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Chlorella sp. extraction and estimation of fuel properties of lipids derived from FFA profiles

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Abstract

Lipid extraction from marine *Chlorella* sp. was performed with various techniques including Soxhlet, heating and stirring with a hot plate magnetic stirrer, heating and shaking in a water bath and sonication. Cell disruption methods with sonication and microwave heating were also investigated. A single extraction in an ultrasonic bath was found to be the most suitable method. The optimum solvent system, extraction time and biomass preparation were investigated considering the extraction yield and quality parameters predicted by fatty acid compositions. Extraction in a binary solvent system, n-hexane/methanol (1:2 v/v), for 80 min using freeze-dried algae was optimal as the extraction yield was higher and the fuel properties estimated by the fatty acid composition meet biodiesel standards.

Keyword: Microalgae, *Chlorella* sp., Extraction, Crude lipid, Ultrasonic, Biodiesel

1. Introduction

Fuels from algae have received a growing interest as they have certain advantages compared to other feed stocks. Algae have higher biomass production due to their rapid growth rate [1], produce more lipids per area compared to traditional oil crops [2], do not compete for farmland and can grow anywhere there is moisture [3]. However, there are technical methods that need further development and economic challenges to be overcome before commercialization of algal fuels can be achieved. *Chlorella* sp. is a class of single-cell green freshwater and marine microalgae with a small size, 2–15 µm. It has a high oil content, about 28 to 32% of its dry weight [4]. Our previous study showed that marine *Chlorella* sp. Cultivated using a fertilizer medium in an open pond could provide an economically viable feed stock for biodiesel production [5]. As the microalgae are small with the complex cell membranes as well as thick and rigid cell walls, a suitable oil extraction method is unlike those used for terrestrial oilseed crops. Algal cell disruption techniques including mechanical, chemical, physical and biological methods that are usually applied before lipid extraction. Prabakaran and Ravindran [6] examined five cell disruption methods (autoclaving, bead milling, microwave treatment, sonication and osmotic shock using a 10% NaCl solution) for lipid extraction from fresh water microalgae (*Chlorella* sp., *Nostoc* sp. and *Tolypothrix sp*.). Sonication was found to be the most promising technique. Zheng et al. [7] compared grinding, ultrasonication, bead milling, enzymatic lysis and microwave treatments for disruption of marine *Chlorella vulgaris*. Grinding in liquid nitrogen gave the highest lipid yield followed by enzymatic lysis by cellulose, microwave treatment, sonication and bead milling.

The extraction methods for microalgal lipids are categorized as mechanical (expeller, microwave assisted extraction and ultrasonic assisted extraction), chemical (Soxhlet extraction, supercritical fluid extraction, and accelerated solvent extraction or pressurized fluid extraction) methods. Dai et al. [8] reported that crude oil yields extracted from dry powders of microalgae using three methods, heating with a reflux condenser and magnetic stirrer, ultrasonication, and microwave extraction were 14, 5 and 18 wt%, respectively. Hussain et al. [9] studied three lipid extraction methods, hexane Soxhlet (Sox-Hex), Halim (HIP) and Bligh and Dyer (BD) performed on freeze-dried (FD) and oven-dried (OD) *Chlorella vulgaris.* They found that HIP was the most suitable method with the highest lipid yields of 20 and 22% on FD and OD biomass, respectively. Microalgal biomass contains a high water content, 80-90% [10]. Oils are difficult to extract from wet biomass with solvents without cell disruption, but are more easily extracted from freeze dried biomass [11]. Lipids can be classified into three categories as polar lipids such as phospholipids and glycolipids, neutral/ non- polar lipids such as mono- , di- and tri-acylglycerols, and free fatty acids [12]. The solvents normally used for algal lipid extraction are n- hexane, ethanol, 1-butanol, dimethyl ether, and mixtures of chloroform/methanol, n-hexane/ ethanol, n-hexane/isopropanol, dichloroethane/methanol, dichloroethane/ethanol, and acetone/dichloromethane. Chloroform/ methanol (1: 2 v/ v) is the most widely used

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organic solvent system for lipid extraction from biomass. The Bligh and Dyer method [13] and its modified forms have been extensively applied. The combination of polar and non-polar solvents assists lipid extraction as the ability of a polar solvent to release lipids from protein-lipid complexes facilitates their dissolution in a non-polar solvent [12]. The effect is greater when lipid extraction is done with wet algal biomass. This is because the polar solvent can access the water layer and make the lipids more accessible to non-polar solvent solvation.

