Microwave-Assisted Extraction of Polysaccharides from *Chlorella pyrenoidosa* and Its Characterization

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Abstract: *This study explored an efficient method for extracting polysaccharides from* Chlorella pyrenoidosa *using microwave-assisted extraction (MAE) with water as the solvent, a technique consistent with green chemistry principles. The goal was to enhance the yield and quality of polysaccharides for their potential applications as multifunctional active ingredients in the pharmaceutical and functional food industries. Key extraction parameters, including extraction time (10, 20, and 30 min), temperature (80 °C), and solid-to-liquid ratios (1:20, 1:30, 1:40 m/v), were systematically evaluated. The results indicated that a solid-to-liquid ratio of 1:40 m/v at 80 °C for 10 min yielded the highest polysaccharide content (56.64%). FTIR analysis confirmed the presence of pyranose rings in D-glucose and hydroxyl groups, while HPLC identified D-mannose (58.12%) as the predominant sugar, followed by D-glucose (34.46%), D-galactose (3.61%), and L-rhamnose (3.81%). Purified polysaccharide was composed of major mannose and glucose, a biomolecule very important given that it has wide applications in medical and food industries.*

Keywords: Chlorella pyrenoidosa*; glucose; mannose; microwave-assisted extraction; polysaccharide*

INTRODUCTION

Currently, there is a large interest in developing nutritious functional foods as a result of rising public awareness towards healthy food [1]. Functional foods are defined as food products enriched with additives that offer high nutritional and energy value while also delivering physiological benefits to human health due to the biological activity of their active ingredients [2]. Among these, polysaccharide is often considered as a key active component and thus causes higher demand in the market [3]. Polysaccharides composed of C5 and C6 sugars serve as essential precursors for producing sugar-based platform chemical building blocks such as furfural, glutamic acid, xylonic acid, levulinic acid, xylitol, and sorbitol. These compounds have been recognized by the U.S. Department of Energy as "Top Value-Added Chemicals from Biomass" [4]. Consequently, investigating optimal methods for extracting polysaccharides is of great importance.

Polysaccharides can be sourced from various biomass types. Signoretto et al. [5] classified biomass into three distinct generations. The use of polysaccharides derived from first-generation biomass has faced criticism, particularly in the chemical industry, as it competes directly with food supplies when sourced from food crops. Second-generation biomass, which includes non-food materials like lignocellulosic biomass (e.g., wood, forest residues, and agricultural waste such as bagasse, plant residues, rice straw, banana waste, and rice husks) [6], presents its own challenges. While it is non-edible, cultivating these plants requires large land areas [7] and can negatively impact soil fertility [8]. These limitations of the first and second generations have driven the development of third-generation biomass sourced from algae, including both macroalgae and microalgae. This third-generation biomass offers several advantages, including its lack of competition with food supplies, ease and cost-effectiveness of cultivation, and high concentration of active ingredients resulting from the production of complex compounds [9]. Generally speaking, the structural properties of polysaccharides determine their extraction, separation, and purification. Natural resource-derived polysaccharide main structures are remarkably varied and complicated [10].

One type of microalgae with great potential as a raw material for polysaccharides is *Chlorella pyrenoidosa* [9,11]. According to Yuan et al. (2020), the biological activities of polysaccharides in *C. pyrenoidosa* include antioxidant, immunomodulatory, hypolipidemic, antitumor, anticancer, anti-aging, anti-asthma, and antiviral properties [12]. *C. pyrenoidosa* is photoautotrophic organism, relying on log meaning it requires light as an energy source for cell growth and the synthesis of various essential substances so it can be plant biostimulants [13], bioremediation [14] and plant fertilizer [15]. The characteristics of the light source, such as wavelength and intensity, are critical factors influencing the production of *C. pyrenoidosa* and microalgae in general [16]. *C. pyrenoidosa* was first described by Chick (1903) [17], who distinguished it from *Chlorella vulgaris* based on the presence of pyrenoids in its chloroplasts. Pyrenoids are subcellular microcompartments found in the chloroplasts of algae, certain land plants, and hornworts. They are associated with the carbon concentration mechanism. The presence of pyrenoids in *C. pyrenoidosa* contributes to its significant carbohydrate content, which includes polysaccharides.

