THE EFFECTS OF ENZYME CELLULASE AND MICROWAVE ON FUCOIDAN EXTRACTION FROM *Ceratophyllum demersum*

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ABSTRACT

Ceratophyllum demersum has many bioactive ingredients, notably fucoidan. Fucoidan is a fucose-rich polysaccharide often present in seaweed, having many functions related to physiological activities, demonstrated through biological activities such as anti-cancer, immune, anti-inflammatory or antioxidant, ect. Therefore, this compound is often used a lot in functional foods. This aimed study is conducted to determinate the effects of the solvent system of impurities, enzyme, microwave effect on fucoidan content extracted from *C. demersum* seaweed. The results showed that obtained fucoidan content in solvent system 1 was 794.894 ± 6.054 µg/g_{dm}, higher than that of solvent system 2 (574.851 ± 5.569 µg/g_{dm}). In addition, in microwave-assisted method, the fucoidan content was 986.968 ± 3.514 µg/g_{dm} while the figure was 1049.046 ± 9.838 µg/g_{dm} in enzyme-assisted method at 50 °C for 3 hours and the ratio of enzyme/ material 2/20 (v/w). Moreover, the the combination of enzyme and microwave in fucoidan extraction with the microwave-enzyme sequence resulted in fucoidan content of 1246.871 ± 8.352 µg/g_{dm}, which is significantly higher than the reverse sequence investigated in this study.

Keywords: Ceratophyllum demersum, enzyme, fucoidan, microwave.

1. INTRODUCTION

Fucoidan is a complex and heterogeneous sulfate polysaccharide commonly found in seaweed. Its structural properties and composition differ among seaweed species. But the determination of fucoidan is based on the content of sulfate, galactose, xylose, mannose and uronic acid [1]. This polysaccharide has many different biological activities including antioxidant, anti-cancer, antibacterial, anti-virus, anti-inflammatory, immune, anti-diabetic, anti-obesity, anticoagulant activity, etc. [2]. The biological activities of fucoidan are related to monosaccharide structure, composition, sulfate content, the position of sulfate ester groups and its molecular weight. In particular, the extraction methods significantly affect fucoidan efficiency recovery, more specifically, it also greatly affects the structure of monosaccharides and sulfate components of fucoidan [3]. Comparing with other polysaccharides, fucoidan is widely used due to it is available in many inexpensive sources. And seaweed is one of the richest sources of fucoidan. In our country, seaweed resources are profuse and plentiful, but not fully exploited. The fucoidan extraction method is usually performed according to different pre-treatment steps, using solvents for extraction and precipitation. Consequently, fucoidan would be purified by using ion-exchange chromatography. Pre-treatment is required to remove chlorophyll, mannitol, NaCl and other small compounds. The solvent system MeOH-$CHCl_3$-H_2O with the ratio (4: 2: 1) [4] or ethanol 80-85% are the two methods commonly used to treat raw materials before performing the elicitation [5]. Extraction with acid solvent [6] or hot

water at 60-100 ℃ [7] and $CaCl_2$ is sometimes used to precipitate alginate during extrication [8]. Recently, fucoidan extraction processes are also complemented by ultrasonic waves, microwaves, the action of the enzyme and supercritical fluids. In particular, the methods of microwave-assisted extraction (MAE) and enzyme-assisted extraction (EAE) have gained much attention, probably because this technique is highly efficient and eco-friendly. Moreover, it is easy to do under laboratory conditions, and it also greatly increases extrication efficiency.

The seaweed *C. demersum* belongs to the family Ceratophyllaceae, a family of seaweed with a single-celled structure [9]. *C. demersum* exists underwater, widely distributes in freshwater ponds, swamps, to be suitable for growing in all terrains and not harmful to other organisms as well as the surrounding environment. In particular, the climatic conditions in Vietnam are very suitable for the development of this seaweed. In this study, the effects of the impurity-removing solvent system, the action of enzyme and microwave on fucoidan extraction from *C. demersum* were presented.