Microalgal lipids must comply with some set specifications stipulated by current standards before they can be used as fuels. These parameters include its ignition quality, heat of combustion, cold flow, oxidative stability, viscosity, and lubricity. These properties can be estimated from the amounts of specific fatty acids present in the lipid [14].

This work includes a comparative study of lipid extraction from marine *Chlorella* sp. using various techniques, with and without cell disruption. Once the suitable technique is decided, the optimum solvent system, extraction time and biomass preparation are investigated considering the extraction yield, energy consumption and quality parameters predicted by the fatty acid composition. The procedure of lipid extraction from marine *Chlorella* sp. cultivated with a fertilizer medium in an open pond system is then proposed for economically feasible biodiesel production.

2. Materials and methods

2.1 Microalgal preparation

The marine *Chlorella* sp. used in this study was cultivated in 25-tonne open ponds at the National Institute of Coastal Aquaculture (NICA), Songkhla, Thailand using $CO(NH_2)_2$ and 16-16-16 fertilizer (consisting of 16% nitrogen, 16% phosphorus, and 16% potassium) in a culture media using bubbling air at atmospheric pressure. The microalgae were harvested after two weeks of cultivation using aluminum sulfate for flocculation. The microalgal flocs were cleaned with deionized water and filtered. *Chlorella* sp. was harvested and approximate 4 kg (dry basis) per algal pond was obtained. The microalgae were divided into two portions, one was kept as freeze-dried sample and ground into powder in a ceramic mortar. The other one was gathered as a microalgae paste.

In the freeze-drying method, *Chlorella* cells were thinly coated on the inside of a bottle and frozen at -30 °C. A FTS Systems Dura-Dry, Flexi-Dry μ P with a vacuum of 500 Torr was used during a 12 h drying period. An algae paste was prepared by dewatering using vacuum filtration with Whatman No. 4 filter paper and vacuum a pump. The moisture content of the paste was determined before it was stored at -5 °C.

2.2 Lipid extraction

2.2.1 Cell disruption and extraction equipment tests

For disruption, hexane was the selected solvent because it is normally used for lipid extraction. Freeze-dried algae was employed in this test. Two cell disruption methods, sonication in an ultrasonic bath and microwave irradiation, were studied along with the extraction methods. Microwave irradiation was done in a 0.021 m^{-3} microwave oven (LG MS2120VW, LG Electronics, Korea) at 1,100 W for 5 min, while sonication was accomplished using an ultrasonic bath (CP-2600 HT Ultrasonic Cleaner, Crest Ultrasonics, USA, internal dimensions: $49.5 \times 29.7 \times 20$ cm) operating at 42 kHz with an input power of 300 W for 5 min.

Four lipid extraction methods using various types of equipment including Soxhlet, heating and stirring with a hot plate magnetic stirrer, heating and shaking in a water bath, and sonication in an ultrasonic bath were done. A 10 g mass of freeze-dried *Chlorella* sp. was placed in a 750 mL vessel and mixed with n-hexane at a ratio of 10:500 g/mL. The mixture was extracted at 60 °C with stirring and shaking methods at 250 rpm. In the Soxhlet method, extraction was done at the solvent boiling point. Although there was no heat application with sonication, the temperature of the solvent was increased due to cavitation and acoustic streaming, but to not more than 50 °C. Sonication was done for 20 min, while the other three extraction methods ran for 4 h. All extraction experiments were conducted in triplicate. A diagram of the experimental setup is shown in Figure 1.

2.2.2 Solvent test

The freeze-dried *Chlorella* was mixed with 350 mL of solvent at a ratio of 10:100 g (dry basis)/mL in a 500-mL Duran bottle. It was then extracted in an ultrasonic bath working at 42 kHz and 300 W for 20-80 min, resulting in solvent temperatures of 40-50 °C. The investigated solvents were n-hexane, acetone, n-hexane/acetone (1:2 v/v), n-hexane/methanol (1:2 v/v), chloroform/methanol (1:2 v/v) and chloroform/methanol/water (1:2:0.8 v/v/v). The optimum equipment, time and solvent for dry cell extraction were used for algal paste extraction. A 10 g mass (dry basis) of algae paste (81.9% moisture) was also used for comparative extraction with a dried cell extract. Both freeze-dried and paste algal extractions were performed in triplicate.

