

Optimising Extraction Methods for Enhanced Lipid Recovery from Macroalgae

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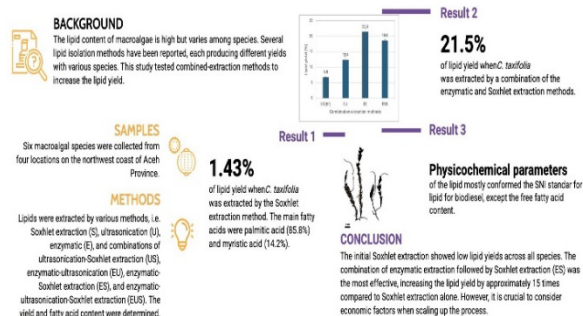
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Abstract: Biodiesel feedstocks have evolved from the use of food-grade vegetable oils to lipids from microalgae and microorganisms. The challenges faced in the production of biodiesel from this third-generation lipids lie in the efficient extraction techniques and production costs. This study aimed to optimise extraction techniques to enhance lipid recovery from macroalgae. Six macroalgal species from the northwest coast of Aceh, Indonesia, were initially screened to evaluate their lipid yields using the Soxhlet extraction (S) method. *Caulerpa taxifolia* was selected for further optimisation based on its high lipid yield and favourable fatty acid profile, consisting of 85.8 % palmitic acid (C16:0) and 14.2 % myristic acid (C14:0). Various extraction methods were tested, including ultrasonication (S), enzymatic extraction (E), and combinations of ultrasonication-Soxhlet extraction (US), enzymatic-ultrasonication (EU), enzymatic-Soxhlet extraction (ES), and enzymatic-ultrasonication-Soxhlet extraction (EUS). The ES method produced the highest lipid yield of 21.5 %, a 15-fold increase compared to that of the S method alone. Physicochemical analysis of the extracted lipids showed properties suitable for biodiesel production, although further refinement is required to fully meet the quality standards. This study demonstrates the potential of optimised extraction techniques to significantly enhance lipid recovery from macroalgae, thereby contributing to the development of sustainable marine-biofuel resources. Further research is required to refine these methods and explore their large-scale applicability in the field.



Keywords: macroalgae, *C. taxifolia*, ultrasonication, enzymatic, soxhle

1 Introduction

Macroalgae, commonly known as seaweeds, are promising resources for various applications. They are multicellular algae that thrive in shallow coastal waters on sandy or rocky substrates and bask in sunlight¹. Their rich content of lipids, polysaccharides, proteins, and various secondary metabolites² makes them a source of food, energy, cosmetics, and pharmaceutical products³.

The lipid content of macroalgae varies by species and habitat, with some green and brown species having more than 10 % lipid content on a dry weight basis⁴⁻⁶. For instance, *Dictyota sandvicensis* has an approximate lipid content of 20 %⁷. In contrast, some species have low lipid contents, such as *Caulerpa* sp. (1.23 %), *Sargassum* sp. (0.58 %), and *Turbinaria* sp. (5.69 %) ^{8, 9}. Red macroalgae, such as *Gracilaria corticata* and *Hypnea valentiae*, contain less than 3 % lipids^{10, 11}.

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The utilisation of macroalgal lipids depends on their fatty acid composition, with those high in omega-3, -6, and -9 fatty acids being suitable for food¹²⁾, long-chain polyunsaturated fatty acids as biodiesel feedstock, and stearic, oleic, and myristic acids used in cosmetics¹³⁾.

The fatty acid compositions of lipids also differ among macroalgal species. Green macroalgae typically contain palmitic, stearic, oleic, linoleic, and linolenic acids. In contrast, brown macroalgae are rich in palmitic, arachidonic, eicosapentaenoic, oleic, stearic, linoleic, and linolenic acids. Red macroalgae mainly contain palmitic, myristic, oleic, arachidonic, and eicosapentaenoic acids¹⁴⁾. Variations in lipid and fatty acid content are closely linked to environmental factors such as season, temperature, and salinity⁴⁾.

The extraction method is essentially aimed at breaking down the cell wall to release lipids stored in the form of lipid droplets or the cell membrane component. The simplest method is the Bligh and Dyer method, which is based on the principle of differences in lipid solubility within a solvent mixture. This method has been used to extract lipids from macroalgae with high consistency⁴⁾, but less environmentally friendly compared to other methods^{15, 16)}. The most used method is Soxhlet extraction¹⁷⁾, which gives a relatively high yield. For example, lipid extraction from the filamentous green algae *Spirogyra* using Soxhlet extraction with n-hexane as the solvent has been reported to produce yields of more than 80%¹⁸⁾. The drawbacks of this method are the high costs for solvent, energy, and waste management. Pressing method gives an alternative to overcome the environmental problems caused by the use of solvents. Although it is cost effective, the yield is low for macroalgal lipids¹⁹⁾. The supercritical fluid method can produce high yield if the correct solvent mixture is used. The lipid yield from *Chlorella vulgaris* using this method can reach about 40% when using a CO₂-ethanol mixture under supercritical conditions, but only around 2% when using CO₂ alone²⁰⁾. The addition of enzymes, such as cellulase, helps degrade the cell wall structure²¹⁾ allowing intracellular lipids to be extracted. The lipid yield from macroalgae *Codium tomentosum* with enzyme addition has been reported to increase extraction results to between 48–62%²²⁾. The application of ultrasonic waves can also degrade cell wall integrity and depends on the frequency used, ranging from 0.3 to 300 GHz²⁰⁾. Its application in combination with other methods may help save time and increase the yield^{15, 23)}.

