The effect of solvents polarity and extraction conditions on the microalgal lipids yield, fatty acids profile, and biodiesel properties

Mohammad Javad Zarrinmehr [a,b], Ehsan Daneshvar [a,c,*], Subhasha Nigam [d], Kannappan Panchamoorthy Gopinath [e], Jayanta Kumar Biswas [f,g], Eilhann E. Kwon [h], Hailong Wang [i,j], Omidvar Farhadian [b], Amit Bhatnagar [a,c]

[a] Department of Environmental and Biological Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211 Kuopio, Finland
[b] Department of Natural Resources, Isfahan University of Technology, Isfahan 8415683111, Iran
[c] Department of Separation Science, LUT School of Engineering Science, LUT University, Sammonkatu 12, FI-50130 Mikkeli, Finland
[d] Amity Institute of Biotechnology, Amity University, Noida Uttar Pradesh 201313, India
[e] Department of Chemical Engineering, Sri Sivasubramaniya Nadar College of Engineering, Kalavakkam, 603110 Chennai, Tamil Nadu, India
[f] Department of Ecological Studies, University of Kalyani, Nadia, West Bengal, India
[g] International Centre for Ecological Engineering, University of Kalyani, Kalyani, West Bengal, India
[h] Department of Environment and Energy, Sejong University, Seoul 05005, Republic of Korea
[i] Biochar Engineering Technology Research Center of Guangdong Province, School of Environmental and Chemical Engineering, Foshan University, Foshan, Guangdong 528000, PR China
[j] Key Laboratory of Soil Contamination Bioremediation of Zhejiang Province, School of Environmental and Resource Sciences, Zhejiang A&F University, Hangzhou, Zhejiang 311300, PR China

HIGHLIGHTS

- The effect of polar and non-polar solvents on microalgal biodiesel was investigated.
- The amount of extracted lipids by non-polar solvents was higher than polar solvents.
- A higher amount of saturated fatty acids was obtained by non-polar solvents.
- The cetane number was higher for the extracted lipids by non-polar solvents.
- Reaction temperature affected the saturation degree of fatty acids.

GRAPHICAL ABSTRACT
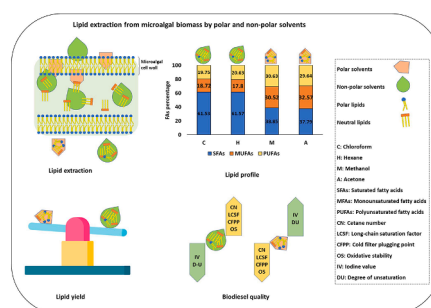
ARTICLE INFO

ABSTRACT

This study reports the effects of polar (acetone/methanol) and non-polar (chloroform/hexane) solvents on lipid yield, fatty acids methyl esters (FAMEs) composition, and biodiesel properties of microalgae. The lipids yield extracted by hexane and chloroform (100.01 and 94.33 mg/g) were higher than by methanol and acetone (40.12 and 86.91 mg/g). The polarity of solvents also affected FAMEs composition of microalgal lipids. Total saturated fatty acids and unsaturated fatty acids of extracted lipids were 61.53% and 38.47% by chloroform and 38.85% and 61.15% by methanol. Moreover, polar and non-polar solvents affected the biodiesel properties such as cetane number and oxidative stability. In addition, higher ratio of chloroform to methanol and higher temperature

* Corresponding author.
  E-mail address: ehsan.daneshvar@lut.fi (E. Daneshvar).

increased the lipid yield and saturation degree of lipids, through ultrasound-assisted lipid extraction method. Overall, the results revealed that the lipids yield, FAMEs composition, and biodiesel quality of microalgal biomass can be significantly affected by solvents polarity and extraction conditions.

1. Introduction

Although microalgae have been highlighted as a promising feedstock for biodiesel production, biodiesel from microalgal lipids needs to meet the required quality authorized by international standards such as European standards (EN 14214) and American Society for Testing and Materials (ASTM D6751) (de Jesus et al., 2020; Vignesh et al., 2020). The quality of microalgal biodiesel towards ignition, lubrication, and oxidation stability is determined based on the key parameters including cetane number (CN), iodine value (IV), saponification value (SV), cold filter plugging point (CFPP), degree of unsaturation (DU), calorific value (CV), and kinematic viscosity (KV). These absolute values depend on the compositional matrix of fatty acids methyl esters (FAMEs) (Rinna et al., 2017). The fatty acids of microalgae are divided into saturated (without double bonds), monounsaturated (one double bond) and polyunsaturated (more than one double bonds) fatty acids with a 12–22 linear carbon length (Wu and Miao, 2014). The properties of biodiesel are highly contingent on the degree of saturation and a carbon length of fatty acid (Deshmukh et al., 2019). Saturated fatty acids provide storage stability to the biodiesel and protect it from autooxidation during a long-term storage, while unsaturated fatty acids with lower viscosity are beneficial in terms of cold flow characteristics (Wu and Miao, 2014).

In this respect, it has been reported that lipid extraction methods greatly affect the yield and quality of lipids that are extracted from microalgal biomass (Ortiz-Martínez et al., 2019). Lipid extraction from microalgal biomass is mainly performed by the chemical and mechanical methods or their combinations (Mubarak et al., 2015). Solvent extraction, supercritical CO_2 extraction, ultrasound-assisted extraction, microwave-assisted extraction, thermochemical liquefaction, and pressurized liquid extraction are some of the common methods of lipids extraction from microalgal biomass (Iqbal and Theegala, 2013). Usually, the process is preceded by mechanical cell disruption followed by chemical solvent extraction. Chloroform, toluene, benzene diethyl ether, n-hexane, methanol, ethyl acetate, acetone, ethyl acetate, and ethanol are some of the common organic solvents for lipid extraction (Mubarak et al., 2015). Although, these organic solvents have been frequently used for lipid extraction, but the effects of their degree polarity and their different mixture ratios on lipid yield, fatty acids composition, and biodiesel quality have rarely been studied.

Accordingly, this study was conducted to investigate the effect of different ratios (1:0, 1:1, 1:1:1, and 1:1:1:1) of polar (methanol and acetone) and non-polar (chloroform and hexane) solvents on the amount of extracted lipids, FAMEs composition, and microalgal biodiesel quality. Subsequently, different mixture ratios of chloroform to methanol were used for the extraction of lipid by ultrasound-assisted method at different sonication time, and reaction temperature. The findings of this study could be useful for improving the quality of microalgal biodiesel, based on the selection of suitable extraction solvents and extraction conditions.

2. Materials and methods

2.1. Chemicals

High-performance liquid chromatography (HPLC) and Gas Chromatography (GC) analysis grade of methanol (≥99.9%), chloroform (≥99.9%), hexane (≥95%), and acetone (≥99.9%) were purchased from Sigma-Aldrich (Finland Oy). Analytical grade of sulfuric acid (Min 95%) was acquired from Fisher Scientific Oy (Finland). Supelco 37 Component FAME Mix and heptadecanoic acid (C17:0) were procured from

Sigma-Aldrich (Finland Oy).

2.2. Microalgal cultivation

Scenedesmus quadricauda (*S. quadricauda*) (Chlorophyceae) was used as model microalgae in this study. The first stock of this species was purchased from Culture Collection of Algae and Protozoa (CCAP, Scotland, UK). Microalgae was cultivated in dairy wastewater, collected from a local factory. *S. quadricauda* was cultivated in 30 L flat photobioreactor at 25 °C. The culture was illuminated via a white LED tube under 12:12 photoperiod and 100 μmol photons/m^2/s light intensity. After 7 days, a partial of culture was centrifuged and microalgal cells were dried at 60 °C for 24 h. The dried biomass was preserved for lipid extraction experiments.

2.3. Experimental design

2.3.1. Phase I. Lipid extraction by polar and non-polar solvents
Methanol (M), chloroform (C), hexane (H), acetone (A) and their binary, ternary, and quaternary mixtures were used for the extraction of lipids from microalgal biomass. For this purpose, 30 polypropylene tubes (15 experimental runs with two replicates) were labeled. 100 mg of microalgal biomass was added to each tube. In the next step, 10 mL of organic solvent was added to each tube. Consequently, the tubes were agitated on a shaker at 80 rpm for 30 min at 25 °C, and the mixture was centrifuged at 7000 rpm for 5 min. Following that, supernatants were collected in clean tubes and the procedure was repeated with the half amounts of extraction solvents. The supernatant was separated after centrifugation and added to previous extracts. Subsequently, 1% (v/v) NaCl (2 mL) was added to each tube to develop phase separation. The mixture was agitated on a roller shaker for 5 min. Finally, the dark green layer was collected and transferred to pre-dried (at 55 °C for 24 h) and pre-weighted tubes. After evaporating the solvents, the values of extracted lipids were calculated gravimetrically (Vignesh et al., 2020).

