Lipid extraction from *Aphanothece sp*. using ultrasounds

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Abstract. The unprecedented reduction in the availability of fossil fuels requires the research community to find safe, clean, renewable and sustainable energy sources. One promising source is microalgae biofuel, which can reduce environmental problems and energy crises due to its closed carbon cycle. Selecting the right strain and optimizing lipid productivity are very important for the success of the biodiesel conversion process economically. In this study, the lipid extraction process from *Aphanothece sp*. dry biomass which was previously grown in photobioreactor systems with atmospheric carbon dioxide feed inputs was carried out using the ultrasound method, with variations of solvent systems. The maximum lipid extract yield of about 40.79 ± 0.76 percent DW, w/w was obtained for the dichloromethane/methanol ratio of 1:2 (v/v).

1. Introduction

Energy is one of the fundamental elements affecting both technological progress and the standard of living of a nation. Unsustainable energy resources such as fossil fuels are not only insufficient to meet future demands but also contribute to pollution and environmental damage [1,2]. This condition has led to increased interest and efforts to replace these energy sources with energy sources that are clean, environmentally friendly and renewable. One source of energy that has great potential is biofuels. Microalgae have been known as the most promising source of biofuels to replace fossil fuels, due to the high productivity of biomass and lipid content of large-scale algae culture. Microalgae has a high growth rate and can be grown in various types of waters so that it has the potential to utilize media and nutrients that are underutilized for biomass production [3]. One of the most important steps in the entire process of microalgae biofuel production is to choose the optimal species that can grow quickly and must be adapted to large-scale cultivation with high-quality lipid yield [4].

Some important characteristics that need to be considered in selecting microalgae strains for the cultivation processes are stability and resistance to various environmental conditions [5]. Cyanobacteria are photoautotrophic prokaryotes inhabiting various types of terrestrial and aquatic ecosystems. Due to their simple growth requirements, high growth rates and tolerance for growing in different habitats including rivers, lakes, rivers, ponds, marine environments, and certain extreme habitats, they have been able to adapt exceptionally to various environmental conditions. In terms of the utilization of biomass-based raw materials, Cyanobacteria is an attractive and high potential candidate compared to eukaryotic microalgae. Thus far, many studies have succeeded in producing a variety of valuable products in cyanobacteria that are metabolically engineered [6].

Cyanobacteria grow diverse in nature and can be found as filaments and unicellular, living in the sea and freshwater, living freely and symbiotic. Cyanobacteria have been named as one of the most important biomass sources on Earth. Of the approximately eleven cyanobacteria species commonly

found in nature, only *Aphanothece halophytica* and *Schizothrix arenaria* are found to survive in salty environments. *Aphanothece halophytica* from the halophilic environment is reported as an alternative potential source to overcome the depletion of fossil energy sources. This species has been proven to be highly resistant to various environmental changes and can utilize nutrients in the environment effectively, both organic and inorganic [7]. Zepka *et al*., (2010) recommend the use of *A. Microscopica Nägeli* microalgae grown in parboiled rice waste as a source of single-cell protein [8].

Microalgae produce lipids such as triacylglyceride (TAG) which can be converted to biodiesel and various high-value products. Microalgae is reported to contain Omega-3, eicosapentanoic acid (EPA) and decosahexaenoic acid (DHA), so that it can be utilized in the food industry and pharmaceutical industry [9,10]. Besides lipids, microalgae also produce other components such as pigments, vitamins, proteins, enzymes, polysaccharides, and also microbicides [11]. For biofuel production at mass scale, it is necessary to explore microalgae that have high growth rates and lipid content. The purpose of this study was to evaluate the total lipid extraction from Aphanothece Sp. dried biomass using different solvents. The extraction method used in this research is ultrasound extraction.