2.3 Analytical methods

The extracted solution was vacuum filtered and then centrifuged for 10 min at 4,000 rpm to precipitate the remaining residue. Solvent was evaporated using a rotary evaporator and the remaining extract was kept in a desiccator at room temperature until it was weighed to calculate the extraction yield (dry basis) as follows:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of dried biomass}} \times 100\% \quad (1)$$

Fatty acid composition was analyzed using GC to determine fatty acid methyl esters (FAMEs) with a gas chromatograph-flame ionization detector (GC-FID, Hewlett Packard 6890, USA) equipped with 30 m CP9080 capillary column (0.32 mm internal diameter and 0.25 µm film thickness). The FAMEs were prepared by placing 150 mg of the extract into a capped test tube where they were transesterified with 1 mL of KOH-CH_3OH. The split ratio was 50:1 and the detector temperature was 300 °C. The oven temperature program started at 210 °C for 12 min, increased at 20 °C/min until 250 °C and kept at this temperature for 8 min. The carrier gas, He, was flowed at a constant rate of 1 mL/min.

The physicochemical properties of oil, saponification value (*SV*), cetane number (*CN*), iodine value (*IV*), degree of unsaturation (*DU*), long chain saturation factor (*LCSF*) and cold filter plugging point (*CFPP*, °C) were calculated from the fatty acid composition using the following empirical equations [15-17].

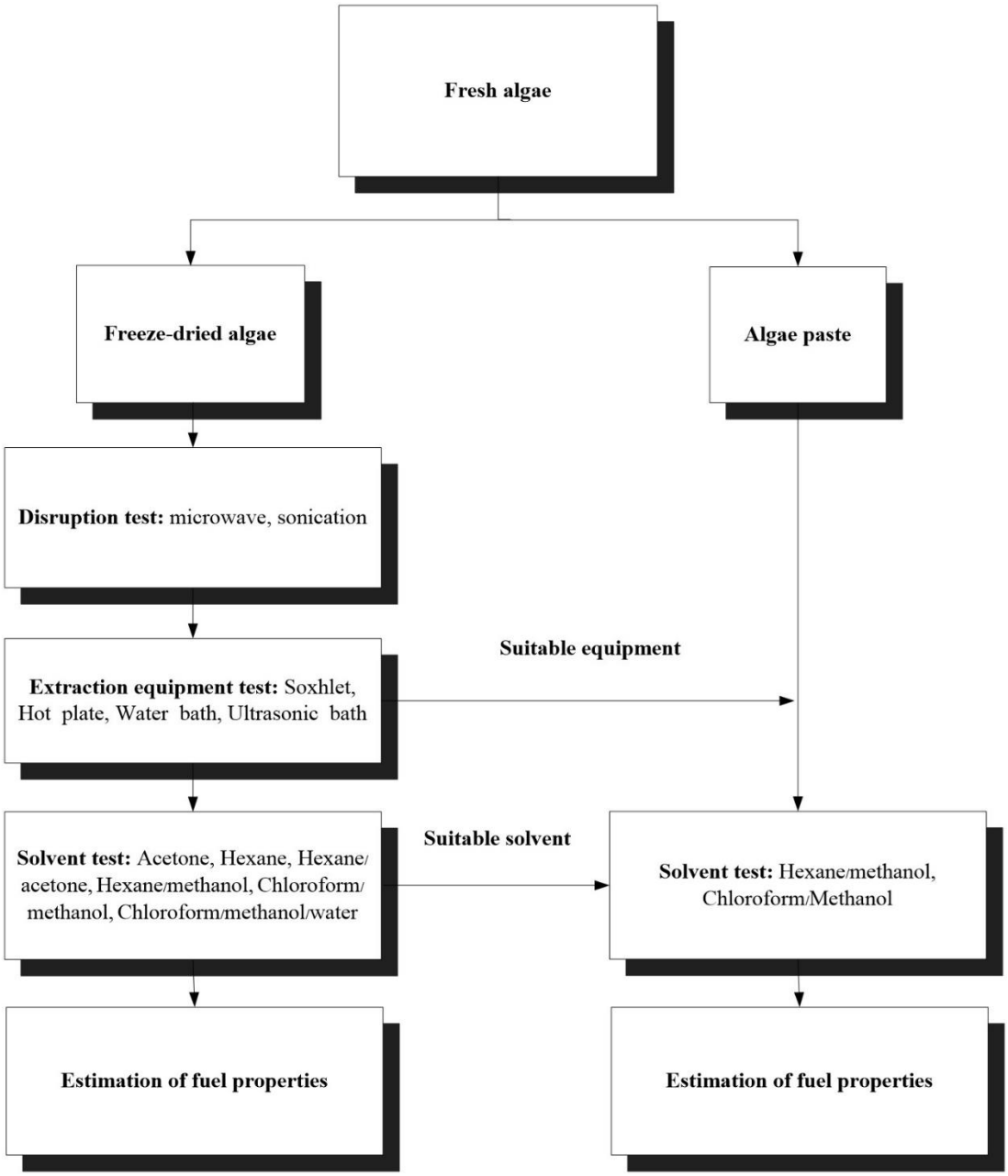

Figure 1 Methodology flow diagram.