Several macroalgal species were isolated from the northwest coast of Aceh Province, Indonesia. The macroalgae were identified in significant abundance²⁴⁾. Studies on macroalgae from Aceh waters have been limited, with the majority focusing on antioxidant content²⁵⁾ and heavy metal absorption²⁶⁾. However, studies on their lipids have not been widely reported.

Therefore, this study aimed to optimise lipid extraction methods from macroalgae for future research. An optimal extraction method would increase the quantity of extracted lipids and promote their application for various purposes.

2 Materials and Methods

2.1 Preparation of macroalgae samples

Samples were collected from four seashores on the northwest coast of Aceh Province, Indonesia, namely Ulee Lheue, Ujung Pancu, Lange, and Lhoknga. Six macroalgae species were used based on their abundance, namely *Sargassum plagiophyllum*, *Turbinaria decurrens*, *Hypnea durvillei*, *Gracilaria edulis*, *Valoniopsis pachynema*, and *Caulerpa taxifolia*. The salinity, pH, and water temperature at each sampling site were determined in situ using the corresponding portable meters. The samples were then washed and dried in sunlight for 48–72 h. The dried samples were ground, sieved using a 100 mesh sieve, and stored at 4 °C before use²⁷⁾.

2.2 Extraction of macroalgae lipid

Fine samples (100 g) were extracted using the Soxhlet extraction method with 300 mL n-hexane at 65 °C for 6 h. After centrifugation at 3000 rpm for 15 min, the topmost layer was added with 0.1 g of Na₂SO₄, and the solution was concentrated using a rotary evaporator. The extract was used to calculate the yield (Equation 1) and for physicochemical analysis (viscosity, density, saponification value, free fatty acids, and moisture content)²⁸⁾. Indigenous amylase producing *Aspergillus terreus* isolates were used for enhanced production of amylases in an optimization study. Physico-chemical parameters of pH, temperature and substrates with three different concentrations were used to evaluate their influence on amylase production. Further, the susceptibility of Ochratoxin A (OTA) producing *A. terreus* to different plant essential oils were also determined.

$$\text{Yield (\%)} = \frac{\text{Weight of lipid (g)}}{\text{Dry weight of macroalgae (g)}} \times 100 \%$$

(1)

2.3 Analysis of fatty acid

The fatty acids of the lipids were analysed using Gas Chromatography-Mass Spectrophotometry (GC-MS)-QP2010 Ultra with an Rtx-5MS column and helium as the carrier gas. The analysis conditions were as follows: separation temperature 31 °C; injection temperature 270 °C, injection time 1.00 min, and flow rate of 1.2 mL/min.

2.4 Optimisation of macroalgae lipid extraction

C. taxifolia was chosen to optimise the lipid extraction method as it had a high yield and low free fatty acid content. In addition to Soxhlet extraction (S), lipids were extracted using ultrasonication (U), enzymatic (E), and combinations of ultrasonication-Soxhlet extraction (US), enzymatic-ultrasonication (EU), enzymatic-Soxhlet extraction (ES), and enzymatic-ultrasonication-Soxhlet extraction (EUS) methods.

2.4.1 Ultrasonication extraction

C. taxifolia powder (10 g) was mixed with 150 mL n-hexane and sonicated in a 40 kHz ultrasonicator (Brason 8510) for 15, 30, 45, and 60 min at 50, 55, 60, and 65 °C. The suspensions were separated by centrifugation at 3000 rpm for 15 min. The supernatant was added with 0.1 g Na₂SO₄, and the solution was concentrated using a rotary evaporator²⁹. The yield was calculated using Equation 1. The treatment that produced the highest yield was applied to the combination extraction experiments.

2.4.2 Enzymatic extraction

Cellulase enzyme (MedChemExpress, specific activity 534 U/mg protein) was dissolved in acetate buffer (pH 4.8) at various concentrations (10, 50, 100, and 200 U/g). *C. taxifolia* powder (10 g) was mixed with 100 mL of the enzyme solution. The mixture was incubated at 50 °C and 150 rpm for 30 min. The enzyme was inactivated by adding trichloroacetic acid. The mixture was then centrifuged at 3000 rpm for 15 min. The topmost layer was separated and added with 0.1 g of Na₂SO₄. The solution was then concentrated using a rotary evaporator³⁰. The concentrated extract was weighed to calculate its yield using Equation 1. The treatment that produced the highest yield was applied to the combination extraction experiments.