2. Materials and methods

2.1. Materials

Algal material used in this research was *Aphanothece sp*. (blue–green algae cyanobacteria) obtained from laboratory of microbiology and bioprocess technology, Chemical Engineering Department, ITB. Reagents used in this experiment were methanol, ethanol, dichloromethane, and n-hexane. All reagents were HPLC grade and purchased from Fisher Scientific Ltd. (Loughborough, UK). Medium for cultivation was trace metal mix A5 in BG-11 growth medium ($NaNO_3$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, Na_2CO_3, $CaCl_2 \cdot 2H_2O$, Na_2-EDTA, $(NH_4)5[Fe(C_6H_4O_7)2]$ and $C_6H_8O_7$).

2.2. Methods

2.2.1. Microalgae preparation. Aphanothece sp. cultivated in two stages. Inoculum was made by adding 100 mL starter inoculum *Aphanothece sp*. to 600 mL BG-11 medium with 0.7 mL of A5 trace metal mix solution. After inoculation, the inoculum was placed in a 5 L photobioreactor accompanied by continuous aeration and illumination of around 5700 lux. Microalgae culture was harvested on day 14, the end of the exponential growth phase. Biomass is harvested and separated using centrifugation at 6000 rpm for 10 minutes. The obtained biomass is then dried at 60 °C in an oven and then mashed and sieved through size 45 mesh (around 354 μm in diameter). Figure 1 shows a path to concentrate the microalgae.

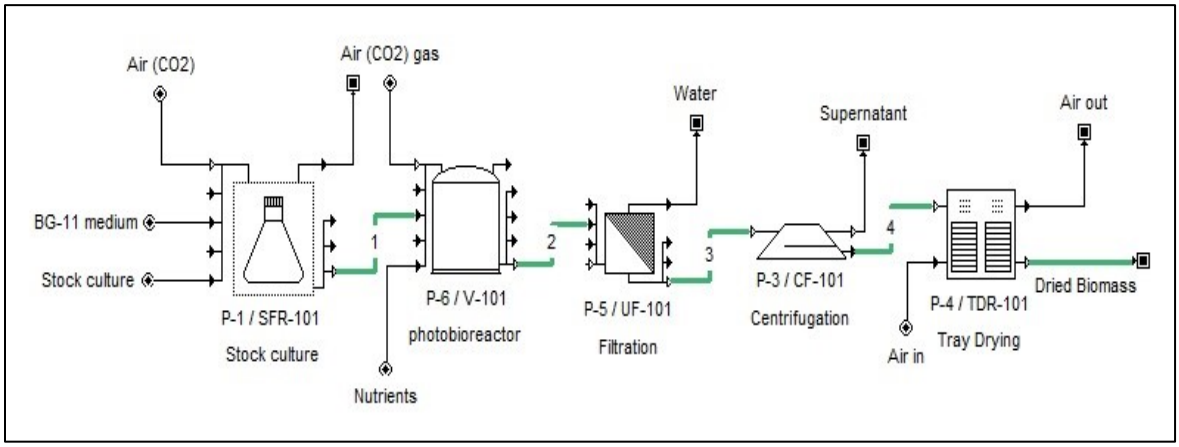

Figure 1. Diagram of dry microalgae production in laboratory.

2.2.2. Lipid Extraction. The extraction processes were performed using various solvents in ultrasound bath: methanol (M); dichloromethane (D); ethanol (E); n-hexane (H); and dichloromethane: methanol (DM) (2:1, v/v). 0.1 g of dried *Aphanothece sp.* biomass were combined with 10 mL of solvent in a 20 *mL vial.* Samples were placed in an ultrasonic water bath (BRANSONIC Model B-2200 E4 2.5 L, working at 47 kHz, US). The ultrasonic extraction process was carried out for 3 hours, with an operating temperature not exceeding 30°C. After that, solids and lipids were separated using centrifugation at 4000 rpm for 15 minutes. The organic phase is transferred to a new glass tube and evaporated using a rotary evaporator. Lipid yield extracted from dry biomass (DW) was calculated by weighing using Equation (1). Analysis of the functional group of dry microalgae biomass used in this experiment was characterized by Fourier transform infrared (FTIR) spectroscopy (Bruker, Model ALPHA II).