2.3.2. Phase II. Ultrasound-assisted lipid extraction
The second phase of the experiment was conducted to investigate the ultrasound-assisted lipid extraction. For this purpose, microalgal lipid was extracted in response to three levels of three variables including methanol to chloroform ratios (2:1, 1:1, and 1:2), sonication time (15, 30, and 60 min), and reaction temperature (25, 40, and 55 °C). The interactive effects of these variables were investigated through 27 experimental runs in duplicate. For this purpose, 10 mL solutions of different ratios of methanol to chloroform were prepared. The solvents mixtures were added to polypropylene tubes containing 100 mg microalgal powder and the same was vortexed for 1 min. Each tube was kept in a water bath sonication (Branson Ultrasonics, 29 × 15 × 15 cm, 5.7 L, 60 W, 40 kHz) for pre-determined time and temperature. After sonication, the mixture was centrifuged at 7000 rpm for 5 min. The supernatant was separated and mixed with 1% (v/v) NaCl (2 mL) to develop the phase separation. Then the mixture was agitated on a roller shaker for 5 min. Finally, the dark green layer was collected and transferred to pre-dried and pre-weighted tubes. After evaporation of the solvents, the values of extracted lipids were calculated gravimetrically (Vignesh et al., 2020). The experimental scheme of phases I and II experiments is shown in Fig. 1.

2.4. Synthesis and analysis of FAMEs

Transesterification of extracted lipids to fatty acid methyl esters

(FAMEs) was performed according to a method reported elsewhere (Karimi, 2017) with minor modifications. Briefly, methanol (2 mL), sulfuric acid 98% (0.092 g), and C17:0 (500 mg/L) as internal standard were added to each tube containing extracted lipid, and the mixture was incubated in a water bath at 55 °C. After 30 min, 1 mL hexane was added to the mixture and vortexed for 1 min. Finally, 200 μL of hexane layer containing FAMEs was collected for the analysis of fatty acids composition. FAMEs composition was analyzed using an Agilent Technologies 7890A gas chromatography (GC) equipment. GC was equipped with a flame ionization detector (FID) and a DB-Wax capillary column (10 m × 0.1 mm internal diameter, 0.1 μm film thickness). The temperatures of detector and injector were set on 250 °C. Helium gas at flow rate of 30.24 cm/s was used as carrier gas. The initial oven temperature was set at 40 °C and held for 30 sec. Then temperature raised to 195 °C (25 °C/min), 205 °C (3 °C/min), and 230 °C (3 °C/min), gradually, and held at 230 °C for 4 min. Supelco 37 component FAME mix was used for the identification and quantification of the peaks obtained from the GC chromatograph.

2.5. Biodiesel quality analysis

For assessing the quality of biodiesel, saponification value (SV), iodine value (IV), cetane number (CN), degree of unsaturation (DU), long-chain saturation factor (LCSF), cold filter plugging point (CFPP), high heating value (HHV), and oxidative stability (OS) were calculated using the following empirical equations (Sandani et al., 2020; Srinuanpan et al., 2018; Valdez-Ojeda et al., 2015):

$$SV \ (mg \ KOH/g \ fat) = \sum[(560 \times FAE\%)/M_i] \quad (Eq. \ 1)$$
$$IV \ (g \ I_2/100 \ g \ fat) = \sum[(254 \times DB \times FAE\%)/M_i] \quad (Eq. \ 2)$$
$$CN = [46.3 + (5458/SV)] - (0.255 \times IV) \quad (Eq. \ 3)$$
$$DU \ (\%wt) = MUFAs(\%) + (2 \times PUFAs(\%)) \quad (Eq. \ 4)$$

$$LCSF \ (\%wt) = (0.1 \times C16) + (0.5 \times C18) + (1 \times C20) + (1.5 \times C22) + (2 \times C24) \quad (Eq. \ 5)$$
$$CFPP \ (°C) = (3.1417 \times LCSF) - 16.477 \quad (Eq. \ 6)$$
$$HHV \ (MJ/kg) = 49.43 - (0.041 \times SV) - (0.015 \times IV) \quad (Eq. \ 7)$$
$$OS \ (h) = (117.9295 \ / \ (\%wt \ C18:2 + \%wt \ C18:3)) + 2.5905 \quad (Eq. \ 8)$$

where, *FAE* (%) is the percentage of each fatty acid ester, M_i is the molecular weight of each fatty acid ester, *DB* is the number of double bonds, *MUFAs* (%) is the percent weight of monounsaturated fatty acids, *PUFAs* (%) is the percent weight of polyunsaturated fatty acids, and C16, C18, C20, C22, and C24 are the weight percentages of saturated chains with 16, 18, 20, 22, and 24 carbon atoms, respectively.

3. Results and discussion

3.1. Lipids yield in microalgal biomass

Fig. 2 shows the effect of extraction solvents on lipids yield of microalgal biomass. As well reflected in Fig. 2, the lipids yield by single extraction solvents was increased in the following order: 40.12 mg/g (M) < 86.91 mg/g (A) < 94.34 mg/g (C) < 100.01 mg/g (H). The results showed that the extracted lipids by hexane and chloroform were higher than acetone and methanol. The higher amount of extracted lipids by hexane and chloroform compared to methanol and acetone could be explained according to the polarity index of these organic solvents. The polarity indexes of hexane, chloroform, acetone, and methanol are 0, 4.1, 5.1, and 5.1, respectively (Ricciutelli et al., 2006). These values show that acetone and methanol are more polar than hexane and chloroform. The ability of a solvent towards dissolving different solutes is determined by the polarity of the solvent. In chemistry, this principle is known as 'like dissolves like'. As such, polar and non-polar solvents are able to dissolve polar and non-polar lipids, respectively (Deshmukh
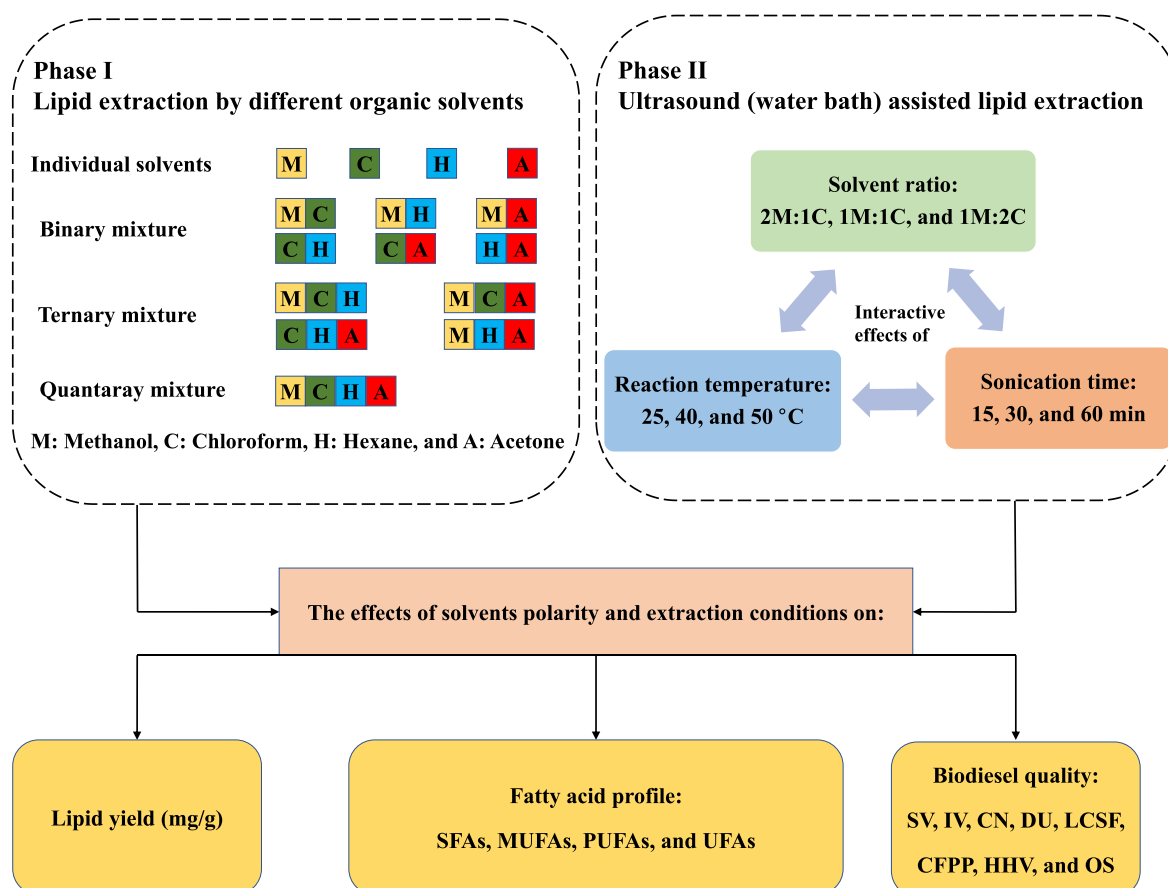

