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Lipid Extraction Maximization and Enzymatic Synthesis of Biodiesel from Microalgae

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Abstract: Microalgae has received overwhelming attention worldwide as a sustainable source for energy generation. However, the production of biofuel from microalgae biomass consists of several steps, of which lipid extraction is the most important one. Because of the nature of feedstock, extraction needs special attention. Three different methods were studied to extract algal oil from two different algae variant, *Chlorella* sp. and *Spirulina* sp. The highest percentage oil yield was obtained by ultrasonication (9.4% for *Chlorella* sp., 6.6% for *Spirulina* sp.) followed by the Soxhlet and solvent extraction processes. Ultrasonication and Soxhlet extraction processes were further optimized to maximize oil extraction as solvent extraction was not effective in extracting lipid. For ultrasonication, an amplitude of 90% recorded the highest percentage yield of oil for *Spirulina* sp. and a 70% amplitude recorded the highest percentage yield of oil for *Chlorella* sp. On the other hand, for Soxhlet extraction, a combination of chloroform, hexane, and methanol at a 1:1:1 ratio resulted in the highest yield of algal oil. Afterward, the crude algae oil from the ultrasonication process was transesterified for 5 h using an immobilized lipase (Novozyme 435) at 40 °C to convert triglycerides into fatty acid methyl ester and glycerol. Thus, ultrasonic-assisted lipid extraction was successful in producing biodiesel from both the species.

Keywords: microalgae; biofuel; enzymatic reaction; ultrasonication; biodiesel; immobilized lipase

1. Introduction

Depleting oil reserves, increased energy dependency, and the environmental impact of fossil fuel use has led to research on sustainable energy resources with a cleaner footprint [1–3]. Biofuel, in particular biodiesel, is a feasible diesel fuel replacement because it can be directly employed without modification of the engine structure [4–6]. Biodiesel generally refers to the mixture of fatty acid methyl esters and are derived from lipid substances originated from triacylglycerol-containing feedstocks [7–9]. A review of life cycle analysis on liquid biofuel systems has concluded that conventional biofuels (from grains and seeds) can provide moderate greenhouse gas (GHG) reduction benefits in measures,

such as 'per GJ fossil fuel displaced', 'per ha land use', etc., owing to high land requirements [10]. The use of oleaginous microalgae biomass will help minimize this arable land requirement and increase GHG emission reductions per hectare for any potential bioenergy application. Microalgae has a high photosynthesis efficiency, oil content, biomass productivity, and growth rate when compared with other terrestrial oilseed crops [11,12]. Despite these advantages, more substantial greenhouse gas emissions, increased nutritional needs, and increased use of water were recorded to algae associated with existing terrestrial bioenergy feedstocks from a life cycle analysis perspective [13–15]. However, those views have been contradicted by others because of limitations in the assumptions of those studies [16].

Microalgae biomass is distinct in many ways from typical feedstock, viz. the cell wall and plasma membrane chemistry, water presence, and tiny cell size [17]. As such, the process of lipid or oil extraction from microalgae needs special attention. Typically, lipid extraction methods use solvents such as toluene, hexane, butanol, ethanol, methanol, or ionic liquids, to extract lipids from intact, chemically treated, or mechanically ruptured cells [18,19]. The mechanical methods include oil press, microwave-assisted extraction, and ultrasonic-assisted extraction [20–22]. The chemical methods include solvent extraction, Soxhlet extraction, supercritical fluid extraction, and accelerated solvent extraction [22,23]. The used organic solvents help in extracting oil by rupturing cell walls and disrupting the interaction forces between the lipid and tissue matrix [24]. An appropriately designed solvent or solvent matrix usage will significantly increase the efficacy of the extraction process.

This work focused on ultrasonic, solvent, and Soxhlet extraction processes. Ultrasonic irradiation has been used to accomplish enhanced mixing between reacting components. Goh et al. [24] employed the ultrasonication to extract lipids from spent coffee ground with three different solvents (hexane, chloroform, or methanol). Wang and Yuan [25] studied the effect of the operating conditions of ultrasonic flow systems on cell disruption of two algal strains, *Scenedesmus dimorphus*, and *N. oculata*. On the other hand, the modified Folch lipid extraction protocol [26] is generally used as a chemical extraction method. Hexane is primarily used in this process due to its less toxicity than chloroform, minimal affinity towards non-lipid contaminants, and apparent higher selectivity towards neutral lipid fractions [27]. However, a combination of several solvents is generally used for maximizing the yield.

This study investigated two different microalgae species, *Chlorella* sp. and *Spirulina* sp., which are widely available in Malaysia and the southeast Asian region and possess low lipid contents. Usually, microalgae contain lipid in the range of 20–50% [28,29]. *Chlorella* is a freshwater green unicellular alga with fast growth and an easy cultivation process. The lipid content of *Chlorella* is about 14–18.7% by the weight of the dry biomass in usual growth conditions [30,31]. On the other hand, *Spirulina* sp. is found in soil, marshes, freshwater, brackish water, and seawater with commercial availability in large quantities [32,33]. It has a lipid content of 6.4–8% by the weight of the dry biomass in usual growth conditions [34]. Liang et al. [30] studied lipid extraction from *Chlorella* sp. using the Bligh and Dyer method [35]. Dry biomass was first ultrasonicated for 30 min in 2:1 (*v*/*v*) chloroform/methanol. Then, water and additional chloroform were added, and the mixture was centrifuged at 4500 rpm for 10 min. Finally, the solvent was collected and dried in a rotary evaporator at 60 °C. Sharma et al. [36] studied lipid extraction and biodiesel production from *Chlorella vulgaris.* Lipid was extracted using chloroform:methanol (2:1). They examined the effects of different reaction conditions, including catalysts, molar ratio, temperature etc., under microwave irradiation on biodiesel outputs and compared them with findings under conventional heating. Parichehreh et al. [37] studied a flux balance approach to optimize the specific growth rate and the lipid production rate of *Chlorella vulgaris* AG10032. They found that the microalgal cells could produce maximal lipid yield under N-starvation conditions. Pohndorf et al. [38] studied the lipid production process of *Spirulina* sp. The dried microalgae (2 g) was placed in a conical flask with 40 mL of chloroform:methanol (2:1 *v*/*v*) added, and stirred for 1 h in a shaker. Thus, maximization of the output from these low lipid content feedstocks has not been studied extensively and needs to be addressed.

The conventional biodiesel production process is alcoholysis of lipids using catalysts, either homogeneous or heterogeneous, as the reaction promoter, or supercritical reactions with or

without the presence of a catalyst [39–41]. Both homogeneous catalyst and heterogeneous catalyst have been studied exhaustively in the literature. On the other hand, enzymatic catalysts are gaining attention as catalysts in transesterification or alcoholysis, especially from microalgae. Immobilized lipases are extensively used in enzymatic alcoholysis of triglycerides into fatty acid methyl esters (FAMEs) [42]. Recently, Sanjib Kumar Karmee [43] reported the optimization of methanolysis of *Manilkara zapota* (L.) using lipases, such as Novozyme 435 (*C. antarctica* lipase-B immobilized on acrylic resin) and CLEA (crosslinked enzyme aggregate) of *C. antarctica* lipase-B as biocatalyst. The author found that under similar operating conditions, 93% biodiesel was obtained after 12 h of reaction using Novozyme 435, whereas CLEA produced 84% biodiesel. Tran et al. [44] studied biodiesel production from *Chlorella vulgaris* ESP-31 microalgae by enzymatic transesterification using an immobilized lipase originating from *Burkholderia* sp. C20. A high conversion efficiency of 97.25 wt.% oil occured when static lipase was utilized. This worked in a high water (>71.39% wt.%) level and high molar ratio settings (>67.93% wt.%).

This work aimed to compare the efficacies of three lipid extraction methods viz. solvent extraction, Soxhlet extraction, and ultrasonication, to obtain microalgal crude oil from *Chlorella* sp. and *Spirulina* sp. The maximization of lipids using Soxhlet and ultrasonic methods was attempted by varying the operating parameters to achieve maximum lipid output. Lipids were then converted to biodiesel using the enzymatic transesterification process. The immobilized lipase was used as a catalyst under constant operating conditions. Finally, the biodiesel was characterized using Fourier transform infrared spectroscopy (FTIR) and gas chromatography (GC).