Polysaccharide extraction is typically performed using conventional methods such as maceration, mechanical calcination, and thermal flux. Each of these techniques has its own set of advantages and disadvantages. Prolonged extraction times and high temperatures can lead to the decomposition of polysaccharides, which may reduce their effectiveness as active ingredients in the pharmaceutical industry [18]. Microwave-assisted extraction (MAE) is a cell disruption method for extracting polysaccharides from microalgae and is considered as a green technology [19]. This technique offers several advantages, including higher yields and better quality of extracts, simple equipment, shorter extraction times, and more efficient solvent use [20]. MAE is also ideal for extracting thermolabile organic materials. The microwave extraction method has been applied to various other biomasses to extract biomolecules, including peel red dragon fruit [21], *Strychnos potatorum* [22], radish leaves [23], and *Ascophyllum nodosum* [24].

The structure of polysaccharides can vary significantly based on cultivation conditions, harvest seasons, and different extraction methods. Previous studies have shown that arabinogalactan can be extracted from hot water extracts of freeze-dried *C. pyrenoidosa* cells [25]. When *C. pyrenoidosa* was extracted using ultrasound-assisted extraction (UAE), D-glucose, D-galactose and D-mannose was obtained [26], while extraction with UAE followed by ultrafiltration resulted in the highest galactose content [27]. These experiments were carried out using water as a solvent.

The objective of this study is to extract polysaccharides from *C. pyrenoidosa* using MAE with water as the solvent. The research aims to evaluate the efficiency of MAE in maximizing the yield of polysaccharides, while maintaining their structural integrity and functional properties. Additionally, the study seeks to optimize the extraction parameters, including extraction time, temperature, and ratio solid-to water ratio establish a green, sustainable, and effective method for polysaccharide recovery.

■ EXPERIMENTAL SECTION

Materials

Dried *C. pyrenoidosa* in powdered form, with a particle size of 100 μm, was obtained from PT Algaepark, Polanharjo, Klaten, Central Java, Indonesia. Additional materials, including ethanol, Cetyltrimethylammonium bromide (CTAB), D-glucose

(Glu), L-rhamnose (Rha), D-galactose (Gal), and D-mannose (Man), from Merck. All chemicals and reagents used were of analytical grade.

Instrumentation

The schematic of the microwave extraction apparatus is presented in Fig. 1. *C. pyrenoidosa* powder was mixed with distilled water in the reactor, with variations in extraction time, solid-liquid ratio, and temperature. Each experiment was conducted in triplicate. The equipment used in this study included a microwave with the following specifications: a maximum rated output power of 800 W, a voltage of 220 V, a rated input power of 1250 W, and a magnetron frequency of 2450 MHz. Pyrex three-neck flasks (1 L) served as the extraction reactors for the MAE method. These flasks were fitted with a condenser for cooling and two openings, one for a thermocouple and another for sampling. The commercial microwave employed in the extraction process was equipped with a thermocouple and a stirrer to ensure uniform mixing and temperature monitoring.

Procedure

Single-factor experiments determine the medium value of the factor's optimal range. The temperature (80 °C), extraction time (10 min), and liquid–solid solvent (40 mL/g) were set as the experimental condition of single-factor. When one factor changes, the other three remain fixed values [28]. All treatments were repeated three times. After the polysaccharides were extracted using microwave-assisted extraction, the sample was centrifuged at 3500 rpm for 10 min, and the supernatant was collected. The extraction residue was then dried at 55 °C for approximately 3 h. After separation, the supernatant was deproteinized and precipitated using CTAB.

Characterization of C. pyrenoidosa *cells*

Proximate analysis was performed to quantitatively determine the chemical composition of the sample. The morphological characteristics of the microalgae particles before and after microwave extraction were examined in dry conditions using scanning electron microscopy (SEM, JEOL, JSM-6510 (LA), Japan).