2. MATERIALS AND METHODS

2.1. Material

The *C. demersum* seaweed was collected in Soc Trang province. After harvesting, it was transported during the day to the laboratory and washed with fresh water to remove impurities such as shells, sand... and stored at -5 °C. After experiments, the materials were thawed and dried at 60 °C until the seaweed under 10% moisture. Because the water content inside the raw seaweed would affect the extraction processes (dilution of the extraction agent causes concentration errors), the raw materials needed to be dried and ground, then it was sieved through a 3 mm sieve to collect the homogenous powder.

2.2. Methods

2.2.1. Fucoidan extraction from C. demersum

Fucoidan extraction: The material powder sample would be soaked with solvent to remove lipids and color compounds. After extracting with 0.1 M HCl solvent at 70 ℃ for 2 hours with the ratio of raw material/ solvent 1:20 (w/v), the extract was filtered to obtain the supernatant. Next, it was continuously precipitated with TCA (Tricloacetic acid) to remove the protein at 4 ℃ for 60 minutes, centrifuged and eliminated the obtained precipitate. Then, the alginate compounds were removed by precipitating with 1% $CaCl_2$, then centrifuged to obtain the clear solution.

Fucoidan precipitation: The fucoidan precipitation was performed with 99% ethanol in two stages. In the first stage, the clear solution would be added 99% alcohol at the rate of 30:69 (v/v) to the alcohol content in the solution to reach 30% and kept at around 4 °C in 2 hours. After that, this solution would be centrifuged to collect clear fluid. In the second step, the clear fluid was continually added 99% alcohol to the solution at the rate of 40:29 to get 70% alcohol concentration, kept cold at 4 °C to precipitate fucoidan, then centrifuged the precipitated solution to obtain fucoidan precipitate. The precipitate of fucoidan was dissolved in the distilled water at the rate of 1:10 (w/v) to obtain the solution quantify fucoidan by UV-VIS spectrophotometric method.

2.2.2. The effects of the solvent system on fucoidan extraction

15g raw material (according to the dry matter content) would be soaked with solvent system 1 (methanol: chloroform: water) or solvent system 2 (hexane, acetone, 80% alcohol, ethanol: water: formaldehyde) [10]. Then, the extraction process would be performed based on section 2.2.1 to select the suitable impurity-removing solvent system for fucoidan extraction.

2.2.3. The effects of enzyme-assisted method on fucoidan extraction

15g raw material (according to the dry matter content) was soaked in the solvent system to remove impurities (section 2.2.2). The experiments would take place at the investigated ratios of cellulase enzyme/ material: 1/20, 2/20, 3/20 (v/w) in the time (1 hour, 2 hours, 3 hours) with temperatures (30 °C, 40 °C, 50 °C, and 60 °C). The extraction was carried out according to section 2.2.1 and determine the fucoidan content.

2.2.4. The effects of microwave-assisted method on fucoidan extraction

15g raw material (according to dry matter) was soaked into a solvent pre-treatment system to remove the impurities (section 2.2.2). The effects of intensity ranges (700W, 1600W, 2500W) corresponding to the levels: Low, Med, High; and time (1 minute; 1.5 minutes; 2 minutes; 2.5 minutes; 3 minutes) were investigated. Then, the extraction procedure was carried out according to section 2.2.1 and determine the fucoidan content.

2.2.5. The effects of enzyme and microwave-assisted method on fucoidan extraction

15g raw material (according to dry matter) was soaked into a solvent pre-treatment system to remove the impurities (section 2.2.2). Combining cellulase enzyme treatment (with the ratio of enzyme/material 1/10 (v/w), extracted in 3 hours, at 50 °C) with microwave procedure (intensity 700W for 1.5 minutes) in the solvent was distilled water, the ratio of material/ solvent was 1/20 (w/v). After that, the extraction process would be done based on section 2.2.1 and determine the fucoidan content.