2. Materials and Methods

2.1. Chemicals and Reagents

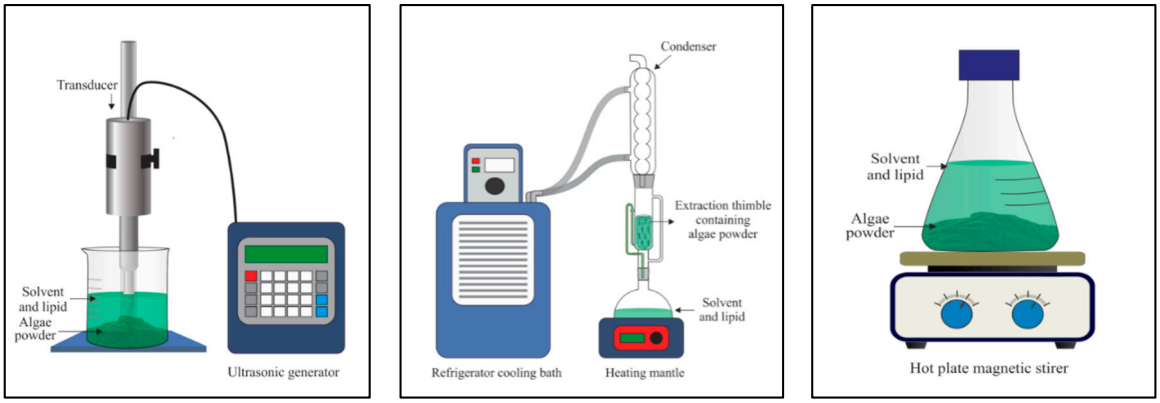
The dried microalgae species used in this study were *Chlorella* sp. and *Spirulina* sp., which were obtained from a local supplier. Commercial immobilized lipase (Novozyme 435) was obtained from Sigma-Aldrich. Novozym 435 is an immobilized lipase, which is based on immobilization via interfacial activation of lipase-B from *Candida antarctica* on a resin, Lewatit VP OC 1600. This resin is a macroporous support formed by poly (methyl methacrylate) crosslinked with divinylbenzene [45]. The Novzyme 435 is extensively used in enzymatic transesterification, converting triglycerides into FAMEs. All organic solvents used in this study were of analytical grade obtained from Fisher Scientific.

2.2. Lipid Extraction

In this study, three extraction processes were studied, including solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction.

2.2.1. Lipid Extraction by Ultrasonication

Ultrasonication was carried out by mixing 30 g of dried algae with distilled water, and processed with a Vibra-cell ultrasonicator (Model VCX500, Sonics & Materials, Inc., Newtown, CT, USA). The equipment has a probe that delivers ultrasound at a frequency of 40 kHz. The probe was immersed into the mixture, and ultrasonication was carried out at 70% amplitude for 15 min to disrupt the microalgae cells. Without separating the biomass from the solution phase, the sample was treated with n-hexane, which acted as an extraction solvent [46]. The suspension was thoroughly mixed and allowed to settle into phases. Centrifugation was necessary to separate the algae residue and solvents containing lipids. This was carried out in a centrifuge (Rotofix 46, Hettich Zentrifugen) at 2000 rpm (358× *g*) for 15 min. The resulting lipid was then placed in a beaker and dried under the hood at room temperature. Additional hexane was then added and evaporated using a rotary evaporator to remove the remaining solvent. This process was performed multiple times to produce a purified product. The crude oil was retained for further transesterification reaction. Figure 1a shows the schematic of lipid extraction using the ultrasonication method.



(**a**) (**b**) (**c**)

Figure 1. Schematic of lipid extraction using (**a**) ultrasonication, (**b**) Soxhlet extraction, and (**c**) solvent extraction method.

2.2.2. Lipid Extraction by Soxhlet Apparatus

Thirty grams of dried algae biomass were used to extract lipid using a Soxhlet apparatus equipped with a condenser and heating mantle. It took 8 h to extract the oil at 60 °C using n-hexane as the solvent. The extracted lipid was then filtered several times to separate the residues. The remaining solvent was entirely removed by a rotary evaporator to obtain crude microalgae oil. The crude oil was retained for further transesterification reaction. The processes were repeated to optimize the solvent concentration for crude oil extraction. Figure 1b shows the schematic of lipid extraction using the Soxhlet method.

2.2.3. Lipid Extraction Using the Solvent Extraction Process

In this process, 150 mL hexane was added to 30 g of dried biomass. The mixture was stirred at 300 rpm and kept at 60 °C for 8 h. After extracting the lipid, the sample was centrifuged at 2000 rpm (358× *g*) for 20 min to remove the residue. In order to eliminate the residual solvent, the top layer mixture was filtered and evaporated multiple times. Figure 1c shows the schematic of lipid extraction using the solvent extraction method.

2.3. Lipid Extraction Maximization from Microalgae

Maximization of the lipids was carried out with ultrasonication and the Soxhlet extraction method only as the remaining process did not lead to a high oil yield. The methods from Sections 2.2.1 and 2.2.2 were repeated with different solvents or amplitude conditions depending on the process.

2.3.1. Ultrasonic Extraction

Different amplitudes were applied for the ultrasonication process with the methanol:chloroform (1:1, *v*/*v*) two-phase system as solvents. The amplitude ranged from a 50% up to a 90% amplitude with an interval of 10%. Ultrasonication was carried out similarly to the process described in Section 2.2.1.

2.3.2. Soxhlet extraction

Meanwhile, the Soxhlet extraction maximization process was carried out using a combination of three different solvents at different volumetric ratios. Solvents of chloroform (C), hexane (H), and methanol (M) were used in this study and combined according to these four different ratios, C:H:M of 1:1:1; 2:1:3; 3:2:1; and 1:2:3. The Soxhlet extraction process was carried out for 8 h at 60 °C.

2.4. Transesterification of Microalgal Oil to Biodiesel

Traditionally, transesterification is carried out with methanol in the presence of homogeneous catalysts, such as KOH, NaOH, etc. [47,48]. In this study, microalgae oil was transesterified using

methanol in the presence of lipase-catalyst to produce FAMEs following s similar procedure reported previously [49]. In this reaction, methanol acts as the acyl acceptor. The extracted lipid was transferred into a glass container in the presence of 5% (*w*/*w*) Novozyme 435 catalyst. The solvents, including methanol and hexane, were then applied to the container to complete the transesterification process. Instead of adding all the methanol at once, it was added in steps to avoid inhibition based on the process described in the literature [50,51]. The reaction was carried out at four different temperatures from 30 °C with a stepwise increase of 5 °C for 5 h at 250 rpm. Samples were drawn out and kept for further analysis by FTIR and GC.

2.5. GC Analysis

A GC (Model: 7890A, Agilent Technologies Inc., Santa Clara, CA, USA) was used to quantify the total FAME content (C6:0 to C24:0) and methyl linolenate content (C18:3) of the methyl esters by comparison with the standard test method. An Agilent HP-INNOWax column (Length × inner diameter × film thickness: 30 m × 0.25 mm × 0.25 µm) was used. The sample was prepared for analysis by measuring 100 mg of ME sample in a 10-mL tube with 100 mg of added methyl nonadecanoate (C19). The mixture was diluted with 10 mL of toluene before transferring into a gas chromatograph vial. About 1 µL of the transferred sample was used for the GC measurements. The temperatures of the injector and the detector were set at 250 °C. In this experiment, the temperature increased at a rate of 10 °C/min until the oven temperature reached 200 °C, and continued to heat at a rate of 5 °C/min until it reached 240 °C, which was maintained for 7 min. After that, the fatty acid composition (FAC of the biodiesel was analyzed according to the EN 14103:2011 standard test method.

2.6. Analysis of Oil Yield and FAME Percentage

The method of calculating the percentage of oil yield for each extraction is as follows:

$$Percentage\ of\ oil\ yield\ (\%) = \frac{Mass\ of\ oil}{Mass\ of\ microalgae} \times 100. \quad (1)$$

The acid value of the produced oil was measured using an acid value tester (Mettler Toledo) following ASTM D664. The measurements were triplicated, and the average was taken. The produced biodiesel was analyzed using FTIR (Model: Spectrum 400, Perkin Elmer, Waltham, MA, USA), according to ASTM D7371. The flowchart of the overall process is presented in Figure 2.

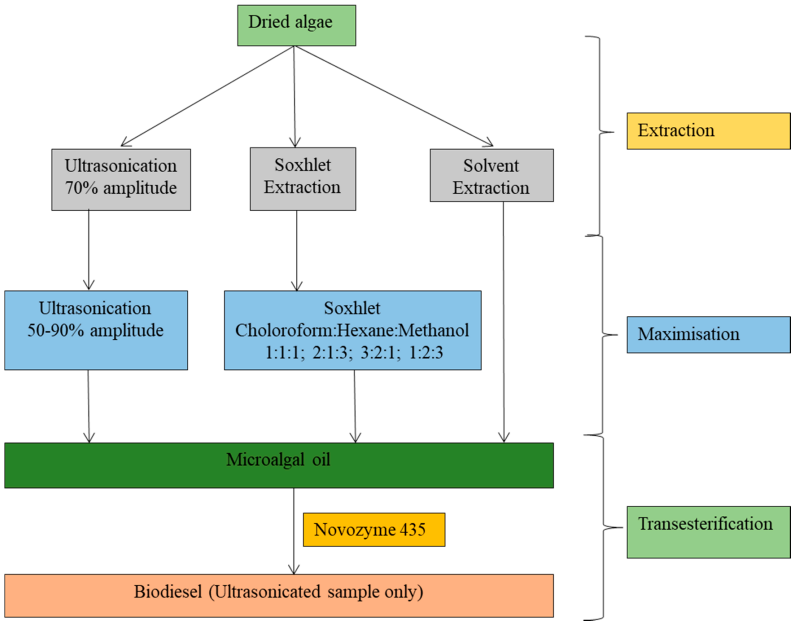


Figure 2. Overall flowchart of lipid extraction of microalgae and biodiesel production.