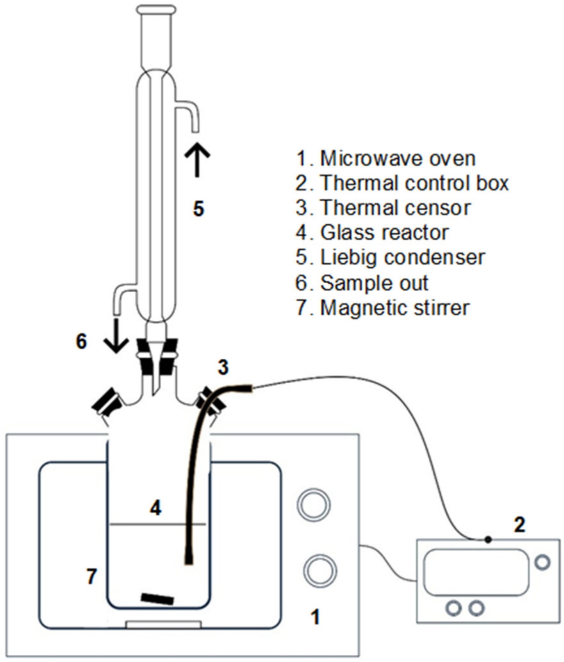

Fig 1. Schematic setup of MAE

The identification of functional groups within the carbohydrate components was carried out using FTIR. FTIR analysis was conducted with a Thermo Scientific Nicolet iS10 equipped with a Deuterated TriGlycine Sulfate (DTGS) detector.

Determination of polysaccharides from C. pyrenoidosa *extract*

The concentration of total sugar content of crude polysaccharide samples was determined using the phenol-sulfuric acid method [29] employing a UV-vis spectrophotometer at a wavelength of 490 nm, with distilled water as the blank solution and glucose as the standard solution. A formula Y (%) = c/w × 100% was used to calculate the yield of polysaccharides, where c represents the weight of polysaccharides and w represents the weight of the dried extracts. The polysaccharide composition was analyzed using high-performance liquid chromatography (HPLC) with an Aminex YMC-Pack Polyamine II/S-5 μm/12 nm detector. The eluent consisted of a 75:25 acetonitrile/water mixture, with a flow rate of 1.00 mL/min at 40 °C [3,30].

Statistical analysis

Data were expressed as means ± standard deviations of three replicated determinations. Statistical analysis was performed using the analysis of variance (ANOVA) test with a confidence level of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Times on Polysaccharide Yield

Polysaccharide yield was determined using the Dubois phenol-sulfuric acid method with a UV-vis spectrophotometer and Glu as the standard. A standard curve was generated based on various Glu concentrations [31]. Fig. 2 shows that the regression equation obtained was y = 2470.2x – 185.3 with an R^2 = 0.9565. These results were used to calculate the polysaccharide concentration in the MAE extract. The R^2 value being close to 1 indicates that the curve can be used as a standard curve for determining yield in this experiment. In this study, the effect of temperature (with a reaction time of 10 min and a solid-liquid ratio of 1:30) on the yield of polysaccharides was observed using the phenol-sulphur method [32]. Fig. 3 shows the impact of temperature on polysaccharide yield at different reaction times.

The effect of various times (biomass-to-aquadest ratio 1:20; temperature 80 °C) on polysaccharide yield is presented in Fig. 3(a). It was verified that times is presented a significant difference yield with 95% confidence (Table 1). Reaction time is a critical factor in determining the efficiency and outcome of chemical processes. In many catalytic reactions, the duration of the process significantly influences the yield and product quality. Reaction kinetics, intermediate formation, and side reactions are all time-dependent factors that must be optimized to enhance process efficiency [33]. For industrial applications, determining the optimal reaction time is essential to balancing yield and cost-effectiveness.

Table 1. ANOVA for Effect of MAE's time

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	12.4863	6.2431	43.0200	0.000
Error	6	0.8707	0.1451		
Total	8	13.3569			

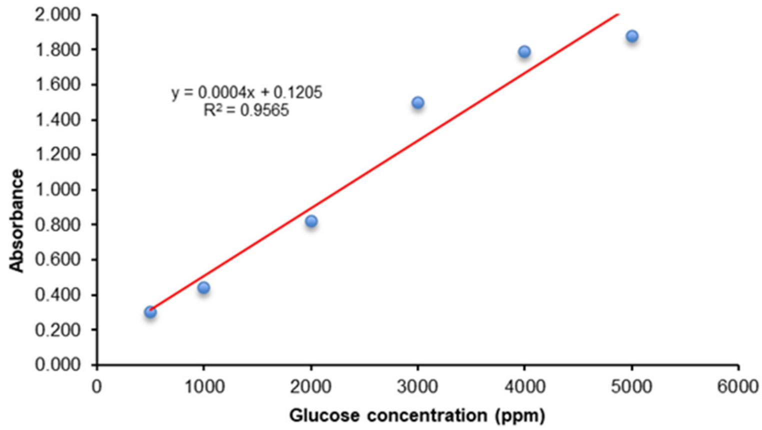

Fig 2. Mean absorbance was measured using a UV-vis spectrophotometer at a wavelength of 490 nm for the standard D-glucose solution, with three repetitions

