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MICROWAVE-ASSISTED ENZYMATIC EXTRACTION OF POLYSACCHARIDES FROM *Ceratophyllum submersum*

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ABSTRACT

Algae primarily consist of polysaccharides, which account for 15-76% of their composition, along with proteins making up 5-47%, minerals ranging from 7-36%, and lipids comprising 1-5%. Algae polysaccharides serve as renewable, abundant, biodegradable, and biocompatible biopolymers, making them extensively utilized in the food industry. Various techniques have been employed for the extraction and preparation of polysaccharides from algae, which have a considerable impact on molecular weight, yield and composition. This study used microwave-assisted enzymatic extraction (MAEE) to isolate polysaccharides from *Ceratophyllum submersum*. The effects of different solid-to-liquid ratios, microwave powers, microwave times, pH values, temperatures, and extraction times were investigated using single-factor experiments. The findings indicated that optimal conditions for extracting *C. submersum* polysaccharides involved a solid-to-liquid ratio of 1:10 w/v, a microwave power of 270 W applied for 3 min at a pH of 5.0, and a cellulase enzyme concentration of 0.1% at 40 °C over a duration of 3 hours. Under the conditions, the polysaccharide content was found to be relatively high (about 46.89 ± 0.19 mg/gDM).

Keywords: Enzyme, microwave, polysaccharides, *Ceratophyllum submersum.*

1. INTRODUCTION

Ceratophyllum submersum, commonly referred to as soft hornwort or coontail, serves as a natural source of bioactive compounds, including polysaccharides, proteins, peptides, lipids, and phenolic substances. It has been shown that *Ceratophyllum* species have 39.37% total carbohydrates, 17.87% fibers, 17.37% proteins, 17.12% ash, and 0.43% total lipids [1]. *Ceratophyllum* species are known for their bioactivities, such as anti-inflammatory, immunomodulatory, antithrombotic, antitumor, antimicrobial, antimutagenic, antiviral activities, etc [2]. Macroalgal polysaccharides are predominantly composed of hexose and pentose monosaccharide sub-units with many glycosidic bonds. It is a structural constituent of the cell wall, energy stores, protective polysaccharides and cell interactions. The industrial use of macroalgae polysaccharides is increasing. Macroalgal polysaccharides, including carrageenan, alginates and ulvan, can be utilized in the creation of environmentally sustainable bioplastic packaging, thereby enhancing food quality and promoting microbial safety [3]. Thus far, polysaccharides extracted from *C. submersum* have been largely overlooked, in contrast to the considerable research interest that has been directed towards polysaccharides obtained from a variety of other natural sources globally.

Polysaccharides derived from algae have demonstrated beneficial effects in areas such as antibacterial activity, anti-inflammatory properties, and immune system regulation, among others [2]. They are also an ideal natural resource for making functional foods and pharmaceuticals [4]. Various extraction techniques influence the chemical composition and characteristics of polysaccharides [5]. Polysaccharides derived from algae, whether for consumption or functional applications, rely on methods that are both compatible and capable of delivering economically sustainable yieldsHot water extraction (HWE), alkali-assisted extraction, acid-assisted extraction, enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), and hot water alkali-assisted extraction are common methods used for isolating polysaccharides from various

materials. Traditional extraction methods often rely on single-phase solvents, such as water or ethanol-water mixtures, to extract polysaccharides from natural sources, typically yielding a single type of polysaccharide. In the EAE process, enzymes break down the cell wall by hydrolyzing cellulose, leading to the release of polysaccharides [6]. Integrating enzymatic-assisted extraction (EAE) with microwave-assisted extraction (MAE) offers a powerful approach for extracting polysaccharides from diverse samples. This method combines the thermal effects of MAE with the enzymatic hydrolysis enabled by EAE, resulting in enhanced extraction efficiency. Additionally, the use of various processing techniques such as microwave, ultrasound, and supercritical fluid extraction—alongside enzymes has proven to be versatile for recovering bioactive compounds from plant-based materials and their by-products [7]. The combination of microwave field intensification and enzymatic cell disruption works synergistically to enhance cell lysis, thereby significantly improving extraction efficiency and reducing extraction duration [8].