3. Results and Discussion

3.1. Lipid Extraction by Different Methods

Choosing the right extraction method is crucial to obtain a high yield of crude oil. The efficiency of each extraction method is generally determined by the percentage of oil yield obtained after each extraction process. In this study, three different modes of microalgae biomass extraction were compared to determine the highest oil yield from two different species of microalgae viz. *Spirulina* sp. and *Chlorella* sp. The oil yields after 15 min of ultrasonication for *Chlorella* sp. and *Spirulina* sp. were 2.83 and 1.98 g, respectively. The oil yield after 8 h of Soxhlet extraction for those microalgae species was 1.05 and 1.25 g, respectively. The oil yield after 8 h of solvent extraction was 0.68 and 0.77 g, respectively. It was reported by Halim et al. [52] that chemical extraction operating in dynamic mode, i.e., the Soxhlet extraction process, resulted in a roughly 280% increase in yield compared to static mode operation, i.e., the solvent extraction process. This significant improvement can be attributed to solvent refluxing, which exposed the cellular matrix to a fresh batch of hexane constantly and enabled continuous re-establishment of mass transfer equilibria [53]. Figure 3 shows the extracted product for the different lipid extraction methods.

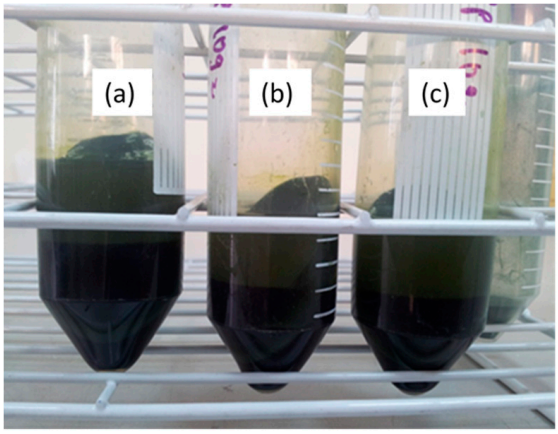


Figure 3. Extracted lipids using (**a**) Soxhlet extraction, (**b**) ultrasonication, and (**c**) solvent extraction.

The percentage of the oil yield of three different methods for these two species of microalgae is depicted in Figure 4. As seen in the figure, for *Spirulina* sp., the rate of oil yield for solvent extraction, Soxhlet extraction, and ultrasonication is 2.5%, 4.1%, and 6.6%, respectively. On the other hand, for *Chlorella* sp., the percentage of oil yield is 2.3%, 3.5%, and 9.4%, respectively. Ultrasonication is the most effective among the three methods. The high efficacy of this method can be attributed to the disruption of the microalgae cells, as reported in other studies [44,54,55]. Sonic waves were passed to the microalgae culture while the ultrasound was done. They made a series of microbubble cavitations, which transferred kinetic energy to the cell surface, having broken up the cells [56]. Thus, ultrasonic waves help to reduce the size of dried algal fractions, which increases the surface area and the number of active sites available for reaction participation. Lee et al. [20] stated that the utilization of microwave and autoclaves are the best technique for disrupting *Chlorella vulgaris* cells, both of which produce a lipid yield of 10%. On the other hand, ultrasonication only resulted in a 5% lipid yield for a 5 min reaction time. In the present study, the 15-min sonication process resulted in a 9% lipid yield for *Chlorella* sp., showing that the longer reaction time was conducive to disrupting the cell walls of *Chlorella* sp., thereby increasing the lipid yield. However, it is to be noted that the effectiveness of cell disruption by ultrasonication will depend on the species of microalgae. For instance, Lee et al. [57] reported that *Botryococcus* species achieved a 16% lipid yield when ultrasonication was used. Thus, it is dependent on the microalgae cell wall thickness and diameter. Table 1 provides a study of oil production by the various methods of extraction. As shown in Figure 4, ultrasonication results in

superior lipid extraction compared to other extraction processes. Structural analysis using SEM was performed to study the extraction methods in-depth. The analysis can be found in Appendix A.

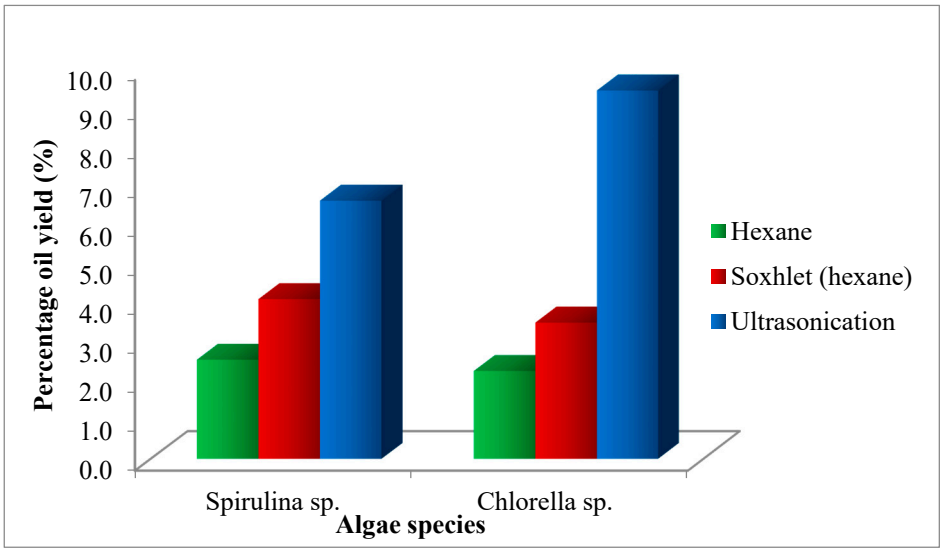


Figure 4. Percentage of oil yield for different extraction processes.

Table 1. Comparison study of the percentage oil yield based on different extraction methods.

Species	Amount of Dried Microalgae Used	Soxhlet Extraction	Ultrasonication	Solvent Extraction	Reference
		Oil Yield in Percentage (%)			
Chlorella sp.	30 g	3.5	9.4	2.7	This study
Spirulina sp.	30 g	4.1	6.6	2.5	This study
Chlorococcum sp.	4 g	0.8	-	-	[52]
Chlorococcum sp.	200 mL stock culture	-	4.5%	-	[56]
Botryococcus sp.	0.5 g	-	<9%	-	
Chlorella vulgaris	0.5 g	-	5%	-	[20]
Scenedesmus sp.	0.5 g	-	<9%	-	
Botryococcus braunii	-	-	16%	-	[57]
Chlorella pyrenoidosa	-	2.2	-	-	[18]

3.2. Lipid Extraction Maximization

As discussed earlier, ultrasonication was found to be more conducive to extracting the microalgae oil compared to other processes. Both ultrasonication and the Soxhlet extraction process were further carried out to maximize the oil yield. Ultrasonication extraction was investigated with varying amplitudes to maximize the oil yield. Different amplitudes were tested, ranging from 50% to 90%. The results of this work are shown in Figure 5. It is to be noted that, for these cases, a percentage increase in the oil yield compared to baseline was presented. The percentage yield of crude oil was observed to increase continuously as the amplitude increased. A higher amplitude enhances the disruption efficiency of the cell wall for both species of microalgae. For *Chlorella* sp., the percentage oil yield continues to increase up to the 90% amplitude, reaching about 36%. On the other hand, at the 70% amplitude, ultrasonication of *Spirulina* sp. resulted in a 30% oil yield. After that, any increase in amplitude did not affect the oil yield significantly and remained nearly identical. Thus, the 70% amplitude maximizes *Chlorella* sp. lipid extraction without significantly affecting output, whereas a 90% amplitude is required for *Spirulina* sp. lipid extraction.

Lipid maximization for Soxhlet extraction was investigated with a combination of solvents at different ratios to enhance the extraction process, and the results are shown in Figure 6. Since hexane resulted in a poor oil yield of both species of microalgae, different solvents were used to extract the microalgae cells. Instead of using a single solvent, a mixture of solvents was used based on the recommendations

of previous research [17,58]. The goal of using a mixture of polar and non-polar solvent was to achieve maximum extraction efficacy compared to an earlier stage where only non-polar hexane was used. As seen in Figure 6, a C:H:M ratio of 1:1:1, 2:1:3, 3:2:1, and 1:2:3 resulted in about a 15.3%, 10%, 14%, and 13.3% percentage oil yield for *Chlorella* sp. On the other hand, a C:H:M ratio of 1:1:1, 2:1:3, 3:2:1, and 1:2:3 resulted in about a 23.3%, 12%, 15%, and 20% percentage oil yield for *Spirulina* sp. respectively. The higher percentage yield of oil was extracted from both species of microalgae compared to the hexane-only extraction. The combination of chloroform, hexane, and methanol at a ratio of 1:1:1 gave a maximum yield of crude oil for both species of microalgae. When comparing the yield of two species, *Spirulina* sp. showed a higher yield for every solvent combination ratio of chloroform, hexane, and methanol, as shown in Figure 6. Appendix B discusses the SEM images of dried microalgae after the Soxhlet extraction maximization process with 1:1:1 C:H:M.