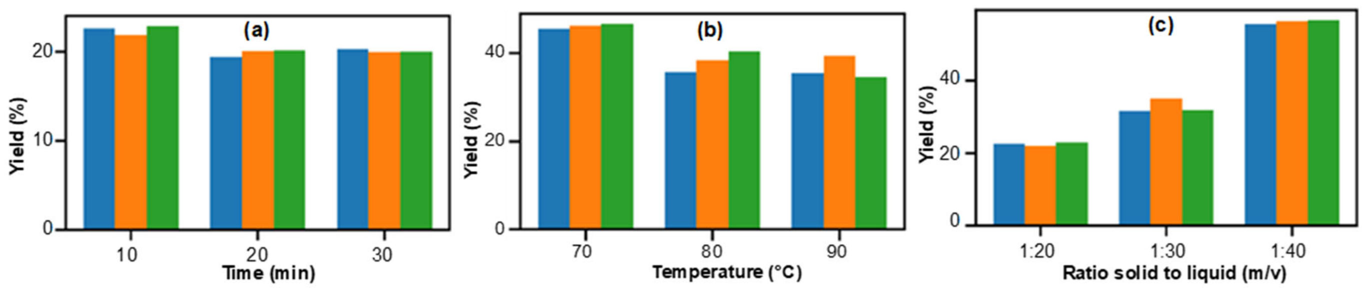

Fig 3. The effect of various (a) reaction time, (b) reaction temperature, and (c) biomass-to-aquadest ratios on polysaccharides yield from microwave-assisted extraction

This study investigates the effect of reaction time on yield percentage at three intervals: 10, 20, and 30 min. The findings aim to provide insights into the optimal time for achieving maximum yield under specified conditions.

Fig. 3(a) illustrates the influence of reaction time on yield percentage, evaluated at three distinct intervals: 10, 20, and 30 min. The highest yields were observed at 10 min (22.25%) and 30 min (20.05%), while the lowest yield (18.74%) occurred at 20 min. The results suggest that the yield demonstrates a non-linear trend with respect to time, peaking at the initial and final measured intervals. This behavior might be indicative of reaction kinetics, where equilibrium is reached at certain time points, leading to either higher product formation or stabilization of intermediate species. The drop in yield at 20 min could be attributed to potential side reactions, incomplete reaction progress, or an intermediate stage before reaching equilibrium [34]. The marginal difference between the yields at 10 and 30 min could signify that extending the reaction beyond 10 min offers minimal improvement in yield. This insight is crucial for optimizing reaction time in industrial processes, as prolonged reactions might unnecessarily increase operational costs without significant gain in yield. Future studies could benefit from exploring additional time intervals and analyzing other factors, such as temperature, catalyst concentration, and reactant ratios, to better understand the reaction dynamics and optimize conditions for maximum yield.

Effect of Various Temperatures

Fig. 3(b) shows the effect of the extraction temperature on polysaccharide yield. The highest yield was reached at an extraction temperature 70 °C. In Fig. 3(b), the highest yield was obtained at an extraction temperature of 70 °C per unit time, which was 46.17%. There was significant difference in yield, with a 95% confidence level (Table 2), which could be explained by the fact that the cells of this microalgae are already broken as much as possible using at a temperature of MAE 70 °C.