$$IV = \sum\left(\frac{254 \times F \times D}{MW}\right) \quad (2)$$

$$SV = \sum\left(\frac{560 \times F}{MW}\right) \quad (3)$$

$$CN = 46.3 + \left(\frac{5458}{SV}\right) - (0.225 \times IV) \quad (4)$$

$$DU = MUFAwt\% + (2 \times PUFAwt\%) \quad (5)$$

$$LCSF = (0.1 \times C16:0) + (0.5 \times C18:0) + (1 \times C20:0) + (1.5 \times C22:0) + (2 \times C24:0) \quad (6)$$

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (7)$$

Where F is the percentage of each fatty acid, D is the number of double bonds of each fatty acid, MW is the molecular weight of a corresponding fatty acid, $MUFA$ is the weight percentage of monounsaturated fatty acid, and $PUFA$ is the weight percentage of polyunsaturated fatty acid.

The ρ (density at 20 °C, g cm^{-3}) and Y (oxidation stability, h) of each sample can be calculated using the following equations [18-19]:

$$\rho = 0.8463 + \frac{4.9}{MW} + (0.0118 \times D) \quad (8)$$

$$Y = \frac{117.9295}{X} + 2.5905 \quad (9)$$

Where X is the percentage of C18:2 and C18:3.

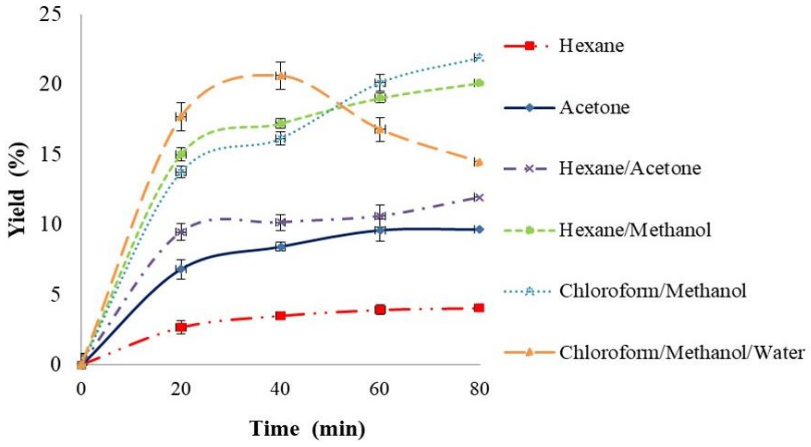

Figure 2 Effect of time and solvent system on the extraction of freeze-dried *Chlorella* sp. using an ultrasonic bath

3. Results and discussion

3.1 Effects of cell disruption and extraction techniques

Little published data is available for comparison of equipment used for lipid extraction from microalgae. Some studies proposed disruption before extraction. The lipid yield from dried algae was greatly impacted by the extraction method used, as is shown in Table 1. Generally, Soxhlet is the conventional method for oil extraction from plants. However, this is not the case for oil extraction from *Chlorella* sp. The extraction yield of the Soxhlet method was lowest, although it was performed at the highest temperature. This is because the cell wall of *Chlorella* sp. is thick and consists of a polysaccharide and glycoprotein matrix that provides it with a formidable defense against its environment [20]. The Soxhlet method uses solvent diffusion without additional mixing, therefore, it gave the lowest yield. Another disadvantage of Soxhlet extraction is lipid degradation resulting from the use of elevated temperatures [12].

Table 1 Extraction yields using various methods with n-hexane solvent. Results are presented as mean ± standard deviation (SD) of triplicate runs.

Method	Yield (%)
Soxhlet 4 h	5.52±0.66
Ultrasonic bath 5 min + Soxhlet 4 h	13.43±0.30
Hot plate magnetic stirrer 4 h	9.78±0.64
Water bath 4 h	6.54±0.76
Microwave 5 min + Water bath 4 h	13.05±0.55
Ultrasonic bath 5 min + Water bath 4 h	15.26±0.34
Ultrasonic bath 20 min	15.90±0.20

Magnetic stirring with better mixing gave increased yields over shaking methods. Cell disruption in an ultrasonic bath for 5 min increased the extraction yields of the Soxhlet method from 5.52 to 13.43%. Cell disruption in an ultrasonic bath and in a microwave field improved the extraction yields of the shaking method. The ultrasonic bath provided better cell disruption than microwave treatment in this study as it gave greater yields with less energy. Prabakaran and Ravindran [6] reported that the algal cell disruption by sonication was more efficient than microwave treatment. The major advantage of the ultrasonication over microwave treatment is lower heat generation, therefore less thermal denaturation of biomolecules.

