, the dried FAMEs were added with 2 mL of hexane. Analysis for gas chromatography-mass spectrometry was proceed after the solution was transferred to the glass vial.

2.4 Gas chromatography (GC) analysis

The FAME profile was classified and measured by using the gas chromatography with a flame-ionization detector (GC-FID: GC-2014, Shimadzu, Japan) and Agilent CP-Sil 5 CB column (50 m x 0.32 mm x 0.12 µm). Helium was used as carrier gas with a constant flow rate in splitless mode. The initial temperature was set to 60 °C for 1.5 min then increased to 130 °C at 20 °C/min. Then, the temperature increased to 250 °C at 4 °C/min. 1 µL of the sample was injected into GC. By comparing the retention times of the peaks of known FAMEs in the standard solution (pentaunsaturated method 2_35min) to the retention times of the FAMEs in the microalgal lipid sample, the FAMEs in the sample were identified. The FAME content was then calculated by using Eq (2).

$$FAME\ Content\ (\%) = \frac{A_{FAME}}{\sum A - A_{C17}}\ x\ 100\ \% \quad (2)$$

Whereby,
A_{FAME} = Peak area of FAME
ΣA = Total area of the peak for FAMEs chromatogram
A_{C17} = Internal standard area peak (C17:0)

3. Results and discussions

3.1 Effect of pretreatment method on lipid extraction

In biofuel production, prior to processing, cell disruption is an important pretreatment from biomass for a more efficient extraction of lipids. However, for low valued biofuels, high-value energy requirements are the issue (Singh et al., 2022). Previous research discovered that washing cells of algae before the second extraction greatly improved the extraction of solvent efficiency of lipid in microalgae (Ren et al., 2021). Assisted treatment with microwave and ultrasonic technique, osmotic and vortex treatment are some of the examples of pretreatment. All of these treatments are believed to enhance the extraction of lipid especially for the yield produced from microalgae oil. In this study, ultrasonic treatment was chosen for the pre-treatment method. This is because ultrasonic has shorter processing time while having a lower energy demand and considered as eco-friendly. Table 1 shows the comparison of lipid content with an addition of pre-treatment and without pre-treatment.

Table 1: Comparison of lipid content with an addition of pre-treatment

Samples	Lipid Yield (%)	
	Without pre-treatment	With pre-treatment
Chloroform:methanol (2:1 v/v)	20.33±1.25	22.67±1.33
2-MeTHF	27.67±1.58	31.00±1.45
2-MeTHF:isoamyl alcohol (2:1 v/v)	30.00±0.89	36.67±1.17

Based on Table 1, it can be seen that the addition of pre-treatment method led to a significant increase in lipid content from *T-iso* (%) in the ranges of 2% to 7 % . The lowest lipid yield is from chloroform:methanol (control) with only 20.33 % without sonication and 22.67 % with sonication. The highest lipid yield was the solvent system of 2-MeTHF:isoamyl alcohol before and after sonication with 30.00 % and 36.67 %. The intricate cell walls of microalgae, which are made of protein, lipid, polysaccharide, cellulose and glycoprotein were aimed to protect the cells inside. However, some lipids in algal cells are linked to the membranes of the cells, making extraction of lipid from these cells to be challenging. Therefore, to maximize the recovery of lipids, the microalgae biomass must be pretreated before direct extraction from lipid to disrupt the cells and violate the walls from the cells (Singh et al., 2022). C17 acted as the benchmarks while calculating the peak of the FAME content for all fatty acids. C17 was not produced by T-iso thus, makes it the most suitable control to be used in this experiment. With this ultrasonic pre-treatment, the disintegration of the cellular structure has great effects on extraction of lipid such as faster releasing for the content of the cell, greater penetration of solvent into the cell, little consumption of solvent and rapid time for extraction (Srivastava et al., 2020). In pretreatment using ultrasonic, an important issue was to choose the appropriate operational parameters like time of pretreatment, input of energy and frequency. Thus, in this experiment, the chosen parameter was frequency and time which was medium frequency and 5 minutes at 30s interval respectively. After several trial, this time and frequency shows the best mode to be done within this experiment. This is due to the too low sonication may not disrupt the cell wall efficiently or too intense sonication (which means higher time or frequency) leads to foaming of liquid,

significant increase in the temperature and denaturation of protein (Liu et al., 2022). The data taken has been done in triplicate manner which targeted on higher precision while conducting this research.