When the temperature was increased to 80 °C and 90 °C, the polysaccharide yield was lower. Polysaccharides in the cell wall will be hydrolyzed at high temperatures and high acid concentrations [35]. The use of MAE allows for precise temperature control, minimizing the risk of polysaccharide degradation and ensuring optimal yield. A stable temperature also helps in producing consistent extract products [36]. A temperature of 70 °C facilitates the rapid breakdown of cell walls and membranes, thus accelerating the polysaccharide extraction process [37]. This rapid heating improves reaction kinetics, allowing the active ingredient to be extracted more efficiently. It also helps maintain the molecular structure of the polysaccharide, preserving its quality and biological activity. Polysaccharides extracted at this temperature have a better potential for pharmaceutical and nutraceutical applications [9]. Temperatures lower than 70 °C can reduce the efficiency of cell wall and membrane breakdown, thus lowering the yield of extracted polysaccharides [38]. Additionally, lower temperatures may require a longer extraction time, increasing the risk of polysaccharide degradation. When the extraction temperature exceeds 70 °C, the yield decreases due to the risk of polysaccharide degradation at higher temperatures. Elevated temperatures can damage the molecular structure of polysaccharides, diminishing the quality of the resulting extract. Furthermore, excessively high temperatures can cause burning or unwanted chemical changes in the polysaccharides [39]. Polysaccharides can undergo hydrolysis when heated in aqueous conditions. Polysaccharides like starch undergo gelatinization at specific temperatures, causing structural changes and potential dissolution in water, making their original form appear reduced [40].

Table 2. ANOVA for Effect of MAE's temperature

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	160.13	80.063	19.05	0.003
Error	6	25.21	4.202		
Total	8	185.34			

Effect of Biomass-to-Water Ratios on Polysaccharide Yield

Fig. 3(c) shows the effect of the solid-liquid ratio of microalgae to water on polysaccharide yield. The yield increases with the solid-liquid ratio [41]. The data presented the yields from the extraction of polysaccharides from *C. pyrenoidosa* at a constant temperature of 70 °C and a 10-min extraction time, using various solid-liquid ratios. The variation in the solid-liquid ratio affected the yield. At a ratio of 1:20, the yield was relatively low, averaging around 22%. The yield significantly improved as the ratio increased to 1:30, reaching values around 32–35%. The highest yield was observed at a ratio of 1:40, with values ranging from 56%.

The ratio was a significant difference in yield, with a 95% confidence level (Table 3). Higher solvent ratios likely improve the solubility of the polysaccharides, leading to increased yields. The 1:40 ratio appeared to be optimal in this experiment, indicating the point at which solvent volume maximizes the yield. As the solid-liquid ratio increases, the diffusion of polysaccharides from the cell wall into the solvent becomes more efficient, suggesting that solvent volume plays an important role in increasing yields [42]. Extraction with a higher water content results in more efficient diffusion of polysaccharides from the surface of solid particles. Increasing the water-to-raw material ratio enhances solvent penetration into the cells and improves the desorption of polysaccharides from the particles.

Proximate Characterization

The selection of *C. pyrenoidosa* as the raw material was based on its carbohydrate content, as summarized in Table 4. Among the microalgae species, *Dunaliella bioculata* has the lowest carbohydrate content at 4%, while *Spirogyra* sp. has the highest, ranging from 33 to 64%. *C. pyrenoidosa* contains approximately 26% carbohydrates, placing it in the medium range compared to other microalgae. Its carbohydrate content is notably higher than that of *C. vulgaris*, by 12–17%. This makes *C. pyrenoidosa* a suitable choice, offering a clear distinction from other *Chlorella* species based on carbohydrate content.

The results of the proximate analysis of the *C. pyrenoidosa* contents used in this study are shown in Table 5. The proximate analysis was conducted to determine the potential of *C. pyrenoidosa* based on the composition of its carbohydrate and polysaccharide

Table 3. ANOVA for the effect of solid-liquid ratio

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	847.700	423.830	11.820	0.008
Error	6	215.100	35.850		
Total	8	1062.800			

Table 4. *C. pyrenoidosa* composition [43-44]

Strain	Protein	Carbohydrate	Lipid
Anabaena cylindrica	43–56	25–30	4–7
Chlamydomonas rheinhardtii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	41–58	12–17	10–22
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Dunaliella tertiolecta	29	14	11
Euglena gracilis	39–61	14–18	14–20
Porphyridium cruentum	28–39	40–57	9–14
Prymnesium parvum	28–45	25–33	22–39
Scenedesmus dimorphus	8–18	21–52	16–40
Scenedesmus obliquus	50–56	10–17	12–14
Scenedesmus quadricauda	47	–	1.9
Spirogyra sp.	6–20	33–64	11–21
Arthospira maxima	60–71	13–16	6–7
Spirulina platensis	42–63	8–14	4–11
Synechoccus sp.	63	15	11
Tetraselmis maculata	52	15	3

Table 5. Sample *C. pyrenoidosa* proximate

Component	*C. pyrenoidosa* (%)
Water	5.57
Ash	4.79
Lipid	1.052
Protein	57.91
Hemicellulose	30.68

contents. The high hemicellulose content, which is part of the polysaccharides to be extracted, makes *C. pyrenoidosa* used in this study noteworthy. As a type of freshwater green algae found in tropical regions, it is likely to have a higher hemicellulose content than microalgae cultivated in other areas [45-46].