In this study, an ultrasonic bath was used in a single-step extraction method. Twenty minutes of extraction in an ultrasonic bath offered the highest yield compared to the other extraction methods, even though they operated with longer times. The process is facile, requiring less time and energy. Ultrasonic waves are those above the audible range (>20 kHz) and below the microwave frequencies (up to 10 MHz). There are two phenomena produced during ultrasonication, cavitation and acoustic streaming. Cavitation produces micro-bubbles that can create pressure within cells to break them up, while acoustic streaming facilitates mixing of the algal culture [21]. A number of factors can affect the formation of cavitation bubbles, including external acoustic and solvent factors. It is established that high density, low viscosity, and mid- level surface tension and vapor pressure are the ideal conditions to produce intense ultrasonic cavitation. The most intense cavitation occurs well below a liquid's boiling point, but not so low as to produce low vapor pressure or high surface tension [22]. Solvent selection is therefore quite important and this is discussed in the next section.

3.2 Effect of sonication time, solvent and biomass preparation

As shown in Figure 2, the extraction yields from freeze-dried algae rapidly increased in the first 20 min for every solvent. The extraction yield by ultrasonication is normally found to increase with time. A longer extraction time can increase mass transfer and enhance it until the solvent achieves saturation, limiting mass transfer. The extraction in this experiment occurred in two stages. The first is a washing step, in which soluble components are dissolved from the surfaces of the matrix. The second stage is slow extraction. Here, mass transfer of the solute from the matrix into the solvent occurs by diffusion. This mechanism is also observed in the extraction of phenolic compounds using ultrasound assisted extraction [23].

Extraction with chloroform/ methanol/ water (1: 2: 0. 8 v/v/v) reached its highest yield at 40 min, while the yields of other solvents still continued increase from 40 to 80 min. This extended time, past 40 min, using chloroform/methanol/water caused a decrease in the lipid content. Prolonged exposure to sonication also stimulated the generation of free radicals deteriorating the lipid quality through oxidation [24].

A binary chloroform/ methanol solvent (1: 2 v/ v) offered the highest extraction yield of 21.91% for 80 min, followed

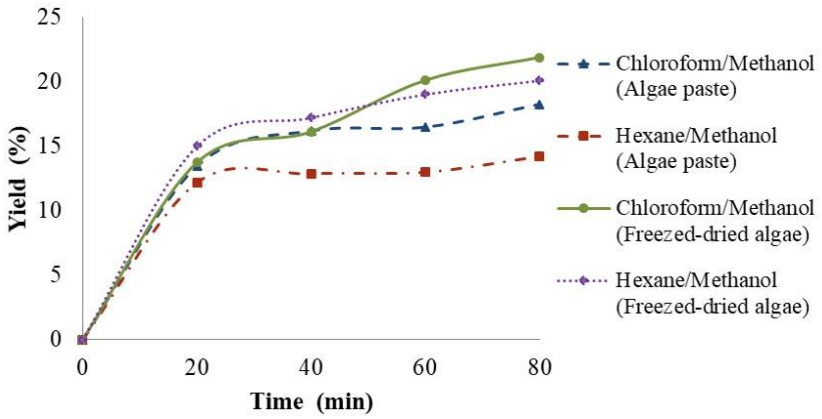

Figure 3 Effect of biomass preparation on the extraction of *Chlorella* sp. using an ultrasonic bath with chloroform/methanol and n-hexane/methanol solvent systems

by a chloroform/ methanol/ water (1:2:0.8 v/v/v) for 40 min and a n- hexane/ methanol (1: 2 v/ v) solvent for 80 min. Chloroform/ methanol (1: 2 v/ v) is the most widely used organic solvent system for lipid extraction from biomass and animal tissues [25]. N- hexane alone presented a very poor yield of 4. 06% at 80 min. Acetone gave better yields than n-hexane, but lower than n-hexane/acetone (1:2 v/v) for every extraction time. Frigo- Vaz & Wang [26] also reported that acetone was better than hexane for lipid extraction from *Chlamydomonas reinhardtii*. The solvents used in the current study can be arranged in order of increasing polarity as methanol>acetone>chloroform>n-hexane. N-hexane, which presents the lowest polarity index in this study, is good only for lipids of low polarity. Microalgal lipids can be grouped into two categories, storage lipids or non-polar lipids and structural lipids or polar lipids [27]. Non-polar lipids are mainly in the form of triacylglycerides (TAGs) that are primarily saturated fatty acids with some degree of unsaturation. Polar lipids generally have a high content of polyunsaturated fatty acids (PUFAs). Algal lipids consist of both non- polar and polar lipids, therefore the extraction yields obtained by a combination solvents are higher than those by the individual solvents. Use of solvent mixtures enhances lipid extraction because a polar solvent can extract more polar lipids and is likely penetrate cell walls, thus making TAGs more available to a non-polar solvent [28]. Nevertheless, conflicting results are presented. Shen et al. [29] found that hexane/ ethanol (1:1 v/v) gave lower lipid yields than hexane for extraction of *Scenedesmus dimorphus* and *Chlorella protothecoides*. The disparate results come from variation of lipid composition from different algal species. Additionally, diverse ratios of solvents also gave various extraction efficiencies. Therefore, only suitable proportions of polar and non-polar solvents can accomplish maximal lipid extraction.