Fig. 1. Experimental scheme of lipid extraction from microalgal biomass by organic solvents and ultrasound-assisted methods.
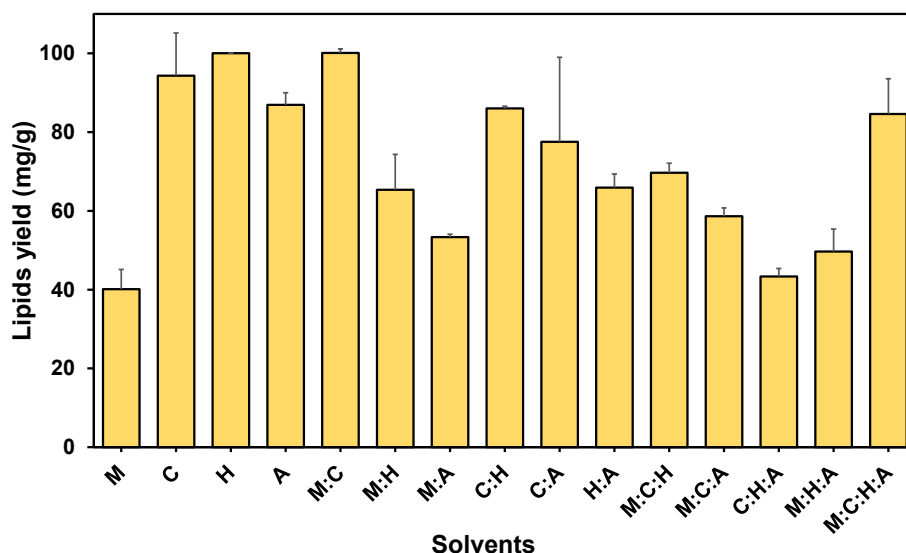
Fig. 2. The effect of polar and non-polar solvents, and their mixtures on the lipids yield of microalgae (M: Methanol, C: Chloroform, H: Hexane, A: Acetone, microalgal biomass: 100 mg, volume of solvents: 15 mL, and T: 25 °C).

et al., 2019). In terms of polarity, microalgal lipids are categorized to polar (e.g., glycolipid, phospholipid) and non-polar or neutral (e.g., triacylglycerol) (Salam et al., 2016). The greater proportion of microalgal lipids is composed of non-polar lipids (mainly triglycerides) (Mata et al., 2010). Accordingly, higher lipids extraction by non-polar solvents (hexane and chloroform) confirmed the negative correlation between the polarity index of solvents and the amount of extracted lipids.

In this study, six binary mixtures of M:C, M:H, M:A, C:H, C:A, and H:A, with the same ratio (1:1) of single solvents (M, A, C, and H) were used for the extraction of lipid from microalgae. Among them, M:C mixture exhibited the highest lipid yield of 100.10 mg/g. The extracted lipid by M:C mixture was higher than that of the extracted lipid by single solvents of methanol (40.12 mg/g) and chloroform (94.34 mg/g) (Fig. 2). In another study, Ryckebosch et al. (2012) examined the effect of different mixtures of organic solvents on the total lipid yield of microalgae. They found the mixture of chloroform:methanol (1:1) as the best extraction solvent. They reported that the mixture of polar and non-polar solvents could extract both polar and non-polar lipids, which increases the amount of total extracted lipids. However, the binary mixture of methanol and chloroform significantly increased the FAMEs yield, but the binary mixtures of methanol and hexane or methanol and acetone significantly decreased the lipids yield.

Ternary and quaternary mixtures of single solvents with the same ratios of 1:1:1 and 1:1:1:1 were also tested for the extraction of lipids. Ternary and quaternary mixtures caused lower FAMEs yield as compared to single solvents of chloroform, hexane, and acetone. Decrease in lipid yield by binary, ternary, and quaternary mixtures of solvents might be explained based on the miscibility of organic solvent. For instance, among the ternary mixtures, M:C:H and C:H:A showed the highest and lowest lipid yield as 69.69 and 43.34 mg/g, respectively. In the first case, methanol is immiscible in chloroform and hexane, while acetone is miscible in both. The miscibility of chloroform, hexane, and acetone might form a homogeneous mixture with different properties of single solvents, which decreases the lipid extraction efficiency. Overall, considering the lipid yield of 15 experimental runs ranging from 40.12 to 100.10 mg/g (Fig. 2); it can be summarized that lipid extraction from microalgae by single polar solvent was not efficient compared to non-polar solvents. Whereas binary mixture of polar and non-polar solvents (e.g., methanol and chloroform) could enhance the lipid extraction efficiently. Also, it should be noted that despite frequent application, organic solvents are considered as toxic and flammable compounds. In this respect, techno–economic comparison between conventional

organic solvents and green solvents such as ionic liquids with lower toxicity (Ozola-Davidane et al., 2021) is highly recommended for future studies.

3.2. Fames composition of microalgal lipid

The effect of four single extraction solvents and their mixtures on FAMEs composition was determined, and the results are tabulated in Table 1. Nine fatty acids with different concentrations were observed in the extracted lipids. Although the derivatives of C16 and C18 were the dominant fatty acids in all treatments (15 experimental runs), the concentrations of individual fatty acids were different depending on the solvents. For instance, the concentrations of C16:0 in the extracted lipids by acetone and hexane were 27.62% and 40.81%, respectively. Another example was the higher (18.32%) and lower (10.88%) concentrations of C18:3 in the extracted lipids by methanol and ternary mixtures of C:H:A. Also, the extraction solvents significantly affected the total percentages of saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) of different treatments. As can be observed from Table 1, the values of SFAs (61.57% and 61.53%) were higher than the values of UFAs (38.43% and 38.47%) in the lipids, extracted by hexane and chloroform. Whereas higher concentrations of UFAs (62.21% and 61.15%) and lower concentrations of SFAs (37.79% and 38.85%) were observed by acetone and methanol. Overall, non-polar solvents (hexane and chloroform) increased the extraction of SFAs, while polar solvents (methanol and acetone) increased the extraction of UFAs. This trend was observed for the binary mixtures of single solvents as well. The values of extracted SFAs by C:H (mixture of non-polar solvents) and M:A (mixture of polar solvents) were 66.02% and 38.39%, respectively. In case of ternary mixtures (M:C:H, M:C:A, C:H:A, and M:H:A), the values of SFAs and UFAs were higher depending on the higher portion of non-polar (C and H) or polar solvents (A and M). For example, higher ratio of non-polar solvents in C:H:A increased the value of SFAs (60.95%) in reference to UFAs (39.05%). At the same time, higher yield of UFAs (58.53%) compared to SFAs (41.47%) was obtained by M:C:A with higher ratio of polar solvents.

Higher percentage of UFAs by methanol and acetone might be related to the fact of dissolving polar lipids in polar solvents. Polar lipids such as phospholipids and glycolipids are well-known as the main structural lipids of microalgal cell wall (Salam et al., 2016). It has been reported that phospholipids and glycolipids of microalgae have high amount of UFAs. Monogalactosyl diacylglycerol, and digalactosyl as the

Table 1
The effect of polar and non-polar solvents and their mixtures on the FAMEs composition (percentage) of microalgal lipids (M: Methanol, C: Chloroform, H: Hexane, A: Acetone, microalgal biomass: 100 mg, volume of solvents: 15 mL, and T: 25 °C).