This study aimed to explore the effects of microwave-enzyme-assisted techniques on polysaccharide yield.

2. MATERIALS AND METHODS

2.1. Materials

C. submersum algae were gathered from Can Duoc commune in Long An province, transported under cool conditions to the laboratory, and then dried in an oven at 60 °C until their moisture content dropped below 10%. The dried material was subsequently ground and sieved to obtain the 80-mesh fraction. The sifted powder was stored in a PET bag (25 cm × 35 cm) and frozen at -20 °C.

The Biogreen Pharmaceutical Chemistry and Biotechnology Joint Stock Company in Vietnam produces the cellulase enzyme. The enzyme activity is 2000 IU/g. The shelf life is 2 years from the manufacturing date. Keep it stored in a cool, dry environment, away from direct light, within a temperature range of 4–25 °C.

UV-VIS spectrophotometer (Model V-730, Japan), Thermostat tank (WNB 14, Germany), Microwave oven (Sharp 23 lit R-370VN-S, China), drying oven (Venticell, Germany), centrifuge (Hermale Z206A, Germany).

2.2. Methods

2.2.1. Polysaccharides extraction procedure

To evaluate the impact of the solid-to-liquid ratio on polysaccharide yield, 2.0 g of *C. submersum* dried powder was dispersed in varying solid-to-liquid ratios (1:5, 1:10, 1:15, 1:20, and 1:25 w/v) using a buffer solution adjusted to different pH levels (4.0, 4.5, 5.0, 5.5, and 6.0). The experiments were carried out under several microwave power conditions (0, 90, 180, 270, 360, and 450 W) and exposure times (1.0, 2.0, 3.0, 4.0, and 5.0 min). Following this, the samples underwent extraction for durations of 1, 2, 3, 4, and 5 hours at temperatures ranging from 35 to 60 °C (35, 40, 45, 50, 55, and 60 °C) with the addition of cellulase enzyme at a concentration of 0.1%. In the final stage of the process, the enzyme was deactivated by heating the mixture to 90–100 °C and cooling it promptly. The treated sample was then centrifuged at 5000 rpm for 15 minutes and filtered to isolate the polysaccharide-enriched supernatant. The polysaccharide content was subsequently measured using the phenol-sulfuric acid method [9].

2.2.2. Phenol-sulfuric acid method

To construct the calibration curve, D-glucose was used as the standard substance at concentration levels of 40, 80, 120, 160, and 200 µg/mL. For each concentration, 2 mL of solution was placed into test tubes. Subsequently, 1 mL of 4% phenol solution and 5 mL of concentrated H2SO4 were added to the tubes, followed by gentle vortexing to ensure proper mixing. The tubes were then maintained at 40 ℃ for 30 min before being cooled in ice water for 5 min. Absorbance readings were taken at 490 nm. Blank samples were prepared using the same procedure. The polysaccharide content was determined based on the standard curve method, replacing the standard solution with the experimental sample where applicable [9].

2.3. Data analysis

The investigation involved conducting each experiment in triplicate, with the results expressed as the mean value ± error. Data calculations were performed using Microsoft Office Excel 2010 and Minitab software. A statistical analysis was carried out through analysis of variance (ANOVA) at a 95% confidence level, and treatment differences were evaluated using the least significant difference (LSD) test.

3. RESULTS AND DISCUSSION

3.1. Effects of solid-to-liquid ratio on polysaccharide extraction

Under the same conditions of microwave power at 270 W, microwave time 2 min, pH 6.0, enzyme concentration 0.1%, extraction temperature 50 °C, and extraction time 2 h, the solid-to-liquid ratio was changed, and its impact on the yield of polysaccharides was studied. The experimental results are shown in Fig. 1.