2.4.3 Enzymatic and ultrasonication extraction

C. taxifolia powder (100 g) was subjected to enzymatic extraction using cellulase at 10 U/g, following the procedure in Section 2.4.2. The mixture was ultrasonicated at 50 °C for 30 min. After centrifugation at 3000 rpm for 15 min, the topmost layer was added with 0.1 g of Na₂SO₄. The solution was concentrated using a rotary evaporator³¹, and the yield was calculated using Equation 1.

2.4.4 Enzymatic and Soxhlet extraction

C. taxifolia powder (100 g) was subjected to enzymatic extraction using cellulase at 10 U/g, following the procedure in Section 2.4.2. The samples were then extracted using the Soxhlet extraction method described in Section 2.2.

2.4.5 Enzymatic, ultrasonication and Soxhlet extraction

Lipids from *C. taxifolia* powder (100 g) were extracted using the procedures described in Section 2.4.3. Subsequently, the crude mixture was extracted using the Soxhlet extraction method described in Section 2.2.

2.5 Physicochemical analysis

The moisture content was determined gravimetrically by heating the sample at 100 °C until a constant weight was reached. The difference in weight before and after heating was used to calculate the water content³².

The specific gravity was determined using a 5 mL pycnometer. The weight of the pycnometer before and after filling it with a particular volume of lipid was used to calculate the specific gravity³².

Viscosity analysis was performed on 5 mL of lipid using a viscometer (NDJ-8S viscometer). The appropriate spindle and rotational speed were used, and the readings were measured in centistokes (cSt)³².

The free fatty acid (FFA) content was determined by dissolving 1 g of lipid in 5 mL of 96 % ethanol at 50 °C. The solution was then titrated with 0.1 N NaOH using phenolphthalein as the indicator³². The FFA count was calculated using Equation 2.

$$\% \text{ FFA} = \frac{V_{\text{NaOH}} (\text{mL}) \times C_{\text{NaOH}} (\text{N}) \times Mr_{\text{palmitic acid}} (\text{g/mol})}{W_{\text{sample}} (\text{g}) \times 1000} \times 100 \% \quad (2)$$

where V_{NaOH} is the volume of NaOH used for titration (mL), C_{NaOH} is the concentration of NaOH used for titration (N), $Mr_{\text{palmitic acid}}$ is the molecular weight of palmitic acid (256.42 g/mol), and W_{sample} is the weight of the lipid sample (g).

$$\text{SV} = \frac{(V_{\text{blank}} - V_{\text{sample}}) (\text{mL}) \times C_{\text{KOH}} (\text{N}) \times Mr_{\text{KOH}} (\text{g/mol})}{W_{\text{sample}} (\text{g})} \quad (3)$$

The saponification value (SV) was determined by dissolving 0.1 g of lipid in 25 mL of 0.1 N alcoholised KOH. The solution was then refluxed for approximately 1.5 h. After cooling, the solution was titrated with 0.1 N HCl using phenolphthalein as the indicator. The blank was prepared similarly but without a sample³². The SV was calculated using Equation 3.

Where V_{blank} is the volume of the blank (mL), V_{sample} is the volume of the lipid sample (mL), C_{KOH} is the concentration of KOH for titration (N), M_{KOH} is the molecular weight of KOH (56.1 g/mol), and W_{sample} is the weight of the lipid sample (g). The iodine value (IV) was determined by mixing 1 g of lipid, 1 mL of chloroform, and 25 mL of Wijs reagent. The mixture was kept in the dark for 30 min and stirred to ensure its homogeneity. Subsequently, 10 mL of 15 % KI solution, 50 mL of distilled water, and 2 mL of amylum solution were added to the mixture. The mixture was then titrated with 0.05N $\text{Na}_2\text{S}_2\text{O}_3$ until the blue colour disappeared. The blank was prepared using a similar composition but without the sample³². The IV was calculated using Equation 4.

$$IV = \frac{(V_{\text{blank}} - V_{\text{sample}}) (\text{mL}) \times C_{\text{Na}_2\text{S}_2\text{O}_3} (\text{N}) \times 12.69}{W_{\text{sample}} (\text{g})} \quad (4)$$

Where V_{blank} is the volume of the blank (mL), V_{sample} is the volume of the lipid sample (mL), $C_{\text{Na}_2\text{S}_2\text{O}_3}$ is the concentration of sodium thiosulphate for titration (N), 12.69 is the conversion factor from milliequivalents of sodium thiosulfate to g of iodine, and W_{sample} is the weight of the lipid sample (g).

2.6 Data analysis

All analyses were performed in triplicate, and the data are presented as mean $\pm$ standard deviation. A Complete Randomised Design (CRD) was used to analyse the effect of temperature and time on the yield in the sonication extraction experiment. One-way ANOVA was performed to analyse the effect of enzyme concentrations and combined-extraction methods on the yield, followed by the Duncan test. The analysis was done using SPSS version 27, with statistically significant differences $p < 0.05$.