Solvents	C13:0	C14:1	C16:0	C16:1	C17:1	C18:0	C18:1	C18:2	C18:3n-3	SFAs[b]	MUFAs[c]	PUFAs[d]	UFAs[e]
M	ND[a]	7.09 ± 0.05	31.1 ± 0.30	1.83 ± 0.01	1.36 ± 0.01	7.74 ± 0.10	20.24 ± 0.26	12.30 ± 0.12	18.32 ± 0.18	38.85 ± 0.40	30.52 ± 0.33	30.63 ± 0.30	61.15 ± 0.63
C	ND	4.09 ± 0.94	39.40 ± 0.62	1.28 ± 0.09	0.64 ± 0.91	22.13 ± 1.80	12.71 ± 0.49	8.07 ± 0.83	11.68 ± 0.98	61.53 ± 2.42	18.72 ± 0.61	19.75 ± 1.81	38.47 ± 2.42
H	ND	5.63 ± 0.81	40.81 ± 1.65	ND	ND	20.76 ± 1.35	12.17 ± 0.64	8.65 ± 0.44	11.98 ± 1.11	61.57 ± 2.99	17.80 ± 1.44	20.63 ± 21.55	38.43 ± 2.99
A	1.22 ± 0.01	17.49 ± 0.72	27.62 ± 0.29	ND	1.76 ± 0.14	8.95 ± 0.44	13.32 ± 0.73	11.23 ± 0.22	18.41 ± 0.23	37.79 ± 0.74	32.57 ± 1.59	29.64 ± 0.45	62.21 ± 2.04
M:C	ND	9.46 ± 0.20	35.83 ± 0.04	1.76	1.41 ± 0.08	10.70 ± 0.37	14.03 ± 0.03	10.41 ± 0.05	16.40 ± 0.05	46.54 ± 0.41	26.65 ± 0.31	26.81 ± 0.10	53.46 ± 0.41
M:H	ND	5.56 ± 1.06	36.04 ± 1.66	1.39 ± 0.02	1.11 ± 0.07	18.07 ± 1.20	14.44 ± 0.23	9.29 ± 0.57	14.09 ± 1.05	54.11 ± 2.86	22.50 ± 1.38	23.39 ± 1.62	45.89 ± 3.00
M:A	ND	10.77 ± 0.04	30.8 ± 0.49	1.88 ± 0.01	1.40 ± 0.09	7.59 ± 0.02	18.29 ± 0.39	11.57 ± 0.13	17.70 ± 0.11	38.39 ± 0.51	32.35 ± 0.53	29.26 ± 0.24	61.61 ± 0.77
C:H	ND	5.34 ± 0.30	37.48 ± 0.39	ND	ND	28.54 ± 1.26	9.68 ± 0.76	7.64 ± 0.11	11.32 ± 0.48	66.02 ± 1.64	15.02 ± 1.06	18.96 ± 0.58	33.98 ± 1.64
C:A	ND	11.74 ± 0.84	30.88 ± 1.37	ND	1.53 ± 0.15	15.92 ± 0.34	13.25 ± 0.30	10.32 ± 0.40	16.36 ± 0.92	46.80 ± 1.71	26.52 ± 0.39	26.68 ± 1.32	53.20 ± 1.71
H:A	ND	9 ± 0.11	31.40 ± 0.63	ND	1.30 ± 0.11	20.17 ± 0.39	13.97 ± 0.14	9.60 ± 0.05	14.56 ± 0.27	51.57 ± 0.24	24.27 ± 0.08	24.16 ± 0.32	48.43 ± 0.24
M:C:H	ND	5.34 ± 0.40	34.43 ± 0.55	1.35 ± 0.06	1.23 ± 0.11	17.66 ± 1.48	14.58 ± 0.41	9.92 ± 0.41	15.48 ± 0.66	52.1 ± 2.03	22.50 ± 0.97	25.40 ± 1.06	47.90 ± 2.03
M:C:A	ND	8.65 ± 1.05	30.46 ± 0.23	1.59 ± 0.08	1.33 ± 0.16	11.01 ± 0.63	17.81 ± 0.07	11.51 ± 0.30	17.64 ± 0.07	41.47 ± 0.40	29.38 ± 0.73	29.15 ± 1.13	58.53 ± 0.40
C:H:A	ND	5.40 ± 0.08	35.63 ± 0.45	ND	ND	25.31 ± 0.51	14.98 ± 0.49	7.79 ± 0.18	10.88 ± 0.21	60.95 ± 0.96	20.38 ± 0.58	18.67 ± 0.38	39.05 ± 0.96
M:H:A	ND	7.82 ± 0.22	33.04 ± 0.76	1.50 ± 0.04	1.20 ± 0.14	14.47 ± 0.39	16.07 ± 0.43	10.24 ± 0.15	15.65 ± 0.17	47.51 ± 1.15	26.60 ± 0.83	25.89 ± 0.32	52.49 ± 1.15
M:C:H:A	ND	9.97 ± 0.86	33.89 ± 0.60	1.68 ± 0.04	1.49	12.04 ± 0.41	13.98 ± 0.35	10.39 ± 0.03	16.56 ± 0.42	45.93 ± 1.00	27.12 ± 0.55	26.95 ± 0.46	54.07 ± 1.00

[a] ND: Not detected
[b] SFAs: Saturated fatty acids
[c] MUFAs: Monounsaturated fatty acids
[d] PUFAs: Polyunsaturated fatty acids
[e] UFAs: Unsaturated fatty acids

predominant glycolipids have high amount of polyunsaturated fatty acids e.g., C16 and C18-omega 3 (Da Costa et al., 2016). Matsui et al. (2020) analyzed the fatty acids profile of phospholipids of *Nannochloropsis oculate* cultivated under nutrient sufficiency and phosphorous deficiency conditions. They found 56.62% and 66.85% of UFAs and 33.84% and 29.06% of SFAs in phospholipids of microalgae cultivated under nutrient sufficiency and phosphorous deficiency. They also reported higher concentrations of UFAs (56.42% and 61.02%) as compared to SFAs (39.38% and 36.47%) in glycolipids of aforementioned treatments. Therefore, dissolving of phospholipids and glycolipids available in microalgal cell wall with high contents of monounsaturated and polyunsaturated fatty acids could enhance the percentage of UFAs in the extracted lipids by polar solvents.

3.3. Biodiesel quality

The effects of polar and non-polar solvents, and their mixtures on the essential fuel characteristics of biodiesel were also evaluated and the results are presented in Table 2. Among the evaluated biodiesel properties, CN index is considered as the main biodiesel quality indicator. The value of CN presents important information about the ignition delay and combustion quality of fuel (Wu and Miao, 2014). A higher CN value indicates a shorter ignition delay time, more complete combustion, and consequently better engine performance (de Jesus et al., 2020). In this study, the CN values of lipids extracted by acetone, methanol, hexane, and chloroform were found as 45.30, 46.47, 55,61, and 56.01, respectively. The higher values of CN were also observed by the binary mixture of non-polar solvents (e.g. C:H) or ternary mixtures of solvents with higher ratios of non-polar solvents (e.g. M:C:H and C:H:A) (Table 2). Fatty acids profile of the lipids extracted by chloroform and hexane had higher amount of C16:0 and C18:0 compared to the lipids extracted by methanol and acetone (Table 1). Therefore, higher CN values of the lipids extracted by non-polar solvents could be explained according to the higher amount of saturated fatty acids. In agreement to the findings of this study, de Jesus et al. (2020) have also stated that more saturated and longer non-branched carbon chains enhance the value of CN. Likewise, Sandani et al. (2020) have pointed out that higher contents of palmitic acid (C16:0) and stearic acid (C18:0) increase the CN value of biodiesel derived from microalgae. The minimum limits of CN values as per worldwide standards of fuel such as ASTM D675 and EN 14,214 are 47 and 51, respectively. The obtained CN values > 55 by hexane and chloroform confirmed that the lipids extracted by non-polar solvents could meet the requirement of ASTM and EN to be used as biofuel.

IV and DU are other indexes that provide further information about the biodiesel quality by indicating the unsaturation degree of fatty acids. IV has a positive correlation with DU, and its value depends on the number and positions of double bonds of MUFAs and PUFAs within the biodiesel (Wang et al., 2018). The maximum acceptable value of IV according to EN 14,214 is 120 g I_2/100 g FAMEs. Higher values might deteriorate the performance of engine due to the polymerization of unsaturated fatty acids and deposition of lubricants (Yang et al., 2016). In this study, the IV and DU values of all tested extraction solvents were found to be in the ranges of 59.51–103.88 g I_2/100 g FAMEs and 52.94–91.85%, respectively, which meet the EN 14,214 standard. As shown in Table 2, the values of IV and DU of lipids extracted by polar solvents (methanol and acetone) were significantly higher than that of lipids extracted by non-polar solvents (hexane and chloroform). The association of higher values of IV and DU with polar solvents is most likely due to the higher percentages of MUFAs and PUFAs of lipids extracted by polar solvents (Table 2). Higher values of IV because of

Table 2

The effect of polar and non-polar solvents and their mixtures on the properties of microalgal biodiesel (M: Methanol, C: Chloroform, H: Hexane, A: Acetone, microalgal biomass: 100 mg, volume of solvents: 15 mL, and T: 25 °C).

Solvents	SV[1] (mg KOH/g oil)	IV[2] (g I$_2$/100 g oil)	CN[3]	DU[4] (%wt)	LCSF[5] (% wt)	CFPP[6] (°C)	HHV[7] (MJ/kg)	OS[8] (h)
M	209.12 ± 0.03	101.69 ± 0.61	46.47 ± 0.15	91.78 ± 0.47	6.98 ± 0.08	5.50 ± 0.25	39.33 ± 0.01	6.44 ± 0.01
C	208.67 ± 0.33	64.48 ± 4.91	56.01 ± 1.29	58.22 ± 4.24	15 ± 0.96	30.74 ± 3.03	39.91 ± 0.09	8.56 ± 0.55
H	209.40 ± 0.12	65.70 ± 5.31	55.61 ± 1.37	59.06 ± 4.54	14.46 ± 0.84	29.03 ± 2.64	39.86 ± 0.08	8.32 ± 0.43
A	213.91 ± 0.44	103.98 ± 0.51	45.30 ± 0.18	91.85 ± 0.15	7.24 ± 0.19	6.30 ± 0.60	39.10 ± 0.03	6.57
M:C	211.10 ± 0.11	90.04 ± 0.56	49.19 ± 0.15	80.28 ± 0.51	8.93 ± 0.19	11.64 ± 0.59	39.42 ± 0.01	6.99 ± 0.02
M:H	208.93 ± 0.24	77.05 ± 5.26	52.77 ± 1.37	69.27 ± 4.49	12.64 ± 0.77	23.30 ± 2.41	39.71 ± 0.09	7.65 ± 0.35
M:A	210.85 ± 0.12	101.11 ± 0.77	46.4 ± 0.18	90.88 ± 0.75	6.87 ± 0.06	5.16 ± 0.18	39.27 ± 0.01	6.62 ± 0.03
C:H	208.44 ± 0.10	59.51 ± 2.52	57.31 ± 0.65	52.94 ± 2.33	18.02 ± 0.67	40.23 ± 2.10	39.99 ± 0.04	8.81 ± 0.19
C:A	210.77 ± 0.16	89.99 ± 3.78	49.25 ± 0.98	79.88 ± 3.03	11.05 ± 0.31	18.29 ± 0.97	39.44 ± 0.06	7.02 ± 0.22
H:A	209.39 ± 0.19	81.11 ± 0.73	51.68 ± 0.16	72.58 ± 0.56	13.23 ± 0.13	25.14 ± 0.42	39.63	7.47 ± 0.06
M:C:H	208.56 ± 0.15	81.94 ± 3.51	51.57 ± 0.91	73.30 ± 3.10	12.27 ± 0.80	22.15 ± 2.50	39.65 ± 0.06	7.24 ± 0.19
M:C:A	209.62 ± 0.40	97.68 ± 1.94	47.43 ± 0.45	87.68 ± 1.53	8.55 ± 0.29	10.44 ± 0.92	39.37 ±	6.64 ± 0.16
C:H:A	208.14 ± 0.03	63.41 ± 1.42	56.35 ± 0.36	57.72 ± 1.34	16.22 ± 0.30	34.57 ± 0.94	39.95 ± 0.01	8.91 ± 0.13
M:H:A	209.58 ± 0.01	87.24 ± 1.55	50.10 ± 0.39	78.38 ± 1.47	10.54 ± 0.27	16.69 ± 0.85	39.53 ± 0.02	7.15 ± 0.06
M:C:H:A	210.94 ± 0.33	90.98 ± 1.91	48.98 ± 0.53	81.02 ± 1.46	9.41 ± 0.26	13.13 ± 0.83	39.42 ± 0.04	6.97 ± 0.07
EN 14,214 (Nayak and Ghosh, 2019)	–	≤120	≥51	–	–	−20 to 5*	–	≥6
ASTM D6751 (Nayak and Ghosh, 2019)	a	a	≥47	–	a	–	–	a