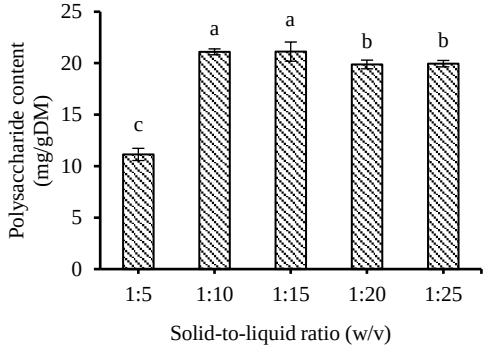

Fig. 1. Effects of solid-to-liquid ratio on polysaccharide extraction

As can be seen from Figure 1, the polysaccharide yield increased as the solid-liquid ratio increased and reached saturation at 21.10 ± 0.29 mg/gDM at a ratio of 1:10 w/v. Based on the results of ANOVA analysis, there was no significant difference when the ratio of solid-to-liquid increased to 1:15 w/v (values in the columns with different exponents represent a significant difference at $\alpha = 0.05$). At ratios 1:20 and 1:25 w/v, the polysaccharide content decreased slightly but not significantly (at around 19.9 mg/gDM). A possible explanation is that the extraction efficiency did not increase when the solvent was increased beyond a certain point because the material contained a limited number of substances. Even if more solvent is used, the amount of extracted substances cannot be increased. Therefore, it is crucial to select an appropriate solid-liquid ratio. In this study, a solid-to-liquid ratio of 1:10 w/v was found to be the most suitable.

3.2. Effects of microwave power on polysaccharide extraction

In enzyme and microwave co-assisted extraction, microwave power has a direct impact on the process. The essence of microwaves is the use of non-ionizing electromagnetic radiation, which disrupts hydrogen bonds and the movement of dissolved ions. The survey was conducted at six microwave power levels of 0, 90, 180, 270, 360, and 450 W. The results are shown in Fig. 2.

Figure 2 illustrates that increasing the power from 0 to 270 W causes a continued improvement in yield because of an increase in mass transfer rates. The yield reached the maximum value (21.39 ± 0.22 mg/gDM) at 270 W. An inconsequential decrease in yield was noted when the power was raised further ($\alpha = 0.05$). Microwave power significantly contributed by delivering localized heating to the sample, thereby facilitating the mechanochemical activation energy necessary for the disintegration of the plant matrix. This process enabled the analyte to diffuse and dissolve effectively in the solvent [10]. The intense energy of the microwave could lead to the depolymerization of polysaccharides, promote aggregation, and reduce viscosity, ultimately resulting in a diminished extraction yield [11]. The result of this study was similar to those of Peng H. *et al*., who obtained polysaccharides from *Chlorella* sp. When performing extraction, the polysaccharide yield increased with increasing microwave power from 150 to 350 W (yield increased from 0.5 to 0.8%), whereas it

decreased as the microwave power continued to increase (350-550 W) [12]. According to the survey results in this study, the suitable microwave power was 270 W.

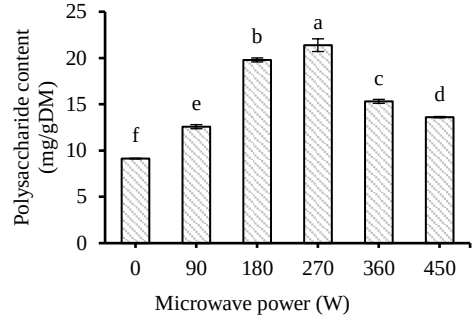

Fig. 2. Effects of microwave power (W) on polysaccharide extraction

3.3. Effects of microwave time on polysaccharide extraction

The length of time that samples are exposed to microwave radiation significantly influences the effectiveness of polysaccharide extraction. If the duration is excessively prolonged, the polysaccharide may partially degrade [13]. Fig. 3 shows the effects of microwave time on the polysaccharide extraction rate.