SEM Characterization

Fig. 4 illustrates the microalgae *C. pyrenoidosa* before and after MAE. Microalgal cell walls contain a diverse range of polysaccharides [45,47]. The rapid heating during MAE generates high internal pressure, weakening the cell wall of *C. pyrenoidosa* and making it more prone to breakage. This disruption enhances the efficiency of extracting active compounds such as polysaccharides and lipids.

Fig. 4(a) shows *C. pyrenoidosa* powder used as the raw material, analyzed by SEM at 10,000× magnification, while Fig. 4(b) depicts the crude polysaccharide obtained after MAE, also at 10,000× magnification. The images reveal that the particle size after extraction becomes smaller, more fragile, and agglomerated. The application of MAE leads to a reduction in the particle size of *C. pyrenoidosa* cells, increasing the surface area available for interaction with the solvent and thereby enhancing the extraction yield. Furthermore, microwaves improve cell membrane permeability, facilitating the release of intracellular components. This enhanced permeability reduces the diffusion barrier between the cell's active ingredients and the solvent, expediting the extraction process [36,46].

FTIR Characterization

Fig. 5 presents the infrared spectra of the MAE purification results for the *C. pyrenoidosa* polysaccharide fraction, which has undergone deproteinization and precipitation using CTAB. The spectra were recorded in absorbance mode within the mid-infrared region (4000–400 cm^{-1}), with triplicate recordings performed for each sample. Infrared spectroscopy of mono- and oligosaccharides plays a significant role in elucidating small molecule structures and analyzing polymers

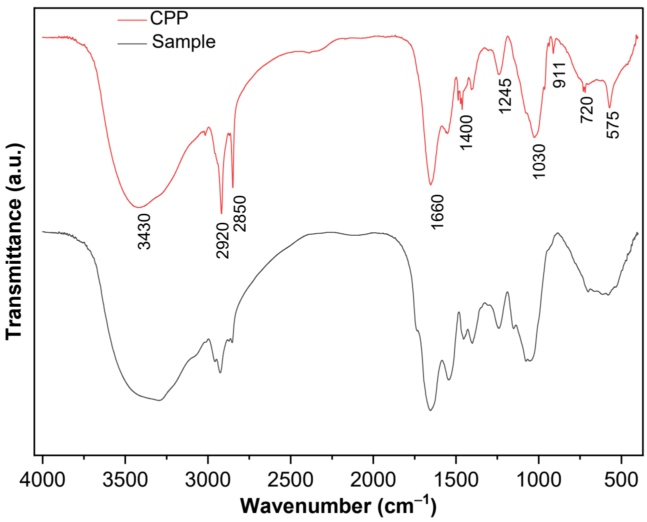

Fig 5. The FTIR spectra of polysaccharide of *C. pyrenoidosa* before (sample) and after (CPP) MAE

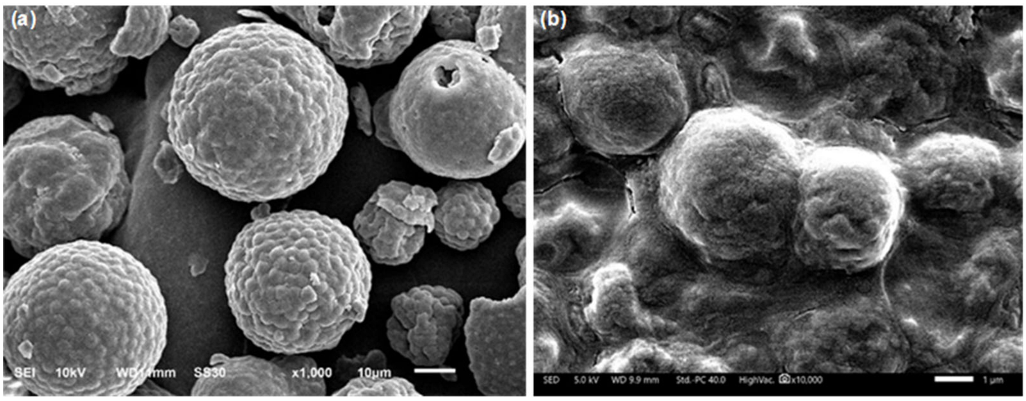

Fig 4. (a) *C. pyrenoidosa* powder as the raw material and (b) *C. pyrenoidosa* crude polysaccharide obtained after MAE