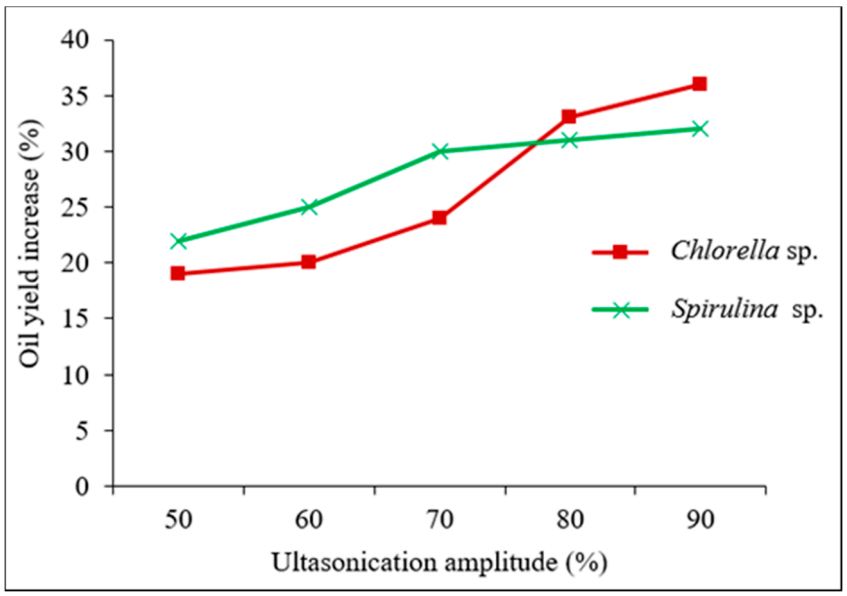


Figure 5. The percentage yield of crude oil for ultrasonication extraction.

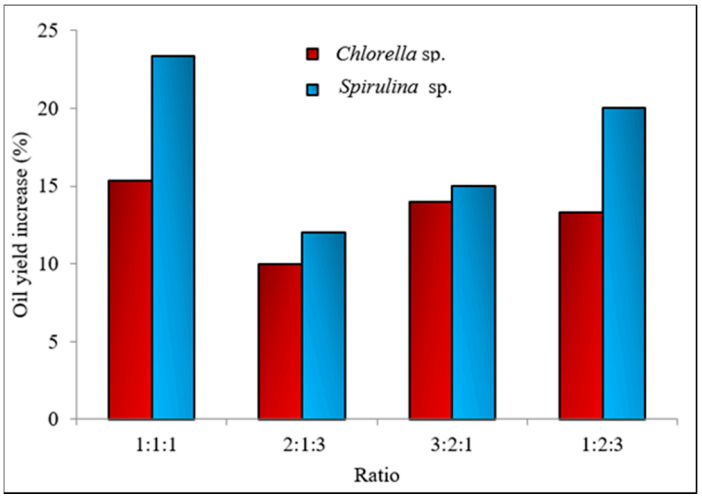


Figure 6. The percentage yield of microalgae by Soxhlet extraction with a solvent system of chloroform, hexane, and methanol (C:H:M) with different ratios.

3.3. Conversion to FAME by Lipase-Catalyzed Transesterification

Table 2 presents the acid value of extracted crude oil using three different methods. As seen from the table, the oil from the ultrasonic extraction process achieved the lowest acid value. Thus, transesterification was carried out for the sample extracted by ultrasonication only. Methanol was added at a molar ratio of methanol to the oil of 3:1 [44] (an excess amount of the stoichiometric ratio) into a mixture of fresh microalgae oil along with immobilized lipase in a stepwise manner to complete the transesterification process. In this study, one-third of methanol was added at the initial stage, one-third at 4 h, and the rest was added at 8 h. Methanol is known as a lipase inhibitor [59]; as such, high concentrations of methanol are unfavorable to lipase, catalyzing the effect and causing reduced enzyme activity and stability [60,61]. Thus, the stepwise addition of methanol is the standard choice since this not only avoids methanol's unfavorable effect but also expands the contact area of oil and methanol to achieve a high yield [50,62].

Table 2. Comparison study for the acid value of crude microalgae oil from three different methods of extraction.

Species	Acid Value(mg KOH/g)		
	Soxhlet Extraction	Ultrasonication	Solvent Extraction
Chlorella sp.	20.38	10.82	30.58
Spirulina sp.	22.05	16.24	38.47

3.4. Analysis of FTIR Peak for FAME

In the present study, the mid-FTIR region was selected to identify the functional groups in the biodiesel sample. The mid-IR range between the 4000 and 400 cm^{-1} wavenumbers. A summary of the IR spectroscopy band assignments for both microalgae species after ultrasonically assisted lipase-catalyzed transesterification is presented in Table 3. The bands were assigned to specific molecular groups based on biochemical standards and previous studies [63,64]. There were three significant peaks identified for characteristic bonds in the FAMEs for *Chlorella* sp. and *Spirulina* sp., which were the functional groups for alkanes, esters, and alkyls. The patterns of band distribution and peaks obtained were found to be similar for both species with closely matching peaks.

Table 3. Bands assignment of FTIR spectra for both species of microalgae.

Functional Group	Assignment	*Chlorella* sp.	*Spirulina* sp.
		Wavenumber, cm^{-1}	
Alkanes	C-H Stretching vibration	2922	2923
Esters	C=O stretching vibration	1741	1740
Alkanes	C-H bending vibration	1462	1462
Esters, alcohols	C-O stretching vibration	1166	1027

The region in the spectra from 3000 to 2800 cm^{-1} indicates the presence of lipids in the sample and is due to the symmetrical and asymmetrical stretching vibrations of $–CH_2–$ groups [65]. These $–CH_2–$ groups form the backbone of the lipids. Both *Chlorella* sp. and *Spirulina* sp. showed a transmittance peak at 2923 cm^{-1}, indicating the presence of the lipid backbone. An absorbance peak for *Spirulina* sp. was shown at a similar wavenumber by Nautiyal et al. [66]. The bands of interest to locate ester bonds in the compounds can be found in two regions: 1750–1735 cm^{-1} and 1320–1000 cm^{-1}. In particular, the peaks between 1750 and 1735 cm^{-1} were identified as C=O bonds of ester groups primarily from lipids and fatty acids, which indicates the conversion of oil to biodiesel [67]. Ester bond peaks were found at 1735, 1740, and 1741 cm^{-1} for these biodiesels, and show the presence of esters in the FAME analyzed. On the other hand, asymmetric deformation vibration of C-H [68] from proteins is usually found at ~1455 cm^{-1}, which represents the presence of alkanes.

3.5. Analysis of Ester Content by GC

The successful detection of ester bonds in the sample by FTIR analysis led to GC analysis to determine the FAME compound in the sample in detail. Table 4 shows the composition of the FAME ester content of the obtained biodiesel for each sample at different temperatures. Different concentrations of unsaturated fatty acids (FA) were observed for each biodiesel sample at different operating temperatures. It is to be noted that palmitic (C 16:0) and stearic (C 18:0) acids were the major components for both *Chlorella* sp. and *Spirulina* sp. biodiesel. The saturated FA percentage of both samples varies around 60–75%, whereas the unsaturated FA percentage varies around 25–40% of the FAME content. Palmitic and stearic acid contents were observed to be increased with the increase in temperature. The highest percentage of palmitic acid for both microalgae biodiesels as well as the highest stearic acid for *Chlorella* sp. was obtained at 45 °C. However, for *Spirulina* sp., the highest content of stearic acid was found at 40 °C, and the percentage decreased at 45 °C. Nautiyal et al. [66] also studied the ester content of *Spirulina* sp. and pond water algae-based biodiesel. Palmitic, linolenic, and linoleic acids were the major FA components in those samples. Adam et al. [55] studied the FAC of ultrasound-assisted *N. oculata* microalgae-based biodiesel. They found only three fatty acids in the biodiesel sample viz. myristic, palmitic, and stearic acids. Similar to the previous study, palmitic acid was the dominant ester content in this biodiesel.

Table 4. Percentage of ester content analyzed by GCMS at different transesterification operating temperatures.