[a] No limit for physical properties
[1] Saponification value (SV)
[2] Iodine value (IV)
[3] Cetane number (CN)
[4] Degree of unsaturation (DU)
[5] Long-chain saturation factor (LCSF)
[6] Cold filter plugging point (CFPP)
[7] High heating value (HHV)
[8] Oxidative stability (OS)
[*] Country specific

higher content of PUFAs have been reported by other researchers also (Sandani et al., 2020; Wang et al., 2018). Since IV value represents the unsaturation of biodiesel, it has a negative correlation with oxidative stability (OS) of biodiesel. Therefore, biodiesel with a lower IV value has more oxidative stability and hence, is more suitable for long term storage than another biodiesel with a higher IV value (Wu and Miao, 2014). The findings of this study revealed higher OS values of the extracted lipids by non-polar solvents, which could be related to the higher concentrations of SFAs (Table 1) and lower IV values (Table 2). The same trends of higher IV and DU were observed for the binary mixture of polar solvents (e.g. M:A) and the ternary mixtures with the higher ratios of polar solvents (e.g. M:C:A and M:H:A) (Table 2).

CFPP was calculated to predict the flow performance of biodiesel at low temperature. As it can be seen from Eq. 6, the value of CFPP depends on the value of LCSF. In this sense, the values of both CFPP and LCSF are positively correlated with the content of SFAs of biodiesel (Zhang et al., 2018). In the current study, the values of CFPP for the extracted lipids by methanol, acetone, hexane, and chloroform were found to be 5.50 °C, 6.30 °C, 29.03 °C, and 30.74 °C, respectively. The higher values of CFPP can be explained according to the higher concentrations of C16:0 and C18:0 of lipids extracted by chloroform and hexane (Table 2). The widely used biodiesel quality parameter of CFPP predicts the

temperature at which fuel loses filterability. The CFPP of biodiesel has not been specified by international standards due to the effect of local climatic conditions on the fluidity of fuels. Therefore, lipids extracted by non-polar solvents (with higher CFPP) are more suitable for biodiesel production in tropical regions, while extracted biodiesel by polar solvents (with lower CFPP) are more applicable for cold climate. Similar to the findings of this study, de Jesus et al. (2020) reported a significant difference between the CFPP values of biodiesel obtained by chloroform (21.6–25.5 °C) and biodiesel extracted by green solvents (-0.3–1.8 °C).

SV and HHV are biodiesel properties that show the saponification value and heat of combustion of a fuel, respectively. SV and HHV showed a negligible fluctuation in response to polar and non-polar solvents or their mixtures (Table 2). The values of SV were observed in the range of 208.14 – 213.91 mg KOH/g, which are close to those of *Micractinium reisseri* SIT04 (208.02–214.51 mg KOH/g) and *Scenedesmus obliquus* SIT06 (210.95–216.73 mg KOH/g) (Srinuanpan et al., 2018). The HHV values of lipids extracted by all solvents were between 39 and 40 MJ/kg. The similar values of 39.95 – 42.07 kJ/kg (He et al., 2016) and 39.89 – 40.66 kJ/kg (Wang et al., 2018) have also been found for the biodiesel of other microalgae species. The reason of similar HHV of biodiesel extracted from different microalgal species could be related to the similar HHV of individual fatty acid esters present in biodiesel as

follows (Valdez-Ojeda et al., 2015): 38.90 kJ/kg (C14:0), 39.45 kJ/kg (C16:0), 40.07 kJ/kg (C18:0), 39.91 kJ/kg (C18:1), and 39.70 kJ/kg (C18:2).

3.4. The interactive effects of extraction conditions

Based on the findings of phase *I*, the mixture of chloroform and methanol with the highest lipid yield was selected for the ultrasound-assisted lipid extraction. In ultrasound-assisted lipid extraction by organic solvents, the ratios of solvents, sonication time and reaction temperature are three main variables that affect the process (Hadrich et al., 2018). Considering this fact, the second phase of this study was conducted to investigate the interactive effects of three levels of these variables on the lipid yield, lipid profile, and biodiesel quality. Fig. 3 (a)

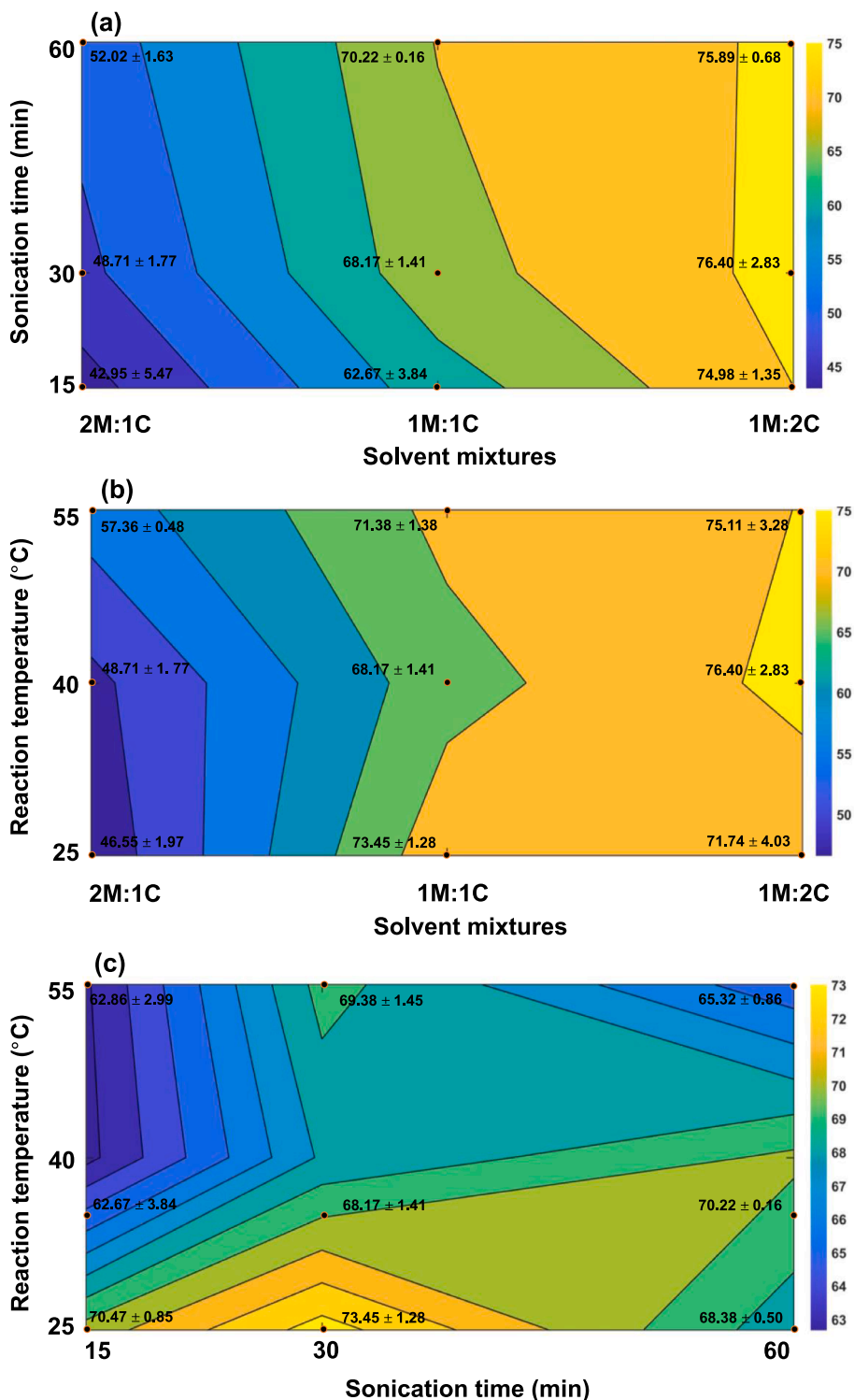**Fig. 3.** The effects of extraction conditions on the lipid yield (mg/g) of microalgal biomass (microalgal biomass: 100 mg, volume of solvents: 10 mL, and T: 25 °C): [(a): interactive effects of chloroform (C) to methanol (M) ratios and sonication time, (b): interactive effects of chloroform (C): to methanol (M) ratios and reaction temperature, (c) interactive effects of sonication time and reaction temperature].