The extraction yields (dry basis) of algae pastes were lower than those from freeze-dried algae, as seen in Figure 3. With the chloroform/ methanol, only a 3. 66% yield from an algae paste was observed, while a 5. 86% yield was obtained with n-hexane/methanol at 80 min. This is due a lower ability of n- hexane in water to dissolve lipids than chloroform. Chloroform is a popular solvent for lipid extraction. However, it is as an extremely hazardous substance and is subject to strict regulations for facilities that produce, store, or use it in significant quantities. Therefore, large-scale use of chloroform is unfeasible due to its environmental and health risks. N- hexane/ methanol (1: 2 v/ v), which is less toxic, is considered an alternative binary solvent.

Use of the Halim method (HIP), a hexane/ isopropanol (3: 2 v/ v) solvent system has also been proposed for lipid extraction from microalgae using ultrasound [9]. This is because of its low toxicity and similar extraction efficiency to the Bligh and Dyer method. The polarity index of the solvent mixture has high influence on lipid extraction from *Isochrysis galbana*, as the lipid yield increases with the polarity index. The polarity index can be calculated as:

$$PI_{mix} = \sum X_i PI_i \quad (10)$$

where PI_{mix} and PI_i are polarity index of the mixture and the component i, respectively, and X_i is the volumetric fraction of component i in the solvent mixture. The polarity index of pure hexane, chloroform, methanol, isopropanol and, water are 0, 4.1, 5.1, 4.3 and 9, respectively [30].

The polarity index of the Bligh and Dyer solvent system is high (5.66) compared to 1:2 v/v hexane/methanol (3.4), and for 3: 2 v/ v hexane/ isopropanol (1.72). Methanol is more inexpensive than isopropanol and it is also a raw material for biodiesel production. A hexane/methanol solvent system can also be applied for single extraction and biodiesel production. Hexane/ methanol (1: 2 v/ v) is then proposed as a suitable solvent system for lipid extraction from *Chlorella* sp. in the current study.

Drying algae is energy intensive and expensive. Use of a wet microalgae biomass has been proposed by many researchers [12, 25, 28]. However, there are reports that moisture seriously hampers lipid extraction and recovery by the restricting contact between hydrophobic extraction solvents and biomass dispersed in a water phase. If a paste sample can be extracted using an appropriate method, energy savings will result [12]. A limitation of wet biomass extraction was also observed in this investigation. Additionally, lipid separation is more difficult with a wet algal paste as evaporation requires longer times.

3.3 Fatty acid profiles and quality parameters

Several studies reported on the fatty acid profiles of lipids extracted from microalgae. However, there is very limited information on lipids extracted with various solvents and algae preparation methods, as was done in the course of the current investigation. The fatty acid profiles of freeze-dried algae and algae paste are presented in Table 2. It was observed that the percentages of unidentified substances, especially from freeze-dried biomass were quite high.

Table 2 The FAME profiles of oils extracted by several solvent systems from freeze-dried algae and algae paste