$$\text{Total yield (\%DW, w/w)} = \frac{\text{weight of the extract (g)}}{\text{weight of biomass (g)}} \times 100 \quad (1)$$

The water content contained in the lipid extracts were determined using oven at a temperature of 105±2 ºC. The calculation of water content is determined using equation (2). In addition, free fatty acid (FFA) determination was carried out through the titration process with KOH (AOCS Method Ca 5a-40). Calculation of free fatty acid content can be determined using equation (3).

$$\text{Water content (\% w/w)} = \frac{\text{initial weight (g) - final weight (g)}}{\text{initial weight (g)}} \times 100 \quad (2)$$

$$\text{FFA (\%)} = \frac{\text{N KOH} \times \text{V KOH} \times 0.256}{\text{weight of lipid (g)}} \quad (3)$$

3. Results and discussion

The transmittance FTIR spectrum of dried biomass of *Aphanothece sp.* is shown in Figure 2. This analysis was conducted to determine the dominant functional groups in the biomass sample. Based on the spectrum in Figure 2, the stretching vibrations of -N-H and -O-H groups are at 3388 cm^{-1} with wide and strong band. The band at wavenumber 2916 cm^{-1} shows stretching of -CH. Whereas the bands peaking at 1649 and 1529 cm^{-1} are asymmetric and symmetrical stretching vibrations from the C=O group. Peaks at wavenumbers 1396 and 1016 cm^{-1} show stretching of C-O groups on the surface of the biomass. Some bands in the fingerprint area are thought to be sulphuric functional groups such as S-OR and S-S. Based on this FTIR spectrum, it can be suggested that the surface of microalgae cells contains various functional groups which can be useful for various applications such as adsorption, and secondary metabolites exploration.