		***Chlorella* sp.**				***Spirulina* sp.**			
Fatty Acids	**Lipid Chain**	**30 °C**	**35 °C**	**40 °C**	**45 °C**	**30 °C**	**35 °C**	**40 °C**	**45 °C**
Hexadecenoic (Palmitic)	C 16:0	28.72	35.62	44.04	45.60	29.06	30.16	33.12	46.81
7-Hexadecenoic (Palmitoleic)	C 16:1	-		-	-	0.325		-	-
7,10-Hexadecaenoic	C 16:2					2.41			
9-Octadecenoic (Oleic)	C 18:0	18.22	14.67			16.34	20.46		
11-Octadecenoic	C 18:1	-		-	17.39	-		26.50	23.50
9,12-Octadecadienoic (Linoleic)	C 18:2	13.95	18.72	27.60	6.56			13.45	9.66
Octadecanoic (Stearic)	C 18:0	27.82	28.05	28.23	30.45	25.51	26.35	26.93	20.03

4. Conclusions

The study compared the efficacy of different lipid extraction methods on two microalgae species viz: *Chlorella* sp. and *Spirulina* sp., since different species behave differently towards an extraction method based on the size, diameter, and cell walls of the microalgae itself. Ultrasonication was found to be the best method to extract lipid from both species of microalgae, with percentage oil yields of 6.6% for *Spirulina* sp. and 9.4% for *Chlorella* sp. On the other hand, the oil yields for these species were 1.05 and 1.25 g for Soxhlet extraction and 0.68 and 0.77 g for solvent extraction, respectively, when 30 g of sample was used. At maximum yield conditions, there was a 30% increase in yield for *Spirulina* sp. and 36% for *Chlorella* sp., respectively, compared to baseline conditions. The transesterification of ultrasonic-assisted extraction samples was successful for both microalgae species, indicating their suitability as a renewable energy source.

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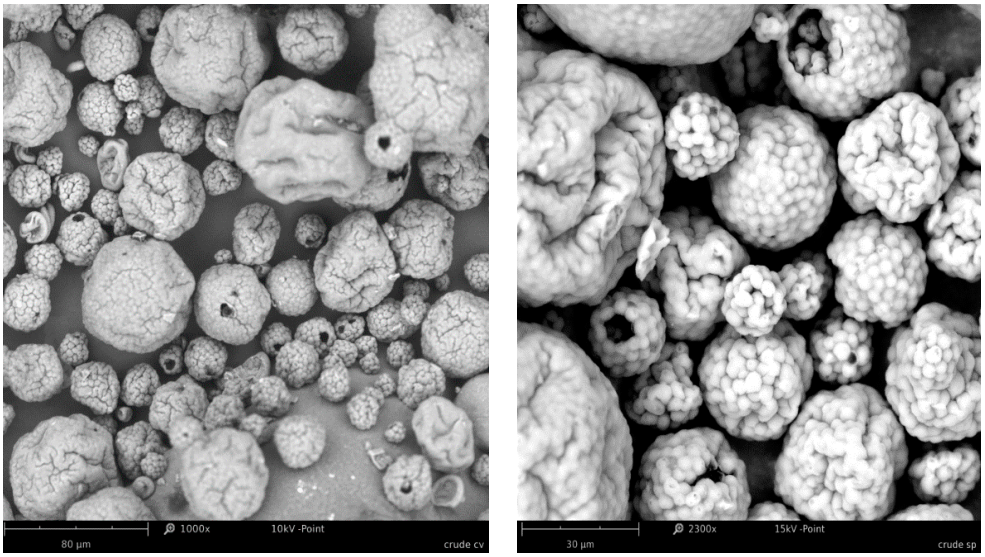
Appendix A. SEM Analysis of Microalgae for Chemical Extraction Methods

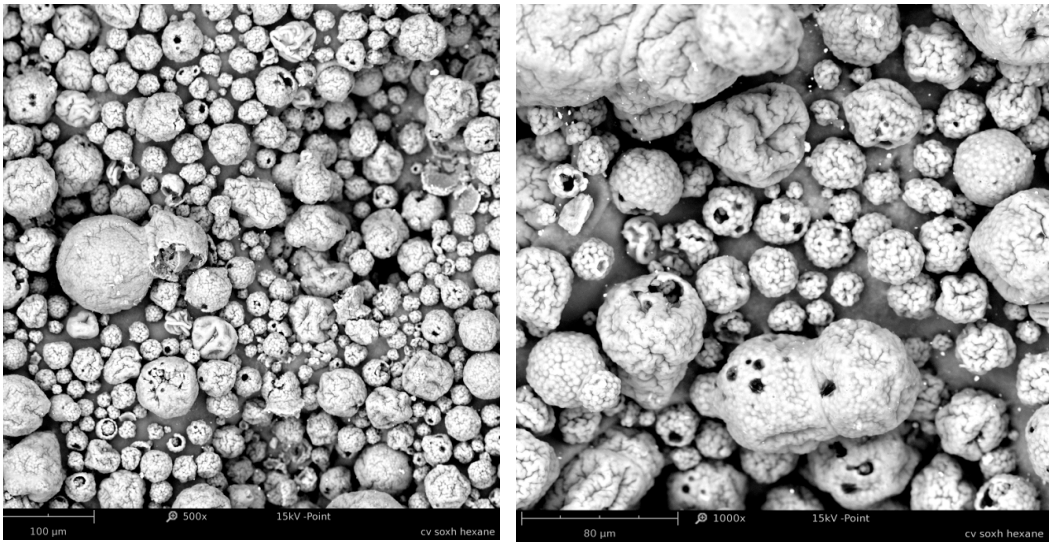
As shown in Figure A1, ultrasonication resulted in superior lipid extraction compared to other extraction processes. Structural analysis using SEM was performed here to study the extraction methods in depth. The structure of dried microalgae used in this study before extraction can be observed in Figure A1. For *Chlorella* sp., the cells have a spherical shape with an uneven surface of the phospholipid membrane, and appear to be clotted. From the EDX analysis, *Chlorella* sp. consisted of 40.2% carbon, 35.7% oxygen, and 23.4% nitrogen for the area analyzed. On the other hand, *Spirulina* sp. contained 43.7% carbon, 30.4% oxygen, and 25.2% nitrogen (25.2%) for the region analyzed.



(**a**) (**b**)

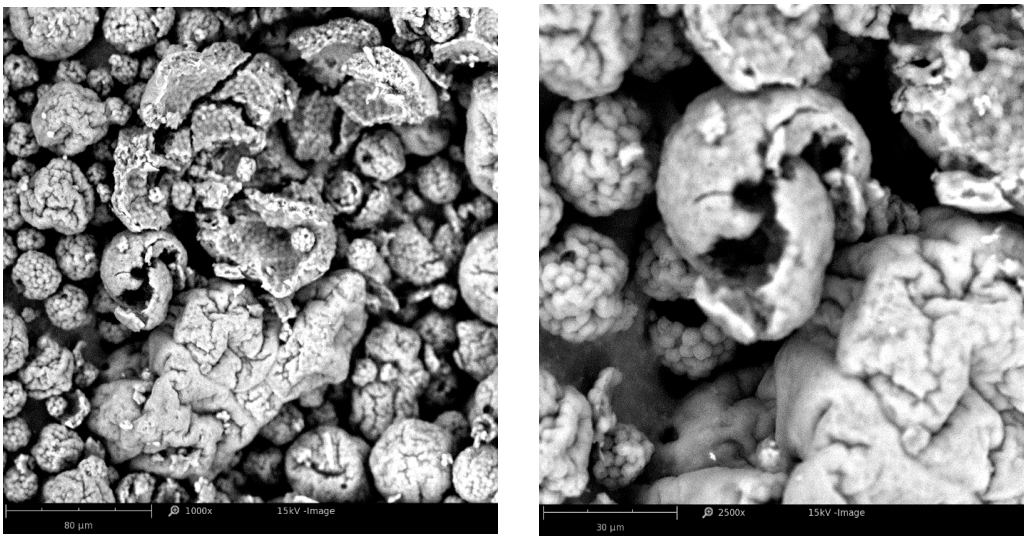
Figure A1. SEM image of dried microalgae, (**a**) *Chlorella* sp. at 1000× magnification 10 kV; (**b**) *Spirulina* sp. at 2300× magnification 15 kV.

Figures A2 and A3 show the structure of dried *Chlorella* sp. and *Spirulina* sp. after the Soxhlet extraction processes. The cells were shrunk compared to the original ones, some cells were broken, and the surfaces of those were distorted. Although Soxhlet extraction affected the structure of some microalgal cells, others were still intact, and the membrane cell did not show any damage. This explains the reason for the lower oil yield for Soxhlet extraction using hexane as the solvent. Hexane is the most popular and economical solvent commonly used in extraction studies. However, hexane was less effective in lipid extraction from *Chlorella* sp. compared to *Spirulina* sp., as seen in Figure A1. Comparing the 1000× magnification images from Figures A2 and A3, it is visible that *Spirulina* sp. cells were more affected by the process than those of *Chlorella* sp., thereby resulting in less yield. Since solvent extraction produced an even worse percentage, the oil extraction sample was not further analyzed. Consequently, a solvent mixture of multiple components was used further to maximize oil production using the Soxhlet extraction process.



(**a**) (**b**)

Figure A2. SEM image of dried *Chlorella* SP. after Soxhlet extraction by hexane at (**a**) 500× magnification, 15 kV; (**b**) 1000× magnification, 15 kV.



(**a**) (**b**)

Figure A3. SEM image of dried *Spirulina* SP. after Soxhlet extraction by hexane at (**a**) 1000× magnification, 15 kV; (**b**) 2500× magnification 15 kV.