Table 6. Yields and sugar composition of *C. pyrenoidosa* extraction using MAE

Sample	Yield (%)	Sugar component (%)			
		D-Glucose	D-Galactose	D-Mannose	L-Rhamnose
Sp-15	56.64	34.46	3.61	58.12	3.81

representing structural units. In carbohydrate analysis, the anomeric region (950–750 cm^{-1}) is particularly important, as it reveals characteristic bands of α and β conformers, as well as vibrations of pyranoid and furanoid rings in mono- and polysaccharides. For instance, the α and β conformers of glucose, galactose, and mannose can be distinguished by bands 2a and 2b at 870–840 cm^{-1} and 890 cm^{-1}, respectively [48]. The FTIR spectrum of the sample shows a peak at 911 cm^{-1}, attributed to the stretching vibration of the pyranose ring, identifiable as D-glucose [48]. Distinguishing oligosaccharides from their hydrolysis products is facilitated by prominent spectra in the regions of 1160–1150 cm^{-1} and 1000–960 cm^{-1}, which can also differentiate non-reducing sugars from reducing sugars [49]. Additionally, a peak at 1651 cm^{-1} corresponds to the stretching vibration of the carbonyl bond in the amide group of the N–H bond, indicating the presence of protein. The band at 3423 cm^{-1} arises from the stretching vibration of the hydroxyl group in polysaccharides, while the band at 2918 cm^{-1} is due to the stretching vibration of C–H bonds [50].

HPLC Characterization

Based on the experimental results, the optimal yield was obtained at a temperature of 80 °C, an extraction time of 10 min, and a solid-liquid ratio of 1:40 m/v (sp-15). Subsequently, characterization was carried out to determine the composition of monosaccharides and disaccharides using HPLC. HPLC calibration was performed using standard saccharide solutions of D-glucose, D-galactose, D-mannose, and L-rhamnose in triplicate to generate a standard curve and determine the retention times for each standard. When sp-15 was injected into the HPLC, the resulting peaks identified the retention times of each component, allowing the calculation of peak areas, which correspond to the concentrations in the sample. The retention time analysis for sp-15 revealed that the polysaccharide composition consisted of D-glucose, D-galactose, D-mannose, and L-rhamnose [46].

The experimental results from the HPLC analysis are summarized in Table 6. It is evident that the dominant monosaccharide in the sample is D-mannose, accounting for 58.12%, followed by D-glucose at 34.46%, L-rhamnose at 3.81%, and D-galactose at 3.61%. The polysaccharide yield was determined to be 56.64%, which was higher than hot water extraction that has yield 22.44%. This hot water extraction taking longer time, 4 h for extraction time using aquadest as solvent. Concerning the energy consumption analysis, the microwave extraction more efficient time with higher yield [51-52].

■ CONCLUSION

The MAE method proved effective in extracting polysaccharides from *C. pyrenoidosa*, achieving a yield of 56.64% under optimal conditions (80 °C, 10 min, solid-liquid ratio 1:40 m/v). D-mannose was identified as the dominant monosaccharide component, highlighting its significant potential for pharmaceutical and nutraceutical applications. The microalga *C. pyrenoidosa* was able to be combined with the microwave technique, which has been demonstrated to be effective in the extraction of polysaccharides from a variety of substrates. This microalga does not compete for agricultural land and it benefits the environment by participating in the bioremediation and biofixing processes of carbon and nitrogen oxides.

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■ CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work presented.

■ AUTHOR CONTRIBUTIONS

The idea was conceived by Arief Budiman, Rochmadi, and Muhammad Mufti Aziz. Margaretha Praba Aulia and Arief Budiman wrote and revised the manuscript. All authors approved the final version of the manuscript.

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