3.2 Effect of 2-MeTHF on lipid extraction

Petroleum derived solvent which came from the non-renewable sources are mainly used for the extraction of oil in the production of biodiesel. These solvents are typically organic volatile compounds which is hazardous to human health and the environment. Typically, in this industry, n-hexane or known as a petroleum fraction can be considered as one of the most commonly used extraction solvents. The solubility for this solvent is one of the major chemical properties factors that is usually providing an ideal functionality for several products like vegetable oil or microalgae oil especially when it is simple to obtain. Nonetheless, n-hexane has recently been classified as carcinogenic, mutagenic and reprotoxic substances 3 (CMR 3) since it is derived from fossil fuels which is potentially carcinogenic (Englezou et al., 2020). Therefore, the solvent of 2-Methyltetrahydrofuran (2-MeTHF) is known for its low-cost (compared to other green solvent), esoteric and bio-based process. In fact, bio-based process occurs by hydrogenation of carbohydrate fractions, obtained by acid hydrolysis of hemicellulose from various feedstock and it is 100% produced from renewable biomass (Pace et al., 2012). According to United States Department of Agriculture (USDA), biobased product is derived from plants and other renewable agricultural, marine, and forestry materials. In this research, 2-MeTHF is a good green solvent as it has a very low toxicity level compared to the conventional solvent systems. 2-MeTHF also has a low boiling point in the ranges of 78°C to 80°C which makes it has a similar chemical properties to chloroform:methanol or hexane and suits the current setting of the rotary evaporator used in this experiment. Higher boiling point will make it hard on evaporating the organic solvent (Lei et al., 2019). Thus, in this research, 2-MeTHF was found to be very helpful in substituting the conventional solvent system which is quite toxic and harmful towards the environment in order to produce fatty acids for further applications like biodiesel processing.

3.3 FAME analysis

FAME analysis has been used for characterization of fatty acids along with gas chromatography analysis. FAME mixtures are much more stable and therefore easier to keep in a single phase and give more consistent results (Niemi et al., 2019). Table 2 shows the fatty acid profile for each fatty acid detected while Figure 1 shows the total FAME content.

Table 2: Fatty acid profile

Fatty Acid	Types of fatty acid	Chloroform:methanol µg/mg (%)	2-MeTHF µg/mg (%)	2-MeTHF:isoamyl µg/mg (%)
C14:0	Saturated	4.71	4.38	5.82
C16:1	Monounsaturated	4.04	3.50	4.55
C16:0	Saturated	5.87	5.27	6.41
C18:4	Polyunsaturated	11.59	14.83	16.28
C18:2	Polyunsaturated	3.69	3.08	3.93
C18:3	Polyunsaturated	3.06	2.87	3.64
C18:1Δ9	Monounsaturated	7.55	6.39	7.68
C18:0	Saturated	0.26	1.04	0.45
C22:5	Polyunsaturated	0.33	0.76	0.64
C22:6	Polyunsaturated	5.12	8.01	6.86

Based on Table 1, C18:4 was observed to produce the highest amount of fatty acid for all of the solvent systems. When compared with previous study by Tang et al. (2020), C18:4 also recorded the highest amount of fatty acid content due to the large peak area produced during gas chromatography analysis. In terms of definition, unsaturated fatty acids have at least one double bond in the fatty acid chain while saturated fatty acids have no double bonds between the individual carbon atoms. Then, according to Figure 1, the total fatty acid content in chloroform:methanol, 2-MeTHF and 2-MeTHF:isoamyl alcohol were 46.21%, 50.14% and 56.27%. The fatty acid profile showed that extractions with 2-MeTHF and 2-MeTHF:isoamyl alcohol had higher percentages of fatty acids with low degree of unsaturation, which is ideal for the production of better quality biodiesel.