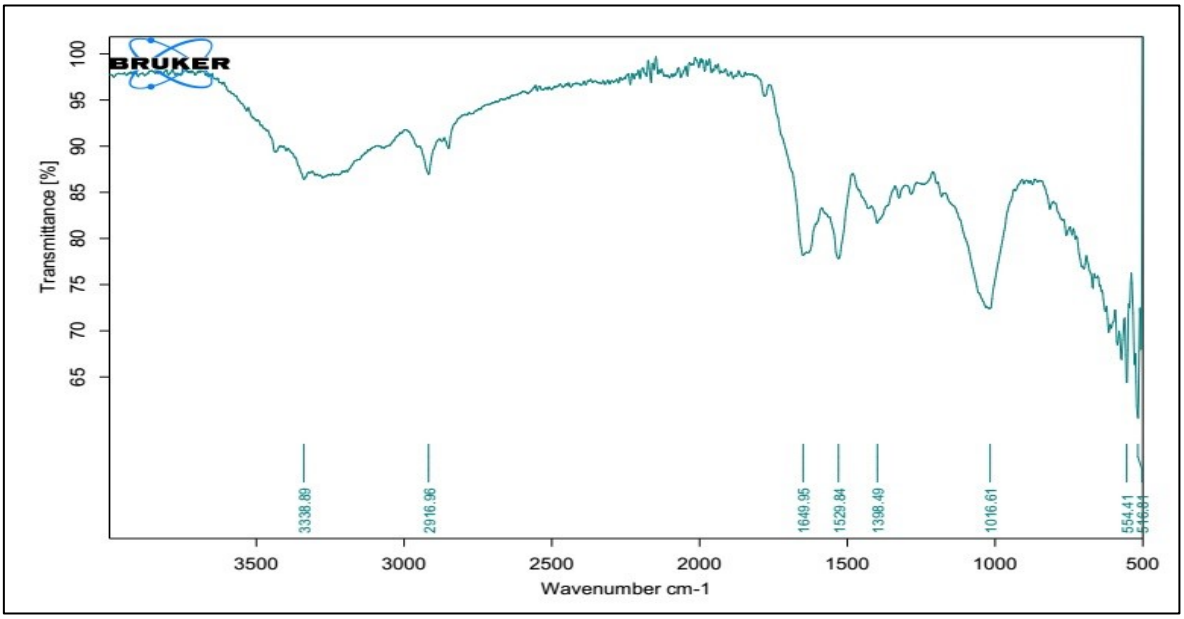

Figure 2. FTIR spectrum of dried *Aphanothece sp.* biomass.

In this research, various solvents were explored in the production of lipid extracts from *Aphanothece sp.* dried biomass. These solvents were used due to their efficiency to extract lipids from microalgae. Ethanol was tested as the solvent because ethanol is a green solvent, environmentally friendly and has good performance in the process of extracting lipids from microalgae [12]. Using these solvents is advantageous for industrial application.

Lipid extracts depend upon algal species and extraction methods, but ultrasound extraction would be the easiest and most efficient method [13]. Ultrasounds generate cavitation which correlated with algal cell disruption [14]. Ultrasonic-assisted extraction is reported as one of the most efficient extraction techniques because it drastically reduces the operation time and volume of the solvent and increases the results of lipid extraction. In addition, this method also has the benefit of high expandability in the industry [15]. Figure 3 shows picture of dried biomass microalgae and organic extract using sonication. As for the results of lipid extraction can be seen in Figure 4 and Table 1. The highest yield was obtained using mix solvent of dichloromethane-methanol (DM) with values of 45.67 ± 0.72%, while ethanol (E) gave total yields of 40.04 ± 1.28%. Lower yields were obtained with methanol (M) (37.16 ± 0.86%), dichloromethane (D) (23.06 ± 0.78%) and n-hexane (H) (8.82 ± 0.39%).

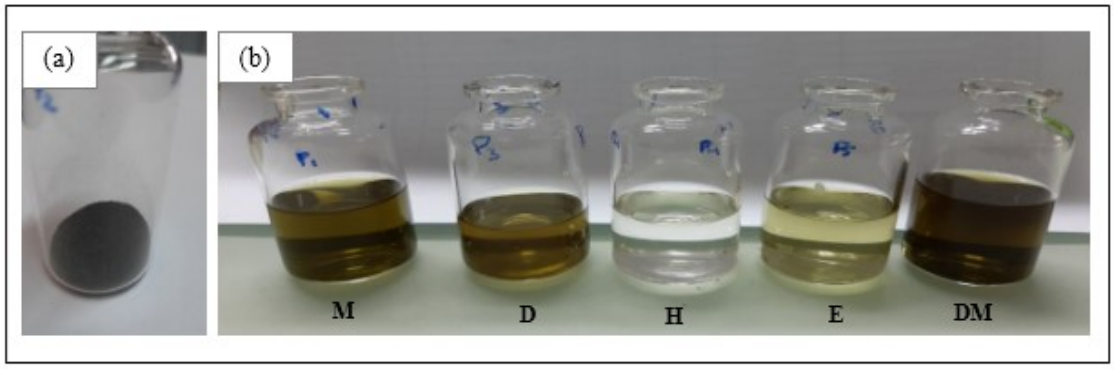

Figure 3. (a) Dried biomass microalgae. (b) Extracts of microalgae using ultrasound extraction.

Table 1. Lipid extraction from different solvent systems.