2.3. Analytical methods

Fucoidan content was determined by UV-VIS spectroscopy:

+ Establishing the calibration curve: The standard substance was fucoidan, used to build the calibration curve with a concentration from 10-100 μg/mL. 1 mL of the solution of concentrations were added to the tubes and cooled at 4 ℃ (for 2-3 minutes). Next, after adding 4.5 mL of sulfuric acid (85%) to the tubes, the tubes were tightly covered to prevent evaporation and placed in a bath of boiling water, for 10 minutes. These tubes were then cooled under running water, and 0.3 mL of 0.1% cysteine hydrochloric acid would be added to the tubes. These were placed in the dark for 2 hours and measured the UV-VIS absorbance at the wavelengths of 390 nm and 430 nm [11].

+ Blank samples were prepared with the same method.

Determination of fucoidan content: It was performed according to the method of making standard curves, replacing standard with experimental samples.

2.4. Data analysis

The experiments were repeated 3 times, the results were processed Microsoft Excel 2016 software, the differences and suitable parameters were selected based on the analysis results of IBM SPSS Statistics 20 software as mean ± error (ANOVA and LSD analysis).

3. RESULTS AND DISCUSSION

3.1. The influence of the impurity-removing solvent system on fucoidan extraction

The influence of the impurity-removing solvent system on the extracted fucoidan content is shown in Table 1.

Table 1. The effects of impurity-removing solvent system on the obtained fucoidan content.

Solvent system	Fucoidan content (µg/g_{dm})
System 1	794.894 ± 6.054
System 2	574.851 ± 5.569

The impurities in seaweed such as polyphenols, lipids, or chlorophyll would affect the extraction efficiency of the target compound. So, the pre-treatment stage before fucoidan extraction plays a key role to enhance the effectiveness of fucoidan extraction [12]. The results in Table 1 show that the fucoidan content after treatment with solvent system 1 (methanol: chloroform: water) was 794.894 ± 6.054 µg/g_{dm}, much higher than that of the solvent system 2 (hexane, acetone, 80% alcohol, ethanol: water: formaldehyde). The system (methanol: chloroform: water) was used to remove lipids and polyphenols from raw materials, and this was also seen in the report of Whyte & Southcott (1970) when isolating lipids from fish [12]. In addition, the high ratio of methanol/water not only prevented the preliminary simultaneous extraction of fucoidan but also eliminated most of the chlorophyll and mannitol [13]. Solvent system 2 had also been found in the process of removing impurities in seaweed materials in the report of Mian & Percival [14], but the formaldehyde component might create covalent bonding between many other compounds, generating high molecular weight complexes, and from this, they could interact with sulfate polysaccharides and lead to precipitation, causing a decline in the obtained fucoidan content [13]. Solvent system 2 suitable was the suitable impurity-removing solvent system for *Ceratophyllum submersum* seaweed [15]. Even though *C. submersum* and *C. demersum* belonged to the family of *Ceratophyllum*, they have differences in structure and habitats. In addition, fucoidan locates close to the cell wall of seaweed. Thus, the effects of solvents in the pre-treatment stage were different for each seaweed [16]. Finally, from the arguments and obtained data, solvent system 1 was chosen for the pretreatment step in this study.

3.2. The effects of enzyme-assisted extraction on fucoidan (EAE)

Various factors have been studied and developed to enhance the recovery yield in fucoidan extraction such as enzymes, microwaves, and ultrasound. In this study, the effects of cellulase enzyme treatment on fucoidan extraction were investigated. The effects of three factors: enzyme/ material ratio, processing time, enzyme processing temperature are shown in Figure 1.