Appendix B. SEM Analysis of Microalgae of Lipid Extraction Maximization Study

Structures of dried microalgae after Soxhlet extraction were also observed under SEM to see the difference. Figures A4 and A5 comprise the structure of microalgae of *Chlorella* sp. and *Spirulina* sp. with 1000× magnification. The cell membranes were damaged after the extraction, broken, shrunk into a smaller size, and the membrane was distorted from the original surface. The Soxhlet extraction with mixtures of chloroform, hexane, and methanol did have a significant effect on the whole structure of microalgae. The highest yield of oil was obtained from the combination of CHM at a 1:1:1 ratio.

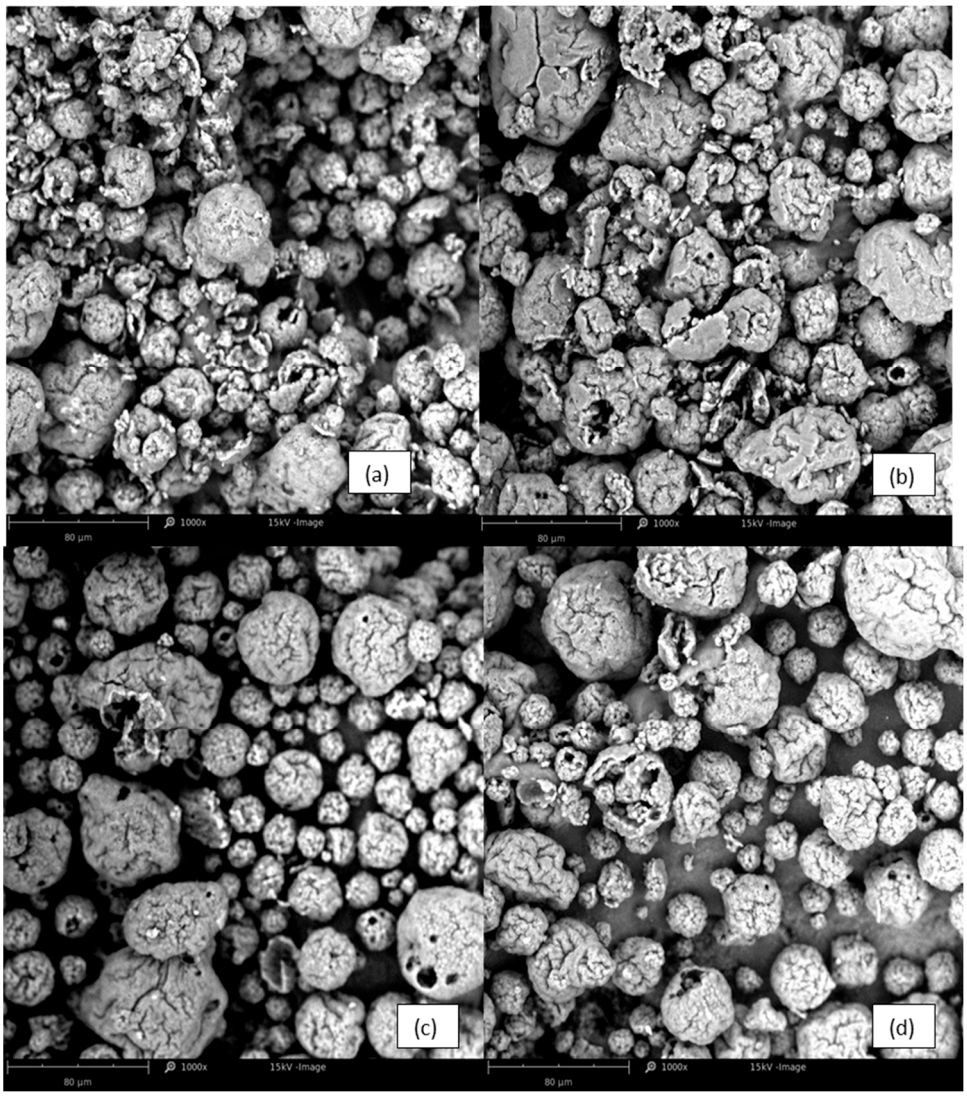


Figure A4. SEM image of dried *Chlorella* sp. after Soxhlet extraction by mixture of C:H:M at ratio of (**a**) 1:1:1; (**b**) 2:1:3; (**c**) 3:2:1, and (**d**) 1:2:3 at 1000× magnification 15 kV.

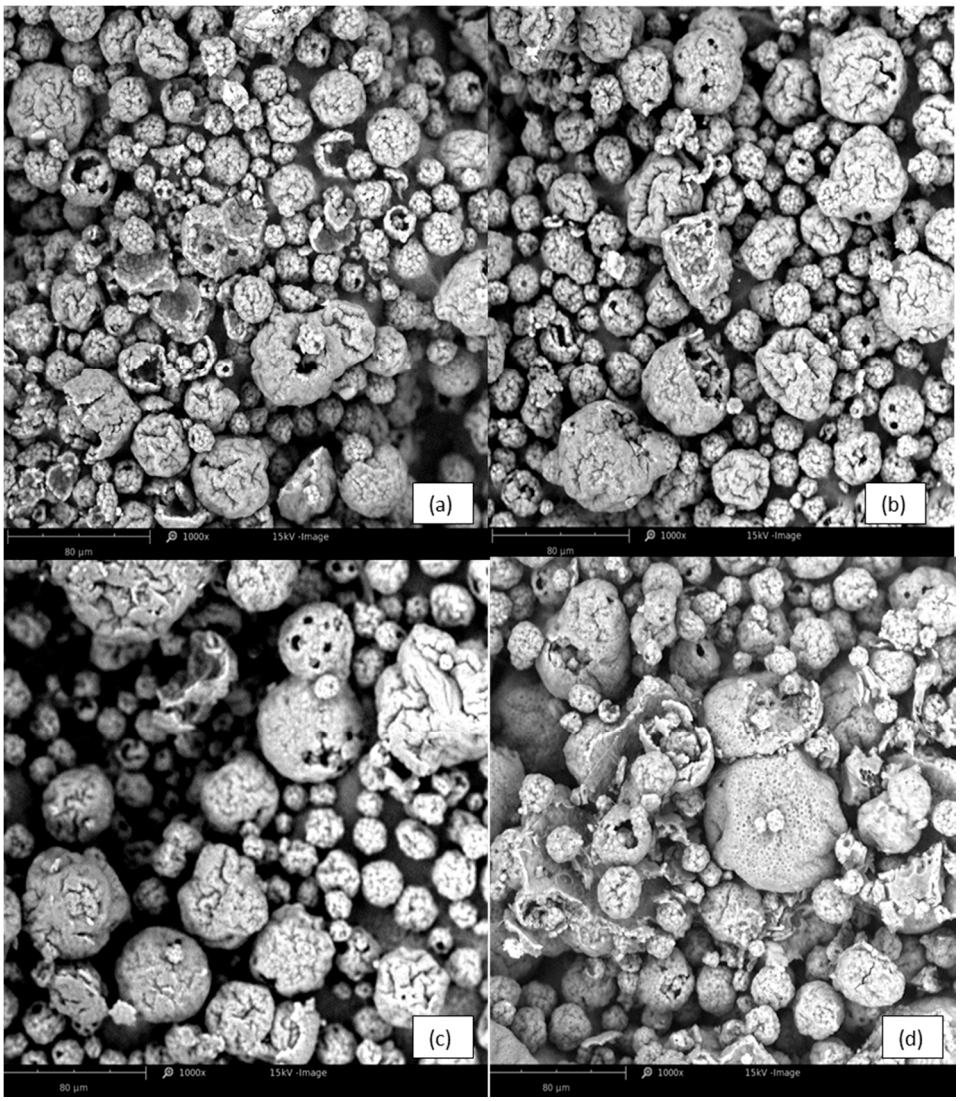


Figure A5. SEM image of dried *Spirulina* sp. after Soxhlet extraction by mixture of C:H:M at a ratio of (**a**) 1:1:1; (**b**) 2:1:3; (**c**) 3:2:1, and (**d**) 1:2:3 at 1000× magnification 15 kV.