Fatty acid	Fatty acid composition (%)							
	Freeze-dried algae						Algae paste	
	Ace	Hex	CH_3Cl+MeOH+H_2O	Hex+Ace	CH_3Cl+MeOH	Hex+MeOH	CH_3Cl+MeOH	Hex+MeOH
C8:0	0.36	0	0.29	0.99	0	0	0	0
C9:0	0	0	0	0	0	0	0	0.01
C10:0	0.34	0	0.25	0.22	0	0	0.03	0.12
C11:0	0	0	0	0	0.18	0	0.05	0.03
C12:0	0.88	1.05	0.60	1.03	0.55	0.50	0.33	0.43
C13:0	0	0	0	0	0	0	0.53	0.64
C14:0	8.66	5.66	6.28	7.57	8.17	7.61	4.53	4.71
C15:0	0	0	0	0	0.49	0.44	0.40	0.37
C16:0	24.17	27.73	19.18	21.06	24.02	22.77	25.25	23.86
C16:1	19.58	15.99	23.13	16.75	22.59	19.83	21.64	20.80
C17:0	0	0	0	0	0.29	0.38	0.14	0.17
C18:0	1.18	2.33	0.02	0.78	0.30	0.45	0.00	0.25
C18:1	3.81	5.69	0.23	4.67	4.27	4.54	0.21	0.18
C18:2	1.11	1.82	2.17	1.65	1.93	2.19	2.15	2.02
C18:3	0	0	0.09	0.04	0	0	0.04	0.04
C20:0	0.22	0	0	0.13	0	0	0	0
C22:0	0	0	0	0	0	0	25.34	27.74
C22:1	0	0	0	0	0	0	0	0.05
C24:0	0	0	0	0	0	0	0.04	0.02
C24:1	0	0	0	0	0	0	0	0.03
Unknown	39.69	39.73	47.76	45.11	37.21	41.29	19.32	18.53

Table 3 The predicted physicochemical properties of oils extracted using several solvent systems from freeze-dried algae and algae paste

Solvent	DU	ρ (g/cm^3)	SV (mg KOH/g)	IV (g I_2/100 g fat)	CN	LCSF	CFPP (Summer/ Winter) (°C)	Y (h)
Biodiesel Standard EN 14214	-	0.86-0.90	-	≤120	≥51	-	≤5/≤-20	≥6
Biodiesel Standard ASTM D6751-02	-	-	-	-	≥47	-	-	-
DOEB B100 Spec., B.E. 2009	-	0.86-0.90	-	≤120	≥51	-	-	≥10
Acetone (Freeze-dried algae)	43	0.88	212	39	63	5	0.3	67
Hexane (Freeze-dried algae)	42	0.88	208	38	64	7	4.1	42
Chloroform/Methanol/Water (Freeze-dried algae)	53	0.88	212	50	61	4	-4.9	30
Hexane/Acetone (Freeze-dried algae)	45	0.88	213	42	63	5	-1.4	41
Chloroform/Methanol (Freeze-dried algae)	49	0.88	210	45	62	4	-3.7	41
Hexane/Methanol (Freeze-dried algae)	49	0.88	209	45	62	4	-3.1	34
Chloroform/Methanol (Algae paste)	33	0.88	194	30	68	50	141.7	46
Hexane/Methanol (Algae paste)	31	0.88	193	29	68	54	153.8	49

One reason for this is that the fatty acids found in the samples are not in the investigated standards. Analysis by gas chromatography-mass spectrometry (GC-MS) is suggested for further study to identify the unknowns. However, the results of this work still indicate the effect of extraction solvents on the fatty acid profiles. Although there were some distinct fatty acid profiles found with the use of various solvents, the majority of fatty acids from freeze-dried algae were the same, i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), myristic acid (C14:0) and oleic acid (C18:1, n-9). Stearic acid (C18:0), linoleic acid (C18:2, n-6), lauric acid (C12:0) were found in lower concentrations. Palmitic acid was the most prominent using most solvents other than chloroform/methanol/water, in which the palmitoleic acid content was more pronounced. Palmitic acid is also the primary microalgae fatty acid found in other studies [31-32]. Additionally, C16 and C18 compounds are the most abundant microalgal fatty acids. These include palmitic, stearic, oleic, linoleic, and linolenic acids. The amounts of other fatty acids such as C14, C20, and C26-C32 are relatively low [33]. Yang et al. [34] reported that lipids with high palmitic and oleic acid contents can be used to produce good quality biodiesel.

The fatty acid profile of the lipids extracted by n-hexane/methanol was similar to that of chloroform/methanol. For algae paste, the most prominent fatty acid was behenic acid (C22:0), while this compound was not found in freeze-dried biomass. However, other fatty acids in lipids from algae paste were similar to those of freeze-dried algae.

The physicochemical properties of the extracted lipids were calculated from their fatty acid composition and are presented in Table 3. Saturated fatty acids (SFA) have a high influence on fuel properties [35]. The CN increment corresponds to high SFA, but IV, CFPP, kinematic viscosity, and density are largely dependent on their degree of unsaturation. To obtain high biodiesel quality, the saturation and unsaturation of FAMEs should be balance. The DUs found in this work are in the range of 31-53, whereas the DU of palm oil is about 67.9 [36]. The greatest DU was found with chloroform/methanol/water while using n-hexane/methanol with algae paste gave the minimum value. The oil extracted

with freeze-dried algae by n-hexane/ methanol gave a DU of 49.