3 Results

3.1 Water quality of the sampling location

Water quality parameters at the four sampling locations were measured to provide an overview of the habitats. Temperature, pH, and salinity were measured in situ before noon (**Table 1**). At Ulee Lheue, the temperature was 25 °C, with a pH of 9.0 and a salinity of 31.3 ppt. Ujung Pancu had a temperature of 25.7 °C, pH of 8.1, and salinity of 30.3 ppt, whereas Lange had a temperature of 25 °C, pH of 8.1, and salinity of 29.3 ppt. Lhoknga showed the highest temperature (32.8 °C), pH (8.8), and salinity (31.7 ppt). The data showed variability in environmental conditions across the sampling sites, which can influence macroalgae growth and properties.

3.2 Initial lipid extraction

Lipids from the six macroalgae were initially extracted using the Soxhlet extraction method. The extracted lipids were analysed for moisture content, density, viscosity, free fatty acids, saponification value, and fatty acid profiles. This analysis aimed to select one macroalga from the six species for further investigations.

The lipid content varied across species, with green macroalgae showing the highest values, *V. pachynema* at 1.47 ± 0.32 % and *C. taxifolia* at 1.43 ± 0.13 %. Viscosity measurements showed that *T. decurrens* had the highest value (17.87 ± 0.18 cSt), whereas *C. taxifolia* had the lowest value (7.96 ± 0.89 cSt). *V. pachynema* had the highest free fatty acid

Table 1 Water parameters of macroalgae sampling locations.

Sampling location	Coordinate	Environmental condition		
		Temperature (°C)	pH	Salinity (ppt)
Ulee Lheue	5°33'37" N 95°17'09" E	25 $\pm$ 0	9.0 $\pm$ 0.3	31.3 $\pm$ 0.5
Ujung Pancu	5°33'52" N 95°14'09" E	25.67 $\pm$ 0.6	8.1 $\pm$ 0.1	30.3 $\pm$ 0.9
Lange	5°30'53" N 95°12'16" E	25 $\pm$ 0	8.1 $\pm$ 0.2	29.3 $\pm$ 0.5
Lhoknga	5°26'49" N 95°14'32" E	32.8 $\pm$ 1.5	8.8 $\pm$ 1.0	31.7 $\pm$ 1.3

content (7.14 ± 1.58 %), and *C. taxifolia* had the lowest (2.55 ± 0.34 %). The lipid density ranged from 784 ± 4 kg/m³ for *T. decurrens* to 861 ± 1 kg/m³ for *V. pachynema*. The saponification values ranged from 238.5 ± 12.5 mg KOH/g for *T. decurrens* to 564.8 ± 10.8 mg KOH/g for *H. durvillei*. The moisture content also varied, with *V. pachynema* showing the highest value at 11.19 ± 0.15 %, and *C. taxifolia* showing the lowest value at 1.27 ± 0.69 % (Table 2). These data indicate a disparity in the processing potential.

The fatty acids of all lipids showed variations in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Fatty acids in *G. edulis* and *C. taxifolia* were all SFA. The SFA in *G. edulis* comprised lauric (17.2 %) and palmitic acids (82.8 %), whereas those in *C. taxifolia* comprised myristic (14.2 %) and palmitic acids (85.8 %). All other species contained abundant SFA, except *V. pachynema*, which contained only 18.3 % SFA. MUFA was most prevalent in *V. pachynema* (81.7 %), but PUFA was not detected in any of the lipids (Table 3).

3.3 Optimisation of the lipid extraction from *C. taxifolia*

To optimise lipid extraction, *C. taxifolia* was selected because its lipid content was the highest among the other macroalgae, and the free fatty acids comprised desirable SFA of myristic and palmitic acids.

Ultrasonication extraction experiments were performed at various temperatures (50, 55, 60, and 65 °C) and extraction times (15, 30, 45, and 60 min). At all temperatures, the optimum yield was achieved within 30 min. At 60 °C, the yield peaked at 4.14 ± 0.16 %, which was the maximum across all temperature and time combinations (Fig. 1A). These results indicate that a higher temperature and moderate ultrasonication time significantly enhanced lipid extraction efficiency. Analysis using CRD indicated that, at a 95 % significance level, varying ultrasonication time resulted in significant differences in lipid yield at each temperature and time, with a *p*-value of 0.0009.

Enzyme-assisted extraction was performed using various enzyme concentrations (10, 50, 100, and 200 U/g). At 200 U/g, the yield was 8.9 ± 0.2 %, while at 100 U/g, it decreased to 7.6 ± 0.4 %. At 50 U/g, the yield increased to 9.2 ± 0.2 %, and at 10 U/g, the highest yield was obtained at 9.6 ± 0.2 % (Fig. 1B). One-way ANOVA followed by Duncan test showed that there was a significant difference in the lipid yield due to cellulase concentrations, with *p*-value < 0.05. These data suggest that lower enzyme concentrations may enhance lipid extraction efficiency.