shows the interactive effects of solvents ratios (2 M:1C, 1 M:1C, 1 M:2C) and ultrasonication time (15, 30, 60 min) on the lipid yield from microalgal biomass at constant temperature of 40 °C. As it can be seen from this figure, the highest amount of lipid (76.40 mg/g) was obtained at higher ratio of chloroform to methanol (2C:1M). By decreasing the amount of chloroform, the lipid yield decreased from 74.98 mg/g (2C:1M, 15 min) to 42.95 mg/g (1C:2M, 15 min). In another study, Hadrich et al. (2018) evaluated the effects of different ratios of chloroform to methanol (1:1, 2:1, and 3:1), ultrasonic time (6, 18, and 30 min), and temperature (30, 45, and 60 °C) on the lipid extraction from *Chlorella* sp. In agreement to the findings of this study, they found the maximum lipid yield at the ratio of 2:1 (chloroform to methanol). dos Santos et al. (2015) also used the ultrasonic assisted method for the lipid extraction from *C. vulgaris* via ethanol, hexane, chloroform:methanol (1:2), and chloroform:methanol (2:1). In their study, 2:1 ratio of chloroform:methanol showed the highest lipid extraction efficiency. They stated that the mixture of chloroform and methanol as non-polar and polar solvents improved the extraction of neutral and polar lipids, which enhanced the extraction of total lipids. In the current study, although increasing the reaction time from 15 to 60 min did not show significant effect on the value of extracted lipid by 2C:1M, it enhanced the lipid extraction from 62.67 to 70.22 mg/g and from 42.95 to 52.09 mg/g by 1C:1M and 1C:2M, respectively. Increasing the amount of extracted lipid at longer sonication time might be related to cell disruption. Ultrasound radiation generates the cavitation of bubbles or cavitation effects in the treated solvents, which provides shear force for the disruption of microalgal cells (Ellison et al., 2019).

Fig. 3 (b) depicts the interactive effects of solvents mixture and temperature on the lipid yield at a constant sonication time of 30 min. Depending on the ratios of chloroform to methanol, an increase in temperature influenced the amount of extracted lipid, differently. For instance, the increase in temperature from 25 to 40 °C of the solvent mixture with a ratio of 1M:2C increased the lipid yield from 71.74 to 76.60 mg/g. Whereas increasing the temperature (25 to 40 °C) of 1C:1M solvent mixture decreased the amount of extracted lipid from 73.45 to 68.17 mg/g. In case of solvent mixture with higher ratio of methanol (2M:1C), lipid yield was significantly increased from 46.55 to 57.36 mg/g by increasing the temperature from 25 to 55 °C. The positive correlation between increase in the lipid yield with increasing reaction temperature has been reported by other researchers also (Mandik et al., 2020; Sivaramakrishnan and Incharoensakdi, 2019). Mandik et al. (2020) observed a drastic enhancement of FAMEs yield from 29.30 to 256.90 mg/g after increasing the reaction temperature from 50 °C to 70 °C. They stated that the reason of increasing FAMEs yield at higher temperature could be related to the increase in kinetic energy and hence, reaction rate. Likewise, in a study conducted by Sivaramakrishnan and Incharoensakdi (2019), increasing the reaction temperature from 35 to 55 °C resulted in the increase of extracted lipid by hexane, significantly. The authors explained that the dissolution capacity of a solvent is increased at higher temperatures, which leads to increase the diffusion between the solid and liquid phases and increases the extraction yield.

Interactive effects of reaction temperature and sonication time on the lipid extraction by equal ratio of chloroform and methanol has been illustrated in Fig. 3 (c). Increasing the temperature from 25 to 55 °C at different sonication times had no positive effect on lipid yield by 1M:1C solvent mixture. This result revealed that lipid extraction from *S. quadricauda* biomass by the same ratio of chloroform and methanol can be optimized at ambient temperature and short time. It is worth noting that although ultrasound-assisted method could enhance the lipid yield, but it also increases the total cost of lipid production from microalgal biomass due to high power consumption. Therefore, optimization of sonication time and reaction temperature is necessary towards obtaining the maximum lipid yield. Evaluating the maximum lipid yield at shorter sonication time in response to different frequency, power, and processing temperature is recommended for future studies.

The interactive effects of aforementioned factors on the lipid profile

were also investigated. Accordingly, the percentage of SFAs, MUFAs, and PUFAs in response to the extraction conditions were calculated (Table 3). As it has been tabulated in Table 3, different ultrasonication times showed no significant effect on the fatty acids profile of microalgal lipid extracted by a certain extraction solvent. On the contrary, different ratios of chloroform to methanol changed the composition of extracted lipids at the same temperature. In this regard, the extracted lipids by higher chloroform to methanol ratio (1M:2C) had higher SFAs as compared to other solvent mixtures (1M:1C and 2M:1C). Moreover, the reaction temperature showed a significant effect on the profiles of microalgal lipids. The extracted lipids at higher temperature had higher

Table 3
The interactive effects of chloroform (C) to methanol (M) ratios, sonication time, and reaction temperature on the FAMEs composition (percentage) of microalgal lipids (microalgal biomass: 100 mg, volume of solvents: 10 mL, and T: 25 °C).

Solvent ratios	Time (min)	Temperature (°C)	SFAs[a]	MUFAs[b]	PUFAs[c]
1 M:2C	15	25	55.67 ± 2.12	23.40 ± 0.58	24.42 ± 0.82
1 M:2C	30	25	54.75 ± 0.45	21.83 ± 0.84	23.97 ± 0.39
1 M:2C	60	25	56.02 ± 0.73	20.71 ± 0.43	23.27 ± 0.03
1 M:1C	15	25	45.49 ± 0.78	27.13 ± 0.16	27.39 ± 0.61
1 M:1C	30	25	43.99 ± 1.33	27.05 ± 0.72	28.96 ± 0.61
1 M:1C	60	25	44.99 ± 0.80	26.88 ± 0.68	28.13 ± 0.27
2 M:1C	15	25	41.02 ± 0.78	29.88 ± 1.45	29.10 ± 1.06
2 M:1C	30	25	39.60 ± 0.93	30.36 ± 1.18	30.04 ± 0.15
2 M:1C	60	25	39.89 ± 1.00	30.44 ± 0.54	29.68 ± 0.11
1 M:2C	15	40	59.20 ± 0.53	18.67 ± 0.15	22.14 ± 0.63
1 M:2C	30	40	58.67 ± 1.98	18.22 ± 0.58	23.11 ± 0.93
1 M:2C	60	40	59.74 ± 1.10	17.38 ± 0.21	22.72 ± 0.30
1 M:1C	15	40	54.57 ± 1.03	21.76 ± 0.66	23.67 ± 1.11
1 M:1C	30	40	49.81 ± 4.49	24.09 ± 5.36	26.10 ± 0.91
1 M:1C	60	40	45.81 ± 1.13	26.21 ± 6.83	27.98 ± 1.55
2 M:1C	15	40	45.72 ± 0.86	26.92 ± 0.68	27.36 ± 1.32
2 M:1C	30	40	44.83 ± 0.54	27.11 ± 0.59	28.06 ± 0.98
2 M:1C	60	40	46.10 ± 0.37	26.09 ± 0.82	27.81 ± 0.51
1 M:2C	15	55	57.73 ± 1.02	20.51 ± 0.33	22.31 ± 0.03
1 M:2C	30	55	59.13 ± 0.36	19.17 ± 0.19	21.70 ± 0.17
1 M:2C	60	55	59.90 ± 0.57	18.89 ± 0.34	21.37 ± 0.27
1 M:1C	15	55	46.52 ± 0.49	25.24 ± 0.15	28.23 ± 0.39
1 M:1C	30	55	46.62 ± 0.83	25.48 ± 0.71	28.73 ± 1.02
1 M:1C	60	55	47.28 ± 2.15	24.98 ± 1.14	27.74 ± 0.61
2 M:1C	15	55	43.72 ± 4.07	25.77 ± 5.36	30.51 ± 0.72
2 M:1C	30	55	42.83 ± 1.11	28.16 ± 0.45	29.01 ± 0.32
2 M:1C	60	55	44.93 ± 1.59	25.18 ± 6.02	29.89 ± 2.01

[a] SFAs: Saturated fatty acids
[b] MUFAs: Monounsaturated fatty acids
[c] PUFAs: Polyunsaturated fatty acids

amount of SFAs than the extracted lipids at lower temperature by the same extraction solvent. For example, the maximum value of SFAs was observed in the extracted lipids by 1M:2C solvent at 60 °C. While the highest amount of MUFAs and PUFAs were obtained by 2M:1C solvent mixture at 25 °C. It has been reported by Xia et al. (2020) that increasing the temperature from 35 to 80 °C increased the lipid yield from 73.20 to 257.3 mg/g. However, at higher temperature of 90 °C, the lipid yield decreased to 193.60 mg/g. The authors explained that decreasing the amount of extracted lipids at higher temperature is likely due to degradation and autoxidation of PUFAs. The protection of PUFAs during lipid extraction process at lower temperature has been reported by Xu et al. (2021) as well.