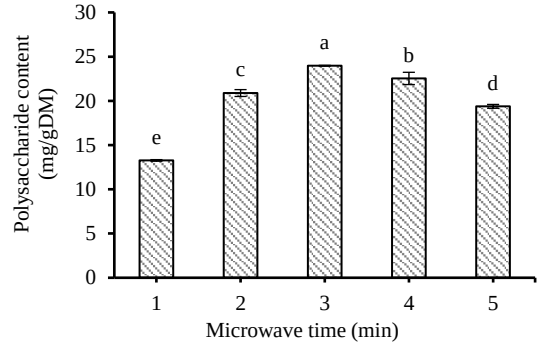

Fig. 3. Effects of microwave time on polysaccharide extraction

The extraction rate exhibited a notable increase (α = 0.05) in response to the duration of microwave exposure (1-3 min), achieving its peak value of 23.98 ± 0.04 mg/gDM at a microwave time of 3 min. As the microwave time was further increased (3-5 min), the extraction yield decreased significantly. As the duration of microwave exposure lengthened, the plant cells underwent an expansion and subsequent rupture as a result of microwave absorption, leading to the release and dissolution of the active compounds within the cells into the solvent. However, prolonged microwave treatment degrades polysaccharides, which results in a decrease in the extraction rate [14]. Therefore, the microwave time was set to 3 min.

3.4. Effects of pH on polysaccharide extraction

The pH level significantly influences enzyme activity, making it essential to explore the optimal pH conditions. In this study, while maintaining constant other variables, a pH range of 4.0 to 6.0 was selected to identify the most suitable pH for maximizing polysaccharide extraction. Fig. 4 showed that the amount of polysaccharide extracted increased with increasing pH to 5.0.

The pH values exhibited a positive linear relationship with the yield of polysaccharides within the range of 4.0 to 5.0 (from 29.46 ± 0.04 to 34.42 ± 0.22 mg/gDM). However, beyond this range, an increase in pH resulted in a decline in yield (Fig. 4). This result may be due to low enzyme activity at higher pH values and its optimum activity at pH 5.0-6.0 [15]. Thus, a pH of 5.0 was the optimal choice for the next experiment.

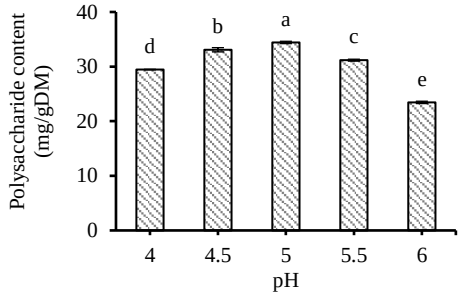

Fig. 4. Effects of pH on polysaccharide extraction

3.5. Effects of extraction temperature on polysaccharide extraction

The effect of temperature on polysaccharide extraction yield is shown in Fig. 5. The extraction process was performed in accordance with the method described in section 2.2.1 for a duration of 2 h, with temperatures ranging from 35 to 60 °C.

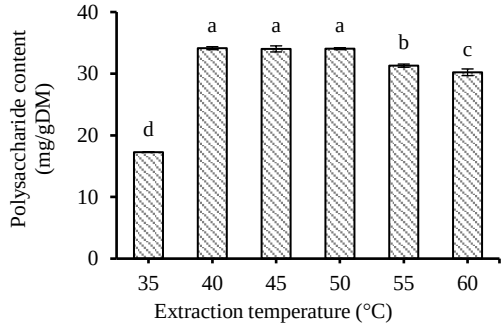

Fig. 5. Effects of temperature on polysaccharide extraction

An elevation in extraction temperature from 35 °C to 50 °C resulted in a notable increase in polysaccharide yield, as higher temperatures can enhance the catalytic activities of enzymes that facilitate polysaccharide release. The maximum yield (34.14 ± 0.25 mg/gDM) was achieved at an extraction temperature of 40 °C. Notably, the yield exhibited a significant decline with any subsequent rise in extraction temperature beyond 50 °C. Elevated temperatures result in a reduction of cavitation bubble formation and diminish the intensity of the cavity's impact.

3.6. Effects of extraction time on polysaccharide extraction

The efficiency of polysaccharide extraction also depends on treatment time. Excessive prolongation of extraction time can cause changes in the polysaccharide molecular structure. Therefore, the extraction time survey was conducted at five different intervals of time: 1, 2, 3, 4, and 5 h. The results are shown in Fig. 6.