Subsequent experiments combined independent extraction methods to increase lipid yield. Four combination methods were used, namely ultrasonication–Soxhlet (US), enzymatic ultrasonication (EU), enzymatic Soxhlet (ES), and enzymatic ultrasonication (EUS).

The US method tested two different Soxhlet extraction times (3 and 6 h). At 3 h, the lipid yield was 5.9 ± 0.14 %, which increased to 6.8 ± 0.09 % at 6 h (Fig. 2). A Soxhlet extraction time of 6 h was used in subsequent experiments. The combined method that resulted in the highest yield was the ES method (21.5 ± 1.16 %). This method outperformed the other methods tested. Following this, EUS yielded 18.6 ± 0.35 % and EU produced a lower yield of 12.4 ± 1.89 %. There was a significant difference in the lipid yield due to the combined-extraction method, as revealed by one-way ANOVA followed by Duncan test, with *p*-value < 0.05. The results indicate the superior efficiency of the ES method in maximising lipid extraction from *C. taxifolia*; thus, this method is recommended for optimal lipid recovery.

3.4 Physicochemical properties of the lipid from *C. taxifolia* by the combined methods

The physicochemical properties of the lipids extracted from *C. taxifolia* using the combined US, EU, ES, and EUS methods were evaluated for density, viscosity, free fatty acid content, saponification, iodine value, and water content. The results were subsequently compared to the Indonesian National Standard (SNI) 7431:2015 for lipid-based biodiesel (Table 4).

The lipid viscosities ranged from 7.3 ± 1.3 cSt for the US method to 15.0 ± 2.1 cSt for the EUS method, indicating that the extraction method significantly influenced the fluidity of the extracted lipids. The lipid density also varied, with the EUS method yielding the lowest value of 830 ± 3 kg/m³, and ES yielding the highest value of 904 ± 1.7 kg/m³. The iodine value ranged from 9.1 ± 0.72 g I₂/100 g for the US method to 16.1 ± 2.02 g I₂/100 g for the EU method. The viscosity, density, and iodine value were within the SNI standard of < 36 cSt, 870-910 Kg/m³, and >115 g I₂/100 g, respectively.

The saponification value was the highest for the EUS method (349 ± 1.6 mg KOH/g), exceeding the SNI standard of 180-256 mg KOH/g. The other methods gave values within the standard range. However, the free fatty acid content for all methods exceeded the permitted standard of a maximum of 4 %, ranging from 6.63 ± 0.96 % in the ES method to 14.7 ± 5.2 % in the US method. The moisture content also exceeded the permitted range of 0.1 %, with the EU and EUS methods showing the highest (4.88 ± 0.11 %) and lowest (1.41 ± 0.51 %) values, respectively.

Table 2 Physicochemical properties of macroalgae lipid.

Parameters	Macroalgae					
	Brown		Red		Green	
	<i>S. plagiophyllum</i>	<i>T. decurrens</i>	<i>H. durvillei</i>	<i>G. edulis</i>	<i>V. pachynema</i>	<i>C. taxifolia</i>
Lipid content (%)	0.92 ± 0.11	1.22 ± 0.41	0.87 ± 0.27	1.33 ± 0.05	1.47 ± 0.32	1.43 ± 0.13
Viscosity (T = 40°C, cSt)	12.78 ± 0.42	17.87 ± 0.18	8.81 ± 0.21	10.71 ± 0.15	9.29 ± 0.02	7.96 ± 0.89
Density (kg/m ³)	802 ± 1	784 ± 4	834 ± 2	840 ± 6	861 ± 1	816 ± 1
Saponification value (mg KOH/g)	350.7 ± 8.7	238.5 ± 12.5	564.8 ± 10.8	311.2 ± 13.1	399.6 ± 3.1	265.5 ± 5.6
Free fatty acids (%)	2.68 ± 1.07	5.49 ± 2.90	3.44 ± 2.74	3.37 ± 1.54	7.14 ± 1.58	2.55 ± 0.34
Moisture content (%)	3.83 ± 0.26	1.80 ± 0.29	6.77 ± 1.07	6.65 ± 0.45	11.19 ± 0.15	1.27 ± 0.69

Table 3 Fatty acid composition in lipid macroalgae was determined using the Soxhlet extraction method.