Density affects fuel atomization and combustion characteristics. The density of biodiesel is higher than diesel resulting in a greater fuel consumption [37]. The densities of palm oil and biodiesel standard EN 14214 are 0.921-0.924 and 0.86–0.90 g/cm^3, respectively. The estimated density of lipids of all extracted lipids in the current study was the same, 0.88 g/cm^3.

SV expresses the amount of potassium hydroxide in mg needed to saponify one gram of oil. It is an index of the average molecular mass of fatty acid in an oil sample. A shorter average chain length (C4-C12) has a higher SV. Islam et al. [35] reported that the SV calculated from FAME profiles of lipids from nine species of microalgae (not including *Chlorella* sp.) were 184-210 mg KOH/ g oil. The predicted SVs in this study are 193-213 mg KOH/g oil, which is similar to palm oil, 195-205 mg KOH/ g oil [38]. The high SV value indicates that the oil is suitable for industrial use. Saponification values of the oils from algae paste are lower than those from freeze-dried biomass. The predicted SV of the lipid using n- hexane/ methanol as solvent is comparable to that of a lipid extracted using chloroform/methanol.

IV is the grams of iodine that react with 100 grams of oil. The IV in this study is 29-50 g I_2/100 g oil. The standard IV of palm oil is in the range of 45-56 g I_2/100 g oil [38]. IV reveals adulteration of oil. High IV indicates a high level of unsaturation and susceptibility to oxidative rancidity. Iodine values of oils from freeze-dried algae are a bit higher than the oils from algae paste. The IVs of the oils extracted using n-hexane/ methanol are almost the same as those extracted with chloroform/methanol.

The cetane number indicates the combustion behavior of diesel fuel and is inversely related to the ignition delay time, the time between injection and ignition. From the biodiesel standards, ASTM D6751-02 and EN14214, the minimum CN should be 47 and 51, respectively. A biodiesel fuel with a greater CN can be blended at higher concentrations with petroleum diesel. The investigated oil shows CN values of 61-68, which are higher than the standard values. CN values of the algae paste oils are slightly higher than those of freeze-dried algae oils. The oils using n-hexane/ methanol have the same CN values as those extracted using chloroform/methanol.

LCSF is used for CFPP calculations. CFPP indicates the lowest temperature at which fuel will still flow. A high CFPP means vehicle fuel lines will clog easily. The EN14214 biodiesel standard sets the CFPP as 5 and -20 °C in summer and winter, respectively. All the oils extracted from freeze-dried algae show CFPP values that meet the standard, while the values of oils from algae paste failed.

The oxidation stability, Y, primary affects the stability of fuel during long-term storage. It may decrease with increasing levels of PUFA and is influenced by several factors, such as presence of air, heat, light, and structural features of the compounds [17]. An oil that is high in linoleic acid, linolenic acid, and other PUFA has poor oxidation stability. The oxidative stabilities of the extracts of the current study shown in Table 3 are 30-67 h, which are above the biodiesel standard EN14214 ($\geq$ 6 h). The oxidative stabilities of oils from eight species of microalgae were reported to be 4.9-5.6 h, except *Nannochloropsis oculata*, which was 95.7 h [37].

Lipid yields from dry and wet algal samples extracted with various solvents were compared with other studies. This work extends these earlier studies by examining the fuel properties estimated from FFA profiles of lipids extracted with various solvents. It is found that all of the lipids from freeze-dried algae can meet the required biodiesel standards, while the lipids from algae pastes failed to meet the CFPP biodiesel standards. The lipids extracted by n-hexane/methanol have similar properties to those extracted with chloroform/ methanol.

4. Conclusions

Comparison of Soxhlet, hot plate magnetic stirrer, and ultrasonic extraction from marine *Chlorella* sp. showed that the ultrasonication process is the most effective method. Disruption and extraction can be performed simultaneously with ultrasonication. This eliminates the need for disruption before extraction. The use of mixed polar and non- polar solvents is recommended to enhance the extraction yield. N-hexane/ methanol (1: 2 v/ v) can be used as an alternative binary solvent system to chloroform/ methanol and Bligh and Dyer solvent systems. N-hexane/ methanol shows a comparative lipid yield and is much less toxic. An optimum extraction time of 80 min using freeze- dried algae is more suitable than algae paste in terms of its yield and estimated fuel properties. All extracted lipids from freeze- dried algae can meet the biodiesel standards, while the lipids extracted from algae paste failed to meet the CFPP biodiesel standards. Use of dried algae is also good for lipid separation as it is easier to evaporate the solvent.

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