Consequently, interactive effects of solvents ratios, ultrasonication time, and reaction temperature on the properties of microalgal biodiesel

Table 4
The interactive effects of chloroform (C) to methanol (M) ratios, sonication time, and reaction temperature on the properties of microalgal biodiesel (microalgal biomass: 100 mg, volume of solvents: 10 mL, and T: 25 °C).

Solvent ratios	Time (min)	Temperature (°C)	SV[1] (mg KOH/g oil)	IV[2] (g I$_2$/100 g oil)	CN[3]	DU[4] (%wt)	LCSF[5] (% wt)	CFPP[6] (°C)	HHV[7] (MJ/kg)	OS[8] (h)
2C:1M	15	25	214.89 ± 8.67	79.88 ± 3.29	51.35 ± 1.87	71.65 ± 3.03	13.20 ± 0.15	25.07 ± 0.46	38.90 ± 0.39	7.42 ± 0.16
2C:1M	30	25	208.56 ± 0.06	77.17 ± 0.98	52.79 ± 0.26	69.22 ± 0.83	12.89 ± 0.08	24.09 ± 0.26	39.21 ± 0.03	7.51 ± 0.08
2C:1M	60	25	208.69 ± 0.14	75.01 ± 0.30	53.33 ± 0.06	67.25 ± 0.33	13.33 ± 0.05	25.47 ± 0.17	39.21	7.66 ± 0.01
1C:1M	15	25	210.67 ± 0.08	91.70 ± 1.60	48.82 ± 0.40	81.90 ± 1.39	8.77 ± 0.13	11.11 ± 0.42	39.14 ± 0.02	6.90 ± 0.10
1C:1M	30	25	210.01 ± 0.05	95.21 ± 2.07	48.01 ± 0.52	84.97 ± 1.94	8.63 ± 0.32	10.70 ± 1.02	39.12 ± 0.02	6.66 ± 0.09
1C:1M	60	25	210.22 ± 0.19	93.25 ± 1.18	48.48 ± 0.32	83.14 ± 1.07	9.04 ± 0.20	11.96 ± 0.63	39.11 ± 0.02	6.78 ± 0.04
1C:2M	15	25	209.56 ± 0.02	97.96 ± 1.93	47.37 ± 0.49	88.08 ± 1.17	7.73 ± 0.24	7.84 ± 0.74	39.14 ± 0.01	6.64 ± 0.13
1C:2M	30	25	209.15 ± 0.33	100.31 ± 0.48	46.82 ± 0.08	90.43 ± 0.58	7.70 ± 0.06	7.74 ± 0.18	39.11	6.52 ± 0.02
1C:2M	60	25	209.54 ± 0.19	99.68 ± 0.47	46.93 ± 0.10	89.79 ± 0.46	7.69 ± 0.30	7.72 ± 0.30	39.10 ± 0.01	6.56 ± 0.01
2C:1M	15	40	207.85 ± 0.25	71.85 ± 2.30	54.24 ± 0.62	64.43 ± 1.82	14.03 ± 0.66	27.69 ± 2.09	39.28	7.70 ± 0.20
2C:1M	30	40	207.85 ± 0.25	71.85 ± 2.30	54.24 ± 0.62	64.43 ± 1.82	14.03 ± 0.66	27.69 ± 2.09	39.28	7.70 ± 0.20
2C:1M	60	40	207.53 ± 0.14	70.03 ± 1.00	54.74 ± 0.27	62.82 ± 0.80	14.83 ± 0.37	30.18 ± 1.17	39.25	7.78 ± 0.07
1C:1M	15	40	207.88 ± 0.12	76.47 ± 2.12	53.06 ± 0.53	69.11 ± 1.72	13.15 ± 0.39	24.90 ± 1.23	39.24	7.58 ± 0.23
1C:1M	30	40	209.49 ± 2.17	85.18 ± 6.33	50.64 ± 1.88	76.29 ± 5.13	10.60 ± 2.17	16.88 ± 0.82	39.19 ± 0.06	7.11 ± 0.16
1C:1M	60	40	208.61 ± 0.49	92.96 ± 0.22	48.76 ± 0.12	83.83 ± 0.20	8.88 ± 0.02	11.46 ± 0.07	39.20 ± 0.02	6.64 ± 0.01
1C:2M	15	40	208.37 ± 0.28	90.20 ± 2.64	49.49 ± 0.64	81.64 ± 2.18	9.51 ± 0.35	13.44 ± 1.10	39.21 ± 0.01	6.91 ± 0.21
1C:2M	30	40	208.03 ± 0.28	92.01 ± 1.87	49.08 ± 0.44	83.23 ± 1.52	9.50 ± 0.16	13.43 ± 0.52	39.19 ± 0.01	6.80 ± 0.15
1C:2M	60	40	208.12 ± 0.12	90.47 ± 0.30	49.45 ± 0.06	81.71 ± 0.14	9.71 ± 0.04	14.09 ± 0.13	39.20	6.83 ± 0.08
2C:1M	15	55	208.42 ± 0.02	71.94 ± 0.98	54.14 ± 0.25	64.57 ± 1.03	14.18 ± 0.19	28.16 ± 0.61	39.23	7.88
2C:1M	30	55	208.69 ± 0.04	69.71 ± 0.56	54.68 ± 0.14	62.56 ± 0.53	14.23 ± 0.04	28.31 ± 0.13	39.25 ± 0.01	8.03 ± 0.04
2C:1M	60	55	208.38 ± 0.14	68.64 ± 0.71	54.99 ± 0.16	61.64 ± 0.68	14.94 ± 0.01	30.55 ± 0.02	39.23 ± 0.01	8.11 ± 0.07
1C:1M	15	55	209.78	91.68 ± 1.01	48.94 ± 0.26	81.71 ± 0.88	9.53 ± 0.17	13.53 ± 0.53	39.13 ± 0.01	6.77 ± 0.06
1C:1M	30	55	209.69 ± 0.22	92.21 ± 2.06	48.81 ± 0.50	82.11 ± 1.52	9.52 ± 0.02	13.47 ± 0.06	39.13 ± 0.03	6.70 ± 0.15
1C:1M	60	55	209.72 ± 0.47	90.19 ± 1.20	49.33 ± 0.25	80.47 ± 1.06	9.92 ± 0.30	14.73 ± 0.93	39.14 ± 0.03	6.84 ± 0.09
1C:2M	15	55	208.36 ± 1.49	96.68 ± 3.05	47.84 ± 0.97	86.79 ± 2.79	8.35 ± 0.24	9.81 ± 0.74	39.18 ± 0.12	6.46 ± 0.09
1C:2M	30	55	209.64 ± 0.19	95.90 ± 0.75	47.88 ± 0.17	86.18 ± 0.71	8.32 ± 0.11	9.70 ± 0.34	39.13 ± 0.02	6.66 ± 0.04
1C:2M	60	55	208.22 ± 2.21	94.69 ± 0.39	48.37 ± 0.18	84.96 ± 0.42	9.06 ± 0.57	12.03 ± 1.80	39.17 ± 0.06	6.54 ± 0.26

[1] Saponification value (SV)
[2] Iodine value (IV)
[3] Cetane number (CN)
[4] Degree of unsaturation (DU)
[5] Long-chain saturation factor (LCSF)
[6] Cold filter plugging point (CFPP)
[7] High heating value (HHV)
[8] Oxidative stability (OS)

were also evaluated. Table 4 represents the effect of these factors on the most important biodiesel characterizations including SV, IV, CN, DU, LCSF, CFPP, HHV, and OS. The results showed that higher ratios of chloroform to methanol (1 M:2C) can improve the quality of biodiesel by increasing the value of CN index. Also, increasing of the reaction temperature increased the CN value, gently. LCSF, CFPP, and OS were the other indexes that showed a positive correlation with higher temperature and higher ratio of chloroform to methanol. In contrast, higher reaction temperature and chloroform concentration decreased the values of IV and DU indexes due to the increasing amounts of UFAs of lipids. As it has been presented in Table 4, ultrasonication time had no significant effect on the biodiesel properties.