References

1. Imtenan, S.; Varman, M.; Masjuki, H.H.; Kalam, M.A.; Sajjad, H.; Arbab, M.I.; Rizwanul Fattah, I.M. Impact of low temperature combustion attaining strategies on diesel engine emissions for diesel and biodiesels: A review. *Energy Convers. Manag.* **2014**, *80*, 329–356. [CrossRef]
2. Fattah, I.M.R.; Masjuki, H.H.; Kalam, M.A.; Hazrat, M.A.; Masum, B.M.; Imtenan, S.; Ashraful, A.M. Effect of antioxidants on oxidation stability of biodiesel derived from vegetable and animal based feedstocks. *Renew. Sustain. Energy Rev.* **2014**, *30*, 356–370. [CrossRef]
3. Masjuki, H.H.; Ruhul, A.M.; Mustafi, N.N.; Kalam, M.A.; Arbab, M.I.; Rizwanul Fattah, I.M. Study of production optimization and effect of hydroxyl gas on a CI engine performance and emission fueled with biodiesel blends. *Int. J. Hydrog. Energy* **2016**, *41*, 14519–14528. [CrossRef]
4. Mahlia, T.M.I.; Syazmi, Z.; Mofijur, M.; Abas, A.E.P.; Bilad, M.R.; Ong, H.C.; Silitonga, A.S. Patent landscape review on biodiesel production: Technology updates. *Renew. Sustain. Energy Rev.* **2020**, *118*. [CrossRef]
5. Ong, H.C.; Masjuki, H.H.; Mahlia, T.M.I.; Silitonga, A.S.; Chong, W.T.; Yusaf, T. Engine performance and emissions using Jatropha curcas, Ceiba pentandra and Calophyllum inophyllum biodiesel in a CI diesel engine. *Energy* **2014**, *69*, 427–445. [CrossRef]

6. Noraini, M.Y.; Ong, H.C.; Badrul, M.J.; Chong, W.T. A review on potential enzymatic reaction for biofuel production from algae. *Renew. Sustain. Energy Rev.* **2014**, *39*, 24–34. [CrossRef]
7. Ong, H.C.; Milano, J.; Silitonga, A.S.; Hassan, M.H.; Shamsuddin, A.H.; Wang, C.T.; Mahlia, T.M.I.; Siswantoro, J.; Kusumo, F.; Sutrisno, J. Biodiesel production from Calophyllum inophyllum-Ceiba pentandra oil mixture: Optimization and characterization. *J. Clean. Prod.* **2019**, *219*, 183–198. [CrossRef]
8. Silitonga, A.S.; Masjuki, H.H.; Mahlia, T.M.I.; Ong, H.C.; Chong, W.T.; Boosroh, M.H. Overview properties of biodiesel diesel blends from edible and non-edible feedstock. *Renew. Sustain. Energy Rev.* **2013**, *22*, 346–360. [CrossRef]
9. Fattah, I.M.R.; Ong, H.C.; Mahlia, T.M.I.; Mofijur, M.; Silitonga, A.S.; Rahman, S.M.A.; Ahmad, A. State of the Art of Catalysts for Biodiesel Production. *Front. Energy Res.* **2020**, *8*. [CrossRef]
10. Larson, E.D. A review of life-cycle analysis studies on liquid biofuel systems for the transport sector. *Energy Sustain. Dev.* **2006**, *10*, 109–126. [CrossRef]
11. Chowdhury, H.; Loganathan, B. Third-generation biofuels from microalgae: A review. *Curr. Opin. Green Sustain. Chem.* **2019**, *20*, 39–44. [CrossRef]
12. Lee, X.J.; Ong, H.C.; Gan, Y.Y.; Chen, W.-H.; Mahlia, T.M.I. State of art review on conventional and advanced pyrolysis of macroalgae and microalgae for biochar, bio-oil and bio-syngas production. *Energy Convers. Manag.* **2020**, *210*, 112707. [CrossRef]
13. Lardon, L.; Hélias, A.; Sialve, B.; Steyer, J.-P.; Bernard, O. Life-Cycle Assessment of Biodiesel Production from Microalgae. *Environ. Sci. Technol.* **2009**, *43*, 6475–6481. [CrossRef] [PubMed]
14. Clarens, A.F.; Resurreccion, E.P.; White, M.A.; Colosi, L.M. Environmental Life Cycle Comparison of Algae to Other Bioenergy Feedstocks. *Environ. Sci. Technol.* **2010**, *44*, 1813–1819. [CrossRef] [PubMed]
15. Silitonga, A.; Shamsuddin, A.; Mahlia, T.; Milano, J.; Kusumo, F.; Siswantoro, J.; Dharma, S.; Sebayang, A.; Masjuki, H.; Ong, H.C. Biodiesel synthesis from Ceiba pentandra oil by microwave irradiation-assisted transesterification: ELM modeling and optimization. *Renew. Energy* **2020**, *146*, 1278–1291. [CrossRef]
16. Starbuck, C.M. Comment on "Environmental Life Cycle Comparison of Algae to Other Bioenergy Feedstocks". *Environ. Sci. Technol.* **2011**, *45*, 833. [CrossRef]
17. Grima, E.M.; González, M.J.I.; Giménez, A.G. Solvent Extraction for Microalgae Lipids. In *Algae for Biofuels and Energy*; Borowitzka, M.A., Moheimani, N.R., Eds.; Springer: Dordrecht, The Netherlands, 2013; pp. 187–205. [CrossRef]
18. Lai, J.-Q.; Hu, Z.-L.; Wang, P.-W.; Yang, Z. Enzymatic production of microalgal biodiesel in ionic liquid [BMIm][PF6]. *Fuel* **2012**, *95*, 329–333. [CrossRef]
19. Gerken, H.G.; Donohoe, B.; Knoshaug, E.P. Enzymatic cell wall degradation of Chlorellavulgaris and other microalgae for biofuels production. *Planta* **2013**, *237*, 239–253. [CrossRef]
20. Lee, J.-Y.; Yoo, C.; Jun, S.-Y.; Ahn, C.-Y.; Oh, H.-M. Comparison of several methods for effective lipid extraction from microalgae. *Bioresour. Technol.* **2010**, *101*, S75–S77. [CrossRef]
21. Milano, J.; Ong, H.C.; Masjuki, H.H.; Silitonga, A.S.; Chen, W.-H.; Kusumo, F.; Dharma, S.; Sebayang, A.H. Optimization of biodiesel production by microwave irradiation-assisted transesterification for waste cooking oil-Calophyllum inophyllum oil via response surface methodology. *Energy Convers. Manag.* **2018**, *158*, 400–415. [CrossRef]
22. Ranjith Kumar, R.; Hanumantha Rao, P.; Arumugam, M. Lipid Extraction Methods from Microalgae: A Comprehensive Review. *Front. Energy Res.* **2015**, *2*. [CrossRef]
23. Crampon, C.; Nikitine, C.; Zaier, M.; Lépine, O.; Tanzi, C.D.; Vian, M.A.; Chemat, F.; Badens, E. Oil extraction from enriched Spirulina platensis microalgae using supercritical carbon dioxide. *J. Supercrit. Fluids* **2017**, *119*, 289–296. [CrossRef]
24. Goh, B.H.H.; Ong, H.C.; Chong, C.T.; Chen, W.-H.; Leong, K.Y.; Tan, S.X.; Lee, X.J. Ultrasonic assisted oil extraction and biodiesel synthesis of Spent Coffee Ground. *Fuel* **2020**, *261*, 116121. [CrossRef]
25. Wang, M.; Yuan, W. Microalgal cell disruption in a high-power ultrasonic flow system. *Bioresourc. Technol.* **2015**, *193*, 171–177. [CrossRef]
26. Folch, J.; Ascoli, I.; Lees, M.; Meath, J.A.; LeBaron, F.N. Preparation of lipide extracts from brain tissue. *J. Biol. Chem.* **1951**, *191*, 833–841.
27. Medina, A.R.; Grima, E.M.; Giménez, A.G.; González, M.J.I. Downstream processing of algal polyunsaturated fatty acids. *Biotechnol. Adv.* **1998**, *16*, 517–580. [CrossRef]