According to Thomas Hahn *et al*., the extraction time and enzyme concentration directly influenced the efficient recovery of fucoidan extraction [13]. The catalytic reaction of an enzyme is formed when its active center is combined with the substrate. The reaction rate can

be fast or slow depends on the enzyme/ substrate concentration. The low enzyme concentration reduces the reaction rate results in a slow or incomplete extraction procedure. The high enzyme concentration and the low substrate concentration led to the high reaction speed. However, the efficient recovery would not be recognized at this time due to the excess enzyme usage. The results in Fig. 1a showed that at the ratios of 2/20 and 3/20 (v/w), the concentration was higher than that of 1/20 (v/w). The highest fucoidan content was at the ratio of 2/20 (v/w) with 886.233 µg/g_{dm}. Thus, the suitable enzyme/material of 2/20 (v/w) was selected for further experiments.

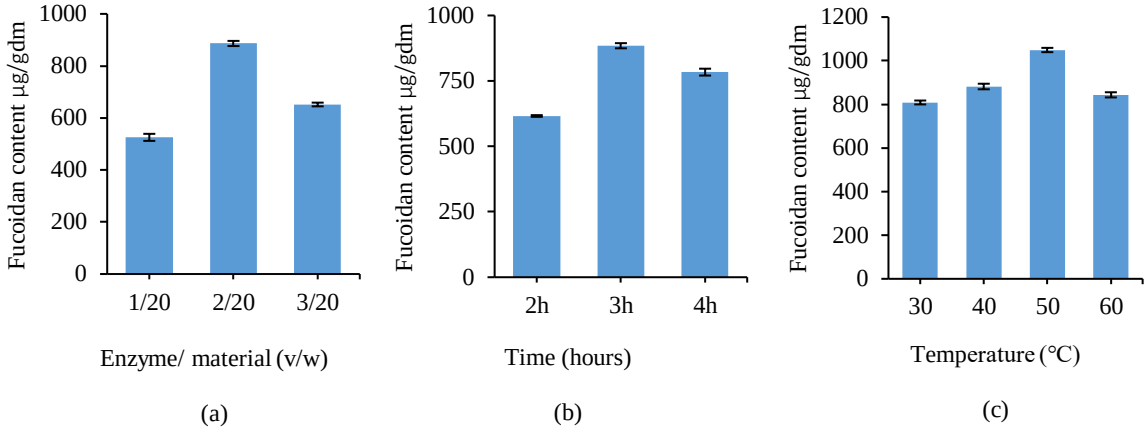

Figure 1. The effects of extraction ratio of enzyme/ material (a), time (b) and temperature (c) on fucoidan extraction

Regarding the extraction time, the contact time for enzyme and material effects disrupt the cell wall and the extraction efficiency. However, the treatment for a long time does not show a better result because substrates have resolved to the limit, while a part of the polysaccharide could also degenerate under the influence of temperature conditions and enzyme catalyst. The highest fucoidan content was 884.374 ± 9.818 µg/g_{dm} at 3 hours (Fig. 1b). Meanwhile, for the same target function, the figures were 615.956 ± 3.281 µg/g_{dm} and 783.621 ± 13.089 µg/g_{dm} at 2 hours and 4 hours, respectively.

Cellulase enzymes increase the permeability of the cell walls, disrupt the membranes and improve the efficient extraction of fucoidan [17]. In theory, the enzyme is a protein in nature, so the temperature has a great influence on its structure and catalytic efficiency. In general, a high temperature would increase the kinetic energy of the enzyme and the substrate. So, the ability to collide between them would be faster, thereby shortening the time to form enzyme-substrate complexes, bringing the optimal extraction efficiency. Each enzyme has a certain optimal temperature, and the cellulase enzyme works effectively at temperatures 50-55 °C [18]. From Fig. 1c, the activity of this enzyme dramatically reduced at 60 °C with the obtained fucoidan of 843.431 ± 11.859 µg/g_{dm}, about 200 µg/g_{dm} lower than the figure at the well-suited temperature (about 50 °C). This could be because the high temperature would cause the denaturation for the enzyme, alter its active center, leading to difficulty or inability to combine with substrates, reducing the efficiency of extracting fucoidan from raw seaweed.