Fatty acid content	Fatty acid (%)					
	Brown macroalgae		Red macroalgae		Green macroalgae	
	<i>S. plagiophyllum</i>	<i>T. decurrens</i>	<i>H. durvillei</i>	<i>G. edulis</i>	<i>V. pachynema</i>	<i>C. taxifolia</i>
Saturated Fatty Acids (SFA)						
C4:0 Butanoic acid (butyric acid)	0.4	-	-	-	-	-
C8:0 Octanoic acid, 1,2,3-propanetriyl (caprylic acid)	-	8.2	-	-	-	-
C10:0 Decanoic acid (CAS) (capric acid)	-	1.3	-	-	-	-
C12:0 Dodecanoic acid (lauric acid)	6.9	19.8	-	17.2	-	-
C13:0 Tridecanoic acid	-	6.0	-	-	-	-
C14:0 Tetradecanoic acid (myristic acid)	-	18.3	-	-	-	14.2
C15:0 Pentadecanoic acid	-	1.0	-	-	-	-
C16:0 Hexadecenoic acid (palmitic acid)	68.4	20.0	57.1	82.8	18.3	85.8
C18:0 Octadecanoic acid (stearic acid)	-	3.1	-	-	-	-
C20:0 Eicosatetraenoic acid (arachidonic acid)	9.0	5.4	-	-	-	-
C21:0 Heneicosanoic acid	-	-	23.0	-	-	-
Total SFA	84.7	83.1	80.1	100	18.3	100
Monounsaturated Fatty Acid (MUFA)						
C18:1n-9 <i>Cis</i> -9-octadecanoic acid (oleic acid)	15.3	16.9	6.6	-	-	-
C19:0 11-Octadecenoic acid	-	-	13.3	-	-	-
C30:0 Eicosyl trans-hex-3-enyl (fumaric acid)	-	-	-	-	81.7	-
Total MUFA	15.3	16.9	19.9	0.00	81.7	0.00
Total PUFA	0.00	0.00	0.00	0.00	0.00	0.00
Total SFA+MUFA+PUFA	100	100	100	100	100	100

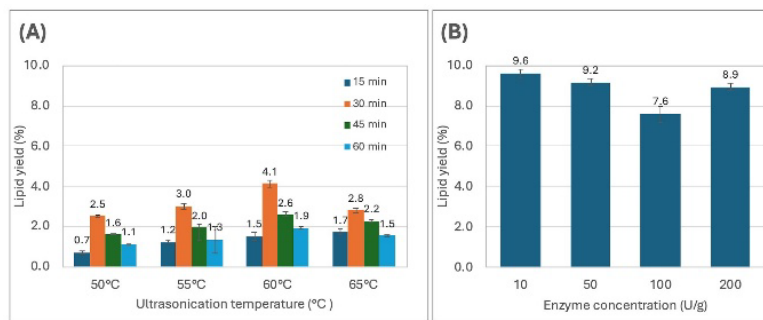


Fig 1 Lipid yield of *C. taxifolia* by (A) Ultrasonication extraction (40 Hz) at various temperatures and times and (B) Enzymatic extraction at various enzyme concentrations at 50 °C for 30 min. One-way ANOVA was performed to evaluate the differences.

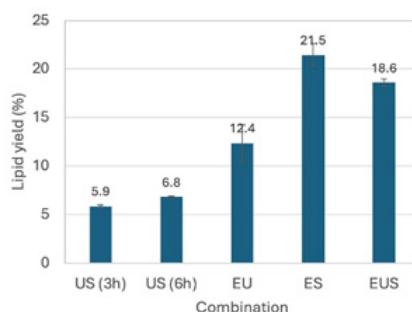


Fig 2 Lipid yield of *C. taxifolia* after extraction by variation in Soxhlet extraction time and combined methods. One-way ANOVA was performed to evaluate the differences.

4 Discussion

Habitat conditions, such as temperature, pH, and salinity, significantly influence the physiology and growth of macroalgae³³. At the sampling sites, temperatures ranged from 25 to 33 °C, which is within the temperature range for tropical marine macroalgae³⁴. Lhoknga had a higher average temperature due to its proximity to industrial areas (Table 1). The pH levels at the sites ranged from 8.0 to 9.0, compared to the normal range of 7.5–8.4³⁵, with Ulee Lheue and Lhoknga showing slightly alkaline pH, likely due to tourism and nearby cement industries. Macroalgae generally tolerate a wide pH range³⁶. The salinity at the sampling site varied from 29 to 32 ppt, which is suitable for macroalgal growth (15–35 ppt)³⁷.

Lipids were initially extracted from macroalgae using the Soxhlet extraction method for species-selection. The yields were low, ranging from 0.87 ± 0.27 % in *H. durvillei* to 1.47 ± 0.32 % in *V. pachynema* (Table 2). Many macroalgal genera, such as *Caulerpa* sp., *Sargassum* sp., *Gracilaria* sp. and *Hypnea* sp., have been reported to have less than 3 % lipid^{8–11}. However, some studies have shown that *C. taxifolia* and *V. pachynema* could have more than 5 % lipid^{38, 39}. These differences are presumably influenced by habitat conditions and nutrient availability⁴¹.