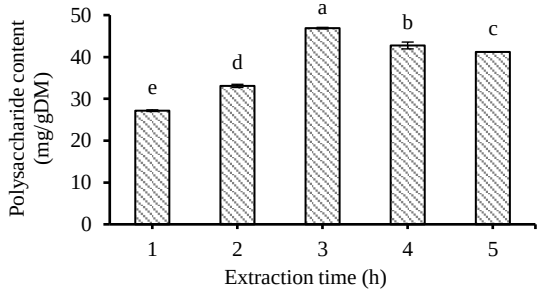

Fig. 6. Effects of extraction time on polysaccharide extraction

Enzymes play a supporting role in weakening the cell wall and promoting the extraction of polysaccharides. If the enzyme contact time with the sample is insufficient, the ability to destroy the cell wall will not achieve good results, thereby affecting the polysaccharide extraction efficiency. This is

proved through the data from the survey results, and it can be seen that, at 3 h, the obtained polysaccharide content is the highest with the data of 46.89 ± 0.19 mg/gDM. After this point, the polysaccharide yield started to decrease with increasing extraction duration. Increasing the extraction duration may enhance the polysaccharide yield. Overextending the extraction duration could lead to alterations in the structure of polysaccharides, which in turn diminishes the yield of polysaccharides obtained. According to this experiment, the most suitable time for polysaccharide extraction was three hours.

4. CONCLUSION

This study successfully integrated the microwave technique with enzymatic processes, demonstrating its effectiveness in extracting polysaccharides from *C. submersum*. The methodology adhered to the tenets of green chemistry, particularly emphasizing the utilization of non-toxic solvents and minimizing both solvent usage and energy expenditure during the extraction process. The results from this study indicated the content of polysaccharides from the *C. submersum* at around 46.89 ± 0.19 mg/gDM. The polysaccharide content obtained was four times higher than the study by Lee M.C. *et al.* (2021) on *Caulerpa mictophysa* algae with polysaccharide content (1457.17 ± 48.25 μg/gDM) [16], and three times higher than the research of Hoang Thi Ngoc Nhon *et al.* (2020) on *C. submersum* (1484.82 ± 8.68 μg/gDM) [17]. These findings provide a theoretical basis for further large-scale extraction studies related to polysaccharides from *C. submersum*.

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TÓM TẮT

NGHIÊN CỨU THU NHẬN POLYSACCHARIDE TỪ RONG *Ceratophyllum submersum* BẰNG PHƯƠNG PHÁP KẾT HỢP VI SÓNG-ENZYME

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Các loại rong tảo chứa chủ yếu các polysaccharide (15-76%), protein (5-47%), khoáng (7-36%) và lipid (1-5%). Các polysaccharide từ rong tảo được sử dụng rộng rãi trong ngành công nghiệp thực phẩm như các polyme sinh học có thể tái tạo, có khả năng phân hủy sinh học và tương thích sinh học. Các phương pháp khác nhau đã được sử dụng để chiết xuất polysaccharide từ rong tảo có ảnh hưởng đáng kể đến hiệu suất, trọng lượng phân tử và thành phần của polysaccharide thu được. Trong nghiên cứu này, phương pháp enzym kết hợp vi sóng (MAEE) đã được sử dụng để chiết tách polysaccharide từ rong *Ceratophyllum submersum*. Ảnh hưởng của tỉ lệ nguyên liệu/dung môi, công suất vi sóng, thời gian vi sóng, giá trị pH, nhiệt độ và thời gian chiết đã được khảo sát bằng các thí nghiệm đơn yếu tố. Kết quả cho thấy, điều kiện thích hợp chiết xuất polysaccharide từ rong *C. submersum* là tỷ lệ nguyên liệu/dung môi 1:10 w/v, công suất vi sóng 270 W trong 3 phút ở pH 5,0 và nồng độ enzyme cellulase 0,1% ở 40 °C trong 3 giờ thu được polysaccharide có hàm lượng 46,89 ± 0,19 mg/gCK.

Từ khóa: *Ceratophyllum submersum*, enzyme, vi sóng, polysaccharide.