In short, from the obtained results and the above arguments, the suitable parameters of the enzyme-assisted method for fucoidan extraction were the ratio of enzyme/material 2/20 (v/w) at 50 °C in 3 hours with the obtained fucoidan content of 1049.046 µg/g_{dm}. And these optimal conditions would be selected for further experiments.

3.3. The effects of microwave-assisted extraction on fucoidan (MAE)

As mentioned above, fucoidan extraction could also be affected by microwave processing. The effects of microwave power and time on fucoidan extraction are shown in Fig. 2 and Fig. 3.

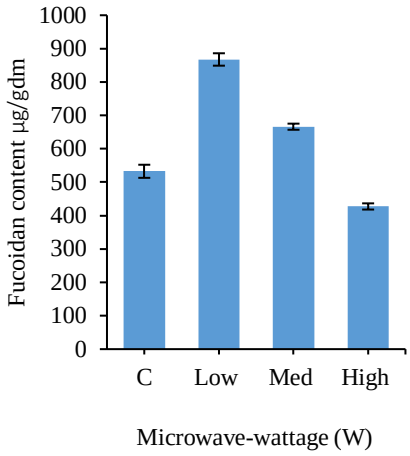

Figure 2. The effect of microwave power (W) on the extracted fucoidan content

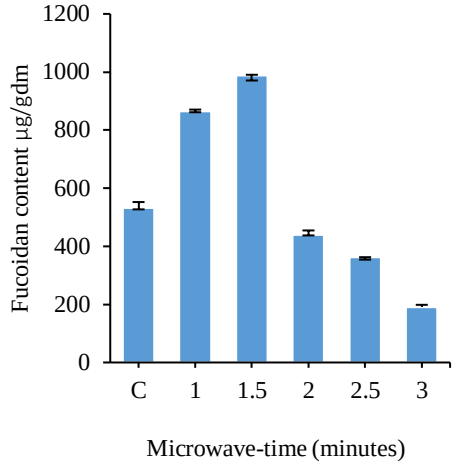

Figure 3. The effect of microwave time on the extracted fucoidan content

The essence of the microwave process for extraction is the microwave energy would cause molecular motion by migration of ions and rotation of dipoles that existing in the solvent, putting pressure immediately on the cell wall of the material, supporting the extraction process [19]. More specifically, the microwave rays induce the vibrations of water molecules in the plant and the solvent, the disordered movement will generate heat and kinetic energy, which motivates these molecules to be more turbulent movement, from this, it is capable of exerting pressure on the cell wall of the material to alter the permeability of the cell surface. As the cell wall breaks, the intracellular contents are released into the medium [13].

Wattage and time for the microwave process were considered to be the two most important factors affecting target compound extraction efficiency [11]. Referring to the wattage, at low microwave power (700W), the obtained fucoidan was 867.583 ± 18.585 µg/g_{dm}, while at Med (1600 W) and High (2500 W) intensity, the obtained fucoidan contents were 666.214 ± 9.144 µg/g_{dm} and 427.357 ± 9.253 µg/g_{dm}, respectively, which is lower than Low intensity (Fig. 2). It can be understood that the higher the microwave intensity, the higher the temperature of the sample, which leads to an increase in the efficiency of fucoidan extraction. On the other hand, high temperatures could cause the change of chemical composition in seaweed, easily leading to degradation of the quality of the extracted compounds, particularly fucoidan [19].

Turning to the microwave time, the highest fucoidan content was 986.968 ± 3.514 µg/g_{dm} at 1.5 minutes (Fig. 3). However, the content of the target compound of longer time levels was significantly reduced. Guillaume Dupont *et al*. reported that the longer time was not highly effective in terms of bio-compound extraction and it also could lead to the deterioration in structure [20]. Rodriguez-Jasso *et al*. reported that the lowest microwave level (30 psi) at the same time ranged from 1 - 1.5 minutes also showed the highest fucose extracted content [11].