The viscosity of the lipid in this study varied from 8 to 18 cSt, with *C. taxifolia* having the lowest and *T. decurrens* the highest (Table 2). Viscosity increases with fatty acid chain length and decreases with the degree of unsaturation⁴⁰. Lipids rich in long-chain SFA have a higher viscosity, whereas short-chain SFA and unsaturated MUFA or PUFA have lower viscosity¹⁰. The presence of multiple double bonds in the fatty acid chain reduces viscosity, as the cis configuration creates bends that inhibit rigid crystal formation^{40, 41}.

Analysis of the fatty acid profile showed that, except for *V. pachynema*, all macroalgae lipids were primarily composed of SFA, with *G. edulis* and *C. taxifolia* containing only SFA. Palmitic acid (C16) was the predominant fatty acid in *C. taxifolia*, whereas *T. decurrens* had a range of SFA from caprylic (C8) to arachidonic (C20). Palmitic, myristic (C14), and lauric (C12) acids each comprised approximately one-fifth of the total fatty acids (Table 3).

Fatty acid content influences viscosity, density, and saponification values⁴². *V. pachynema* lipid had the highest density among samples at 861 kg/m³ despite being dominated by MUFA. This may be due to the presence of complex mixtures of MUFA⁴⁰. Additionally, many fatty acids in *V. pachynema* have shorter carbon chains than those in other

macroalgae species. GC-MS analysis showed a high sterol content in the lipid, which correlated with its relatively low viscosity⁴³. *V. pachynema* also had the highest saponification value among the lipids studied. Lipids rich in short-chain SFA tend to have higher densities than those rich in MUFA or PUFA⁴⁴. Generally, increased density is associated with higher viscosity, which is influenced by the fatty acid composition⁴⁴.

The saponification values of lipids ranged from 238 to 564 mg KOH/g, with *T. decurrens* having the lowest and *H. durvillei* having the highest value (Table 2). This value indicates the amount of KOH required to hydrolyse the lipids and is correlated with the fatty acid content. In general, a higher number of fatty acids results in higher saponification values. *C. taxifolia* had a low saponification value of 265.5 ± 5.6 mg KOH/g, reflecting a higher proportion of long-chain fatty acids, primarily palmitic (C16:0) and myristic acid (C14:0) (Table 3). Lipids rich in SFA typically have higher saponification values owing to their lower molecular weights, which facilitate the saponification reactions. Conversely, lipids with unsaturated fatty acids often have lower saponification values because of their complex long-chain structures, resulting in fewer ester groups per gram of oil and less KOH⁴.

C. taxifolia was used to optimise lipid extraction methods. Its cell wall comprises cellulose, hemicellulose, lignin, and mannan, forming a robust and complex matrix⁴⁵. Its polysaccharide structure mainly contains galactose, along with mannose and xylose⁴⁶. The lipid and fatty acid compositions affect the cell wall structure, contributing to the mechanical and functional properties⁴⁵, thus necessitating unique extraction treatments.

Non-solvent extraction methods have been reported to increase lipid yields. For example, lipid extraction from *U. fenestrata* using enzyme-assisted, ultrasound-assisted, and combined enzyme-ultrasound-assisted methods gives a lipid yield of 9.8 %, 8.65 %, and 17.92 %, respectively³¹. The ultrasonication method used in this study increased the lipid yield from *C. taxifolia* to 4.1 % at 60 °C for 30 min, approximately three times that obtained using Soxhlet extraction (Fig. 1A). Ultrasonic waves (frequency >20 kHz) were applied to generate cavitation, which reduces cell wall integrity, increases cell permeability, and releases intracellular components⁴⁷. The temperature and duration of ultrasonication were varied in this study. In theory, increasing the temperature generally accelerates the rupture of cavitation bubbles and pushes the solvent into the cell tissue, thereby accelerating lipid release. The duration is also expected to increase the yield. However, the results showed that there were optimum conditions in the extraction process, as the lipid structure is likely to break down after reaching a specific temperature⁴⁸. In addition, prolonged time may cause oxidative degradation of lipids due to heat and free radicals generated during the cavitation process⁴⁹.

Cellulase enzymes can degrade the lipid-bound cell wall of *C. taxifolia*⁵⁰, facilitating the release of lipids trapped inside the cell and thereby improving the extraction efficiency⁵¹. Hydrolysis of the cell wall structure increases permeability and allows lipids to escape easily from the cells.

The results showed that the use of enzymes at a concentration of 10 U/g at 50 °C with an incubation time of 30 min resulted in a 9.6 % lipid yield (Fig. 1B). This increase was almost seven times that of the Soxhlet extraction. These results are consistent with those of a study on lipid isolation from *C. lentillifera*, in which cellulase significantly increases the lipid yield⁵². A low enzyme concentration was optimal for cell wall breakage, probably because a high enzyme concentration led to enzyme saturation without a significant increase in cell wall breakage activity. The effectiveness of lipid extraction appears to be relatively fixed, as there is no increase in substrate accessibility²². A high enzyme concentration also accelerates enzymatic reactions that may change the mechanical characteristics of the cell wall, making lipid release more difficult⁵³.