28. Wei, X.; Jie, D. Optimization to Hydrothermal Liquefaction of Low Lipid Content Microalgae *Spirulina* sp. Using Response Surface Methodology. *J. Chem.* **2018**, *2018*, 2041812. [CrossRef]
29. Muhammad, G.; Alam, M.A.; Mofijur, M.; Jahirul, M.I.; Lv, Y.; Xiong, W.; Ong, H.C.; Xu, J. Modern developmental aspects in the field of economical harvesting and biodiesel production from microalgae biomass. *Renew. Sustain. Energy Rev.* **2021**, *135*, 110209. [CrossRef]
30. Liang, K.; Zhang, Q.; Gu, M.; Cong, W. Effect of phosphorus on lipid accumulation in freshwater microalga Chlorella sp. *J. Appl. Phycol.* **2013**, *25*, 311–318. [CrossRef]
31. Ahmad, A.L.; Yasin, N.H.M.; Derek, C.J.C.; Lim, J.K. Microalgae as a sustainable energy source for biodiesel production: A review. *Renew. Sustain. Energy Rev.* **2011**, *15*, 584–593. [CrossRef]
32. Turkkul, B.; Deliismail, O.; Seker, E. Ethyl esters biodiesel production from *Spirulina* sp. and Nannochloropsis oculata microalgal lipids over alumina-calcium oxide catalyst. *Renew. Energy* **2020**, *145*, 1014–1019. [CrossRef]
33. Chia, S.R.; Ong, H.C.; Chew, K.W.; Show, P.L.; Phang, S.-M.; Ling, T.C.; Nagarajan, D.; Lee, D.-J.; Chang, J.-S. Sustainable approaches for algae utilisation in bioenergy production. *Renew. Energy* **2018**, *129*, 838–852. [CrossRef]
34. Olguín, E.J.; Galicia, S.; Angulo-Guerrero, O.; Hernández, E. The effect of low light flux and nitrogen deficiency on the chemical composition of *Spirulina* sp. (Arthrospira) grown on digested pig waste. *Bioresourc. Technol.* **2001**, *77*, 19–24. [CrossRef]
35. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]
36. Sharma, A.K.; Sahoo, P.K.; Singhal, S.; Joshi, G. Exploration of upstream and downstream process for microwave assisted sustainable biodiesel production from microalgae Chlorella vulgaris. *Bioresourc. Technol.* **2016**, *216*, 793–800. [CrossRef]
37. Parichehreh, R.; Gheshlaghi, R.; Mahdavi, M.A.; Elkamel, A. Optimization of lipid production in Chlorella vulgaris for biodiesel production using flux balance analysis. *Biochem. Eng. J.* **2019**, *141*, 131–145. [CrossRef]
38. Pohndorf, R.S.; Camara, Á.S.; Larrosa, A.P.Q.; Pinheiro, C.P.; Strieder, M.M.; Pinto, L.A.A. Production of lipids from microalgae *Spirulina* sp.: Influence of drying, cell disruption and extraction methods. *Biomass Bioenergy* **2016**, *93*, 25–32. [CrossRef]
39. Kusdiana, D.; Saka, S. Effects of water on biodiesel fuel production by supercritical methanol treatment. *Bioresourc. Technol.* **2004**, *91*, 289–295. [CrossRef]
40. Rizwanul Fattah, I.M.; Masjuki, H.H.; Kalam, M.A.; Wakil, M.A.; Ashraful, A.M.; Shahir, S.A. Experimental investigation of performance and regulated emissions of a diesel engine with Calophyllum inophyllum biodiesel blends accompanied by oxidation inhibitors. *Energy Convers. Manag.* **2014**, *83*, 232–240. [CrossRef]
41. Abedin, M.J.; Masjuki, H.H.; Kalam, M.A.; Sanjid, A.; Rahman, S.M.A.; Fattah, I.M.R. Performance, emissions, and heat losses of palm and jatropha biodiesel blends in a diesel engine. *Ind. Crops Prod.* **2014**, *59*, 96–104. [CrossRef]
42. Amini, Z.; Ilham, Z.; Ong, H.C.; Mazaheri, H.; Chen, W.-H. State of the art and prospective of lipase-catalyzed transesterification reaction for biodiesel production. *Energy Convers. Manag.* **2017**, *141*, 339–353. [CrossRef]
43. Karmee, S.K. Enzymatic Biodiesel Production from Manilkara Zapota (L.) Seed Oil. *Waste Biomass Valorization* **2018**, *9*, 725–730. [CrossRef]
44. Tran, D.-T.; Yeh, K.-L.; Chen, C.-L.; Chang, J.-S. Enzymatic transesterification of microalgal oil from Chlorella vulgaris ESP-31 for biodiesel synthesis using immobilized Burkholderia lipase. *Bioresourc. Technol.* **2012**, *108*, 119–127. [CrossRef]
45. Ortiz, C.; Ferreira, M.L.; Barbosa, O.; dos Santos, J.C.S.; Rodrigues, R.C.; Berenguer-Murcia, Á.; Briand, L.E.; Fernandez-Lafuente, R. Novozym 435: The "perfect" lipase immobilized biocatalyst? *Catal. Sci. Technol.* **2019**, *9*, 2380–2420. [CrossRef]
46. Fajardo, A.R.; Cerdán, L.E.; Medina, A.R.; Fernández, F.G.A.; Moreno, P.A.G.; Grima, E.M. Lipid extraction from the microalga Phaeodactylum tricornutum. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 120–126. [CrossRef]
47. Mofijur, M.; Masjuki, H.H.; Kalam, M.A.; Atabani, A.E.; Rizwanul Fattah, I.M.; Mobarak, H.M. Comparative evaluation of performance and emission characteristics of Moringa oleifera and Palm oil based biodiesel in a diesel engine. *Ind. Crops Prod.* **2014**, *53*, 78–84. [CrossRef]
48. Fattah, I.M.R.; Masjuki, H.H.; Kalam, M.A.; Mofijur, M.; Abedin, M.J. Effect of antioxidant on the performance and emission characteristics of a diesel engine fueled with palm biodiesel blends. *Energy Convers. Manag.* **2014**, *79*, 265–272. [CrossRef]

49. Mofijur, M.; Kusumo, F.; Fattah, I.; Mahmudul, H.; Rasul, M.; Shamsuddin, A.; Mahlia, T. Resource Recovery from Waste Coffee Grounds Using Ultrasonic-Assisted Technology for Bioenergy Production. *Energies* **2020**, *13*, 1770. [CrossRef]
50. Shimada, Y.; Watanabe, Y.; Sugihara, A.; Tominaga, Y. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *J. Mol. Catal. B Enzym.* **2002**, *17*, 133–142. [CrossRef]
51. Dizge, N.; Aydiner, C.; Imer, D.Y.; Bayramoglu, M.; Tanriseven, A.; Keskinler, B. Biodiesel production from sunflower, soybean, and waste cooking oils by transesterification using lipase immobilized onto a novel microporous polymer. *Bioresourc. Technol.* **2009**, *100*, 1983–1991. [CrossRef]
52. Halim, R.; Gladman, B.; Danquah, M.K.; Webley, P.A. Oil extraction from microalgae for biodiesel production. *Bioresourc. Technol.* **2011**, *102*, 178–185. [CrossRef] [PubMed]
53. Wang, L.; Weller, C.L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **2006**, *17*, 300–312. [CrossRef]
54. Safi, C.; Ursu, A.V.; Laroche, C.; Zebib, B.; Merah, O.; Pontalier, P.-Y.; Vaca-Garcia, C. Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. *Algal Res.* **2014**, *3*, 61–65. [CrossRef]
55. Adam, F.; Abert-Vian, M.; Peltier, G.; Chemat, F. "Solvent-free" ultrasound-assisted extraction of lipids from fresh microalgae cells: A green, clean and scalable process. *Bioresourc. Technol.* **2012**, *114*, 457–465. [CrossRef]
56. Halim, R.; Harun, R.; Danquah, M.K.; Webley, P.A. Microalgal cell disruption for biofuel development. *Appl. Energy* **2012**, *91*, 116–121. [CrossRef]
57. Lee, S.J.; Yoon, B.-D.; Oh, H.-M. Rapid method for the determination of lipid from the green alga Botryococcus braunii. *Biotechnol. Tech.* **1998**, *12*, 553–556. [CrossRef]
58. Deshmukh, S.; Kumar, R.; Bala, K. Microalgae biodiesel: A review on oil extraction, fatty acid composition, properties and effect on engine performance and emissions. *Fuel Process. Technol.* **2019**, *191*, 232–247. [CrossRef]
59. Shimada, Y.; Watanabe, Y.; Samukawa, T.; Sugihara, A.; Noda, H.; Fukuda, H.; Tominaga, Y. Conversion of vegetable oil to biodiesel using immobilized Candida antarctica lipase. *J. Am. Oil Chem. Soc.* **1999**, *76*, 789–793. [CrossRef]
60. You, Q.; Yin, X.; Zhao, Y.; Zhang, Y. Biodiesel production from jatropha oil catalyzed by immobilized Burkholderia cepacia lipase on modified attapulgite. *Bioresourc. Technol.* **2013**, *148*, 202–207. [CrossRef]
61. Li, X.; Xu, H.; Wu, Q. Large-scale biodiesel production from microalga Chlorella protothecoides through heterotrophic cultivation in bioreactors. *Biotechnol. Bioeng.* **2007**, *98*, 764–771. [CrossRef]
62. Yan, Y.; Li, X.; Wang, G.; Gui, X.; Li, G.; Su, F.; Wang, X.; Liu, T. Biotechnological preparation of biodiesel and its high-valued derivatives: A review. *Appl. Energy* **2014**, *113*, 1614–1631. [CrossRef]
63. Zhang, W.-B. Review on analysis of biodiesel with infrared spectroscopy. *Renew. Sustain. Energy Rev.* **2012**, *16*, 6048–6058. [CrossRef]
64. Stehfest, K.; Toepel, J.; Wilhelm, C. The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. *Plant Physiol. Biochem.* **2005**, *43*, 717–726. [CrossRef] [PubMed]
65. Kar, B.; Sahoo, H.; Rath, S.S.; Das, B. Investigations on different starches as depressants for iron ore flotation. *Miner. Eng.* **2013**, *49*, 1–6. [CrossRef]
66. Nautiyal, P.; Subramanian, K.A.; Dastidar, M.G. Production and characterization of biodiesel from algae. *Fuel Process. Technol.* **2014**, *120*, 79–88. [CrossRef]
67. Dean, A.P.; Sigee, D.C.; Estrada, B.; Pittman, J.K. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. *Bioresourc. Technol.* **2010**, *101*, 4499–4507. [CrossRef]
68. Huang, Y.; Wei, C.; Liao, Q.; Xia, A.; Zhu, X.; Zhu, X. Biodegradable branched cationic starch with high C/N ratio for Chlorella vulgaris cells concentration: Regulating microalgae flocculation performance by pH. *Bioresourc. Technol.* **2019**, *276*, 133–139. [CrossRef]