Solvent	Weight of the total extract (mg)	Total yield (% w/w)
M	33.71 ± 0.62	32.51 ± 0.75
D	13.87 ± 0.85	13.38 ± 0.81
H	8.55 ± 0.78	8.03 ± 0.74
E	38.77 ± 0.59	37.62 ± 0.73
DM	45.81 ± 0.36	40.79 ± 0.76

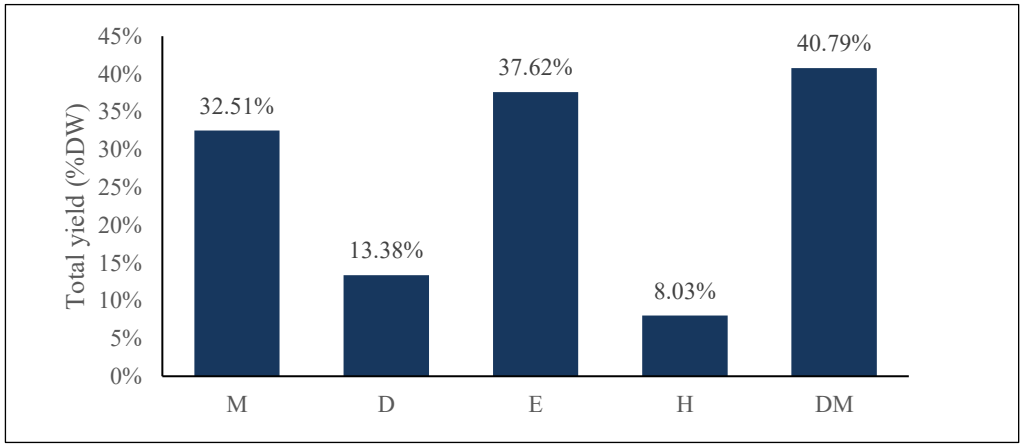

Figure 4. Lipid content using different solvent systems.

These results show that polarity of the solvent directly influences the extraction efficiency. Based on Table 1, it implies that low polarity solvents provide less lipid extract. Nonpolar solvent like n-hexane has zero polarity index, which is not suitable for the extraction of polar lipids such as glycolipids and phospholipids. Lipid extraction and recovery can be improved by increasing the polarity of the solvent, for example by mixing nonpolar solvents appropriately with polar ones, like alcohol. Polar solvents are able to release lipid from protein-lipid complexes, facilitating its dissolution in nonpolar solvents [16-18]. Dichloromethane is a nonpolar solvent with a polarity index of 3.1, while methanol has a polarity index of 5.1, producing a mixture of solvents with sufficient polarity to extract higher amounts of lipids.

Table 2. Lipid extract characteristics.

	Free fatty acid (FFA %)	Water content (%)
M	1.128	0.196
E	1.098	0.247
DM	1.223	0.133

The results of the analysis of water content and free fatty acid levels (FFA) of lipid extracts from dried biomass of *Aphanothece sp.* using methanol (M), ethanol (E) and dichloromethane-methanol solvent mixtures (DM) are shown in Table 2. The water content for each extract does not differ greatly, the highest water content is 0.247% for ethanol extract (E) and the lowest is 0.133% for dichloromethane-methanol (DM) extract. Whereas the highest FFA% is found in DM extract, which is 1.223%. Lipid extract which has high water content and FFA can provide various disadvantages. Studies show that the catalysis reaction for the transesterification will be disturbed (poisoned) by the presence of water (> 0.5%) or FFA (> 5%). Some studies report that the presence of FFA requires the addition of a pre-treatment step before transesterification [19-20]. Van Gerpen [21] stated that the FFA level of 5% can be used in the transesterification process with the help of an alkaline catalyst, but it reduces the biodiesel yield.

4. Conclusions

This study confirms that *Aphanothece sp*. has good lipid production capability and has the potential to be developed as a raw material for making biofuels such as biodiesel. Lipid extractions have been done by ultrasonic extraction method in various solvent systems. The highest lipid yield obtained from the use of dichloromethane-methanol mixed solvents (1:2 v/v), 40.79 ± 0.76%. Water content and free fatty acid content from lipid extract using dichloromethane-methanol solvent mixtures (DM) are 0.133% and 1.223%, respectively.

5. References

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