This study combined independent extraction methods to increase the lipid yield of *C. taxifolia*. Among the various combinations of techniques, enzymatic extraction followed by Soxhlet extraction (ES) produced the highest yield of 21.5 ± 1.16 %, which is approximately 15 times higher than that of Soxhlet extraction alone. Enzymatic extraction was carried out under optimum conditions using 10 U/g enzyme and incubated at 50 °C for 30 min. This step was followed by Soxhlet extraction at 65 °C for 6 h. The combined method of ultrasonication with Soxhlet extraction (US) resulted in a low yield of 6.8 ± 0.085 %. However, it was still better than when these two methods were performed independently, where Soxhlet extraction yielded 1.47 ± 0.32 % and sonication 4.14 ± 0.16 % (Fig. 2). Compared to Soxhlet extraction, combined methods generally resulted in increased viscosity and density, except for the US method, while saponification values decreased, except for the EUS method. Despite the difference in magnitude, the results are consistent with those of several previous studies that report an increase in yield when the extraction method is optimised. For example, the lipid yield is increased by 40 % using the Sono-Soxhlet extraction method⁵⁴.

The extraction process can affect the physicochemical properties of lipids owing to factors such as temperature, time, solvent type, and enzyme type²². The physicochemical parameters of the various combinations of lipid extraction methods in this study were comparable, with slight differences (Table 4). When evaluated according to SNI 7431:2015, none of the methods complied with all the parameters. Non-compliance may be attributed to changes in fatty acid composition, which directly impact density, viscosity, and saponification values⁴². Harsh extraction conditions in methods

Table 4 Physicochemical properties of lipids from *C. taxifolia* extracted using combined extraction methods.

Physicochemical parameter	Combined methods				Standard
	Ultrasonication- Soxhlet extraction (US)	Enzymatic- ultrasonication (EU)	Enzymatic- Soxhlet extraction (ES)	Enzymatic- ultrasonication- Soxhlet extraction (EUS)	(SNI 7431:2015, lipid for biodiesel)
Viscosity (40°C, cSt)	7.3 ± 1.3	10.9 ± 0.5	10.2 ± 1.2	15.0 ± 2.1	Max 36 (cSt)
Density (Kg/m ³)	843 ± 13	851 ± 9	904 ± 1.7	830 ± 3	870-910
Saponification value (mg KOH/g)	163 ± 2.5	187 ± 0.3	232 ± 2.1	349 ± 1.6	180-256
Free fatty acid (%)	14.7 ± 5.2	10.20 ± 1.6	6.63 ± 0.96	10.9 ± 0.8	Maks. 4.0
Iodine value (g I ₂ /100 g)	9.1 ± 0.72	16.1 ± 2.02	14.7 ± 0.66	11.7 ± 0.14	Max 115 g I ₂ /100 g
Water content (%)	3.5 ± 0.3	4.9 ± 0.1	1.7 ± 0.3	1.4 ± 0.5	Maks. 0.1

such as EU or EUS may degrade or modify fatty acids, altering their profiles and how they interact with each other⁵⁵. The increase in saponification value observed in the EUS method suggests a higher proportion of short-chain fatty acids⁴⁰, implying that the extraction method altered the fatty acid composition. Additionally, all extraction methods had higher water content compared to the standard (**Table 4**), which caused lipid hydrolysis. The hydrolysis may explain the higher FFA values observed across all extraction methods. The lipid yield in this study was a product of complex interactions during extraction, which did not correlate linearly. Therefore, the physicochemical parameters varied, and no extraction method satisfied the SNI standard for biodiesel. Nevertheless, The ES method yielded macroalgal lipids with the most desirable characteristics. Therefore, this method is recommended for lipid extraction in biodiesel production, pending further optimisation. Different extraction methods can yield varying lipid properties as shown in this study. Therefore, it is crucial to consider the method when using to different sources.

5 Conclusion

In conclusion, the initial Soxhlet extraction gave low lipid yields across all species. *C. taxifolia* was selected for lipid extraction optimisation experiment mainly due to its relatively high lipid content compared to other species studied, comprising saturated fatty acid of myristic (14.2%) and palmitic acids (85.8%). The extraction optimisation demonstrated substantial improvements in the lipid yield. The combination of enzymatic extraction followed by Soxhlet extraction (ES) was the most effective, increasing the lipid yield by approximately 15 times compared to Soxhlet extraction alone. However, economic factors must be considered when scaling up the process. The costs include not only related to the extraction process but also the handling and processing of byproducts. When evaluated according to SNI 7431:2015, none of the methods produced lipids that fully complied with all the parameters. Further studies are needed to refine these methods and explore the applications of macroalgal lipids in the biodiesel industry.

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Author Contributions

BGB: conceptualisation, investigation, data curation and analysis, writing the original manuscript; MZA: supervision, data validation, reviewing the manuscript; FF: supervision, validation of methodology, organising resources; TMI: conceptualisation, supervision, reviewing and approving the final manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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