4. Conclusions

This study demonstrated that the polarity of solvents and lipid extraction conditions not only affect the amount of lipid extracted from microalgal biomass, but also the FAMEs composition. Since the fatty acids profile determines the properties of biodiesel, therefore, biofuel quality can be controlled by selecting the appropriate extraction solvents. In this respect, solvent mixture with higher ratio of non-polar solvent can be applied at higher temperature to produce biodiesel having more oxidative stability compatible to tropical region. On the other hand, polar solvents at lower temperature can be used to enhance the fluidity of fuels in cold climate.

CRediT authorship contribution statement

Mohammad Javad Zarrinmehr: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. **Ehsan Daneshvar:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Subhasha Nigam:** Writing – review & editing. **Kannappan Panchamoorthy Gopinath:** Writing – review & editing. **Jayanta Kumar Biswas:** Writing – review & editing. **Eilhann E. Kwon:** Writing – review & editing. **Hailong Wang:** Writing – review & editing. **Omidvar Farhadian:** Writing – review & editing. **Amit Bhatnagar:** Funding acquisition, Project administration, Supervision, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

da Costa, E., Silva, J., Mendonça, S., Abreu, M., Domingues, M., 2016. Lipidomic approaches towards deciphering glycolipids from microalgae as a reservoir of bioactive lipids. Mar. Drugs 14 (5), 101. https://doi.org/10.3390/md14050101.

de Jesus, S.S., Ferreira, G.F., Moreira, L.S., Filho, R.M., 2020. Biodiesel production from microalgae by direct transesterification using green solvents. Renew. Energy 160, 1283–1294.

Deshmukh, S., Kumar, R., Bala, K., 2019. Microalgae biodiesel: A review on oil extraction, fatty acid composition, properties and effect on engine performance and emissions. Fuel Process. Technol. 191, 232–247.

dos Santos, R.R., Moreira, D.M., Kunigami, C.N., Aranda, D.A.G., Teixeira, C.M.L.L., 2015. Comparison between several methods of total lipid extraction from *Chlorella vulgaris* biomass. Ultrason. Sonochem. 22, 95–99.

Ellison, C.R., Overa, S., Boldor, D., 2019. Central composite design parameterization of microalgae/cyanobacteria co-culture pretreatment for enhanced lipid extraction using an external clamp-on ultrasonic transducer. Ultrason. Sonochem. 51, 496–503.

Hadrich, B., Akremi, I., Dammak, M., Barkallah, M., Fendri, I., Abdelkafi, S., 2018. Optimization of lipids' ultrasonic extraction and production from Chlorella sp. using response-surface methodology. Lipids Health Dis. 17, 1–9.

He, Q., Yang, H., Hu, C., 2016. Culture modes and financial evaluation of two oleaginous microalgae for biodiesel production in desert area with open raceway pond. Bioresour. Technol. 218, 571–579.

Iqbal, J., Theegala, C., 2013. Microwave assisted lipid extraction from microalgae using biodiesel as co-solvent. Algal Res. 2 (1), 34–42.

Karimi, M., 2017. Exergy-based optimization of direct conversion of microalgae biomass to biodiesel. J. Clean Produ. 141, 50–55.

Mandik, Y.I., Cheirsilp, B., Srinuanpan, S., Maneechote, W., Boonsawang, P., Prasertsan, P., Sirisansaneeyakul, S., 2020. Zero-waste biorefinery of oleaginous microalgae as promising sources of biofuels and biochemicals through direct transesterification and acid hydrolysis. Process Biochem. 95, 214–222.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sustain. Energy. Rev. 14 (1), 217–232.

Matsui, H., Shiozaki, K., Okumura, Y., Ishikawa, M., Waqalevu, V., Hayasaka, O., Honda, A., Kotani, T., 2020. Effects of phosphorous deficiency of a microalga *Nannochloropsis oculata* on its fatty acid profiles and intracellular structure and the effectiveness in rotifer nutrition. Algal Res. 49, 101905. https://doi.org/10.1016/j.algal.2020.101905.

Mubarak, M., Shaija, A., Suchithra, T.V., 2015. A review on the extraction of lipid from microalgae for biodiesel production. Algal Res. 7, 117–123.

Nayak, J.K., Ghosh, U.K., 2019. Post treatment of microalgae treated pharmaceutical wastewater in photosynthetic microbial fuel cell (PMFC) and biodiesel production. Biomass Bioenergy 131, 105415. https://doi.org/10.1016/j.biombioe.2019.105415.

Ortiz-Martínez, V.M., Andreo-Martínez, P., García-Martínez, N., Pérez de los Ríos, A., Hernández-Fernández, F.J., Quesada-Medina, J., 2019. Approach to biodiesel production from microalgae under supercritical conditions by the PRISMA method. Fuel Process. Technol. 191, 211–222.

Ozola-Davidane, R., Burlakovs, J., Tamm, T., Zeltkalne, S., Krauklis, A.E., Klavins, M., 2021. Bentonite-ionic liquid composites for Congo red removal from aqueous solutions. J. Mol. Liq. 337, 116373. https://doi.org/10.1016/j.molliq.2021.116373.

Ricciutelli, M., Di Martino, P., Barboni, L., Martelli, S., 2006. Evaluation of rapamycin chemical stability in volatile-organic solvents by HPLC. J. Pharm. Biomed. Anal. 41 (3), 1070–1074.

Rinna, F., Buono, S., Cabanelas, I.T.D., Nascimento, I.A., Sansone, G., Barone, C.M.A., 2017. Wastewater treatment by microalgae can generate high quality biodiesel feedstock. J. Water Process Eng. 18, 144–149.

Ryckebosch, E., Muylaert, K., Foubert, I., 2012. Optimization of an analytical procedure for extraction of lipids from microalgae. J. Am. Oil Chem. Soc. 89 (2), 189–198.

Salam, K.A., Velasquez-Orta, S.B., Harvey, A.P., 2016. A sustainable integrated in situ transesterification of microalgae for biodiesel production and associated co-product-a review. Renew. Sustain. Energy. Rev. 65, 1179–1198.

Sandani, W.P., Nishshanka, G.K.S.H., Premaratne, R.G.M.M., Nanayakkara Wijayasekera, S.C., Ariyadasa, T.U., Premachandra, J.K., 2020. Comparative assessment of pretreatment strategies for production of microalgae-based biodiesel from locally isolated *Chlorella homosphaera*. J. Biosci. Bioeng. 130 (3), 295–305.

Sivaramakrishnan, R., Incharoensakdi, A., 2019. Enhancement of lipid extraction for efficient methyl ester production from *Chlamydomonas* sp. J. Appl. Phycol. 31 (4), 2365–2377.

Srinuanpan, S., Cheirsilp, B., Prasertsan, P., Kato, Y., Asano, Y., 2018. Strategies to increase the potential use of oleaginous microalgae as biodiesel feedstocks: Nutrient starvations and cost-effective harvesting process. Renew. Energy 122, 507–516.

Valdez-Ojeda, R., González-Muñoz, M., Us-Vázquez, R., Narváez-Zapata, J., Chavarria-Hernandez, J.C., López-Adrián, S., Barahona-Pérez, F., Toledano-Thompson, T., Garduño-Solórzano, G., Escobedo-Gracia Medrano, R.M., 2015. Characterization of five fresh water microalgae with potential for biodiesel production. Algal Res. 7, 33–44.

Vignesh, N.S., Vimali, E., Sangeetha, R., Arumugam, M., Ashokkumar, B., Ganeshmoorthy, I., Varalakshmi, P., 2020. Sustainable biofuel from microalgae: Application of lignocellulosic wastes and bio-iron nanoparticle for biodiesel production. Fuel 278, 118326. https://doi.org/10.1016/j.fuel.2020.118326.

Wang, F., Gao, B., Huang, L., Su, M., Dai, C., Zhang, C., 2018. Evaluation of oleaginous eustigmatophycean microalgae as potential biorefinery feedstock for the production of palmitoleic acid and biodiesel. Bioresour. Technol. 270, 30–37.

Wu, H., Miao, X., 2014. Biodiesel quality and biochemical changes of microalgae *Chlorella pyrenoidosa* and *Scenedesmus obliquus* in response to nitrate levels. Bioresour. Technol. 170, 421–427.

Xia, A.o., Sun, C., Fu, Q., Liao, Q., Huang, Y., Zhu, X., Li, Q., 2020. Biofuel production from wet microalgae biomass: Comparison of physicochemical properties and extraction performance. Energy 212, 118581. https://doi.org/10.1016/j.energy.2020.118581.

Xu, J., Zhao, F., Su, X., 2021. Direct extraction of lipids from wet microalgae slurries by super-high hydrostatic pressure. Algal Res. 58, 102412. https://doi.org/10.1016/j.algal.2021.102412.

Yang, I.-S., Salama, E.-S., Kim, J.-O., Govindwar, S.P., Kurade, M.B., Lee, M., Roh, H.-S., Jeon, B.-H., 2016. Cultivation and harvesting of microalgae in photobioreactor for biodiesel production and simultaneous nutrient removal. Energy Convers. Manag. 117, 54–62.

Zhang, L., Cheng, J., Pei, H., Pan, J., Jiang, L., Hou, Q., Han, F., 2018. Cultivation of microalgae using anaerobically digested effluent from kitchen waste as a nutrient source for biodiesel production. Renew. Energy 115, 276–287.