3.4 Effect of isoamyl alcohol as a mixture solvent

In this research, isoamyl alcohol was added as an additional parameter to investigate the effects on lipid extraction and fatty acid produced. In both cases, the mixture of solvent system with isoamyl alcohol recorded the highest amount of lipid yield and fame content which were 36.67 % and 56.27 %. One issue was detected while using isoamyl alcohol due to the boiling point. Isoamyl alcohol has high boiling point which is 131 °C. This high boiling point makes it hard to dry during drying the lipid samples by using the evaporator. On the first trial,

isoamyl alcohol did not even dried up even after 2 h. As a solution, the temperature of heating has been increased to 80 °C from 40 °C and the pressure has also been lowered into 1 kPa from 10 kPa (Tran et al., 2020). As pressure increases, intermolecular forces between molecules in the liquid state weaken which makes the liquid becomes more volatile (Englezou et al., 2020). Thus, this action has led on faster evaporation for the mixture of 2-MeTHF and isoamyl alcohol. From another point of view, water is partially soluble in 2-MeTHF because the solvent 2-MeTHF and water form an azeotropic mixture. This action has hindered its separation (Laitinen et al., 2021). This is why the fatty acid content in 2-MeTHF is 50.14 % which is lower than the mixture of 2-MeTHF: Isoamyl alcohol that is 56.27%. Overall, the results obtained suggest that 2-MeTHF:isoamyl alcohol (2:1 v/v) can replace the chloroform:methanol system currently used.

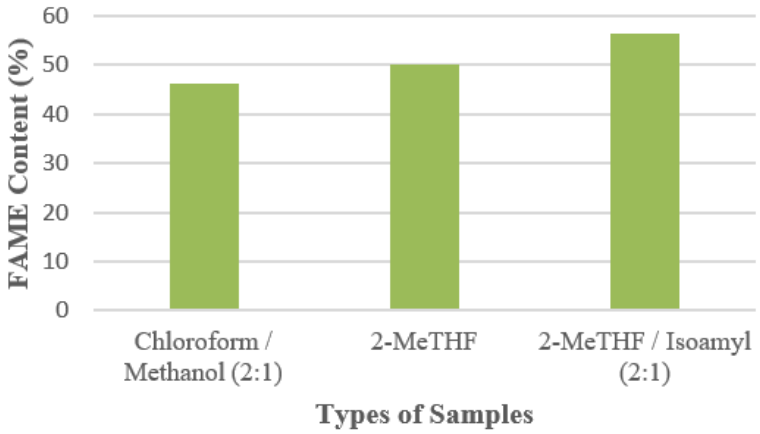

Figure 1: Production of Fatty Acid Methyl Ester (FAME)

4. Conclusions

Today, microalgae have intriguing properties that qualify them as promising feedstocks in alternative measure for a variety of application in industrial and environmental aspects. Nonetheless, numerous efforts are needed to address various challenges, particularly low-cost, high-efficiency biofuels, wastewater treatment, and CO_2 mitigation. Commercial production of microalgal biofuel has the potential to play a significant role in the current global energy scenario and environmental concerns. In this research, ultrasonic pre-treatment method is important to enhance the amount of lipid produced as it is cost and time savings while ensuring a high quality of lipid yield. The effect of pretreatment by washing the algae cells before extraction did boost the lipid yield in each solvent system by 2% to 7%. Green solvent (2-MeTHF) also plays a significant role in this research as it can help on reducing the toxicity exposure that aids in an environmental-friendly process. For future studies, a slightly cheaper green solvent can be aimed to use which may have the same effect as 2-MeTHF, as it is quite expensive to be used on industrial scale. More research on industrial level needs to be done as the set up design for the equipment and the parameters such as pressure and temperature needs to be matched accordingly to find its best fit.

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