Most of all, the results demonstrate that 700W and 1.5 minutes were the suitable parameters for fucoidan extraction from *C. demersum* seaweed.

3.4. The effects of combination treatment between two factors: enzyme and microwave – on fucoidan extraction from *C. demersum*

The effects of combining two enzyme-microwave factors on fucoidan extraction from the *C. demersum* seaweed are shown in Table 2.

Table 2. The effects of the enzyme - microwave combination on the obtained fucoidan content

Combination	Fucoidan content (μg/g_{dm})
Microwave - Enzyme	1246.871 ± 8.352
Enzyme - Microwave	970.095 ± 11.361

The fucoidan yield of samples with enzyme and microwave combination treatment was higher than that of single-factor extraction. This study reports the first time in testing the extraction efficiency from the combination of enzyme and microwave. The fucoidan content using microwave-enzyme treatment was 1246.871 ± 8.352 μg/g_{dm}, higher than the reverse process (970.095 ± 11.361 μg/g_{dm}). This could be because 700W in 1.5 minutes could help to increase the temperature to about 50 - 55°C, which is the optimum temperature for cellulase enzyme, thereby creating more favorable conditions for the extraction of fucoidan by the enzyme. Above all, this sequence combination is even more effective because the microwave process will put pressure on the cell wall of the material sample, soften the cell surface or partially sever the bonds. Cellulose structure was broken down under cellulase enzyme easily and effectively. In fact, this result was similar to the previously stated [15], when the microwave - enzyme - solvent system sequence showed that this combination gave a good efficiency for fucoidan extraction. In this study, the pretreatment solvent was fixed at the first stage before enzyme and microwave treatment that help to remove the impurities from the material, which enhances the yield of fucoidan extraction from *C. demersum*.

4. CONCLUSION

In this study, the effects of different impurity-removing solvents, enzyme and microwave treatment on fucoidan extraction from *C. demersum* were presented. In the pretreatment stage, the system (methanol: chloroform: water) resulted in obtained fucoidan at 794.894 ± 6.054 μg/g_{dm}, higher than the other system. Meanwhile, the samples with enzyme and microwave treatment gave higher than the control. The suitable conditions for enzyme treatment (50 °C for 3 hours with the enzyme/material ratio 2/20 (v/w)) and the microwave treatment (700 W, 1.5 minutes) resulted in 1049.046 ± 9.838 μg/g_{dm} and 986.968 ± 3.514 μg/g_{dm} respectively. In addition, the combination of enzyme - microwave gave better results 1246.871 ± 8.352 μg/g_{dm}, which was higher than that of the opposite procedure as well as the single factor treatment.

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TÓM TẮT

NGHIÊN CỨU ẢNH HƯỞNG CỦA ENZYME CELLULASE VÀ VI SÓNG ĐẾN HIỆU QUẢ TRÍCH LY FUCOIDAN TỪ RONG *Ceratophyllum demersum*

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Rong *Ceratophyllum demersum* chứa nhiều thành phần có hoạt tính sinh học trong đó đáng chú ý phải kể đến fucoidan. Fucoidan là một polysaccharide giàu fucose thường có mặt trong tảo biển, có nhiều chức năng liên quan đến các hoạt động sinh lý, thể hiện qua các hoạt tính sinh học như khả năng chống ung thư, miễn dịch, kháng viêm hay kháng oxy hóa… Do đó, hợp chất này thường được ứng dụng nhiều vào thực phẩm chức năng. Nghiên cứu này trình bày ảnh hưởng của hệ dung môi loại tạp chất, ảnh hưởng của enzyme, vi sóng đến hàm lượng fucoidan trích ly từ rong *C. demersum.* Kết quả cho thấy, hàm lượng fucoidan khi được xử lý với hệ 1 là 794,894 $\pm$ 6,054 µg/g_{dm} cao hơn so với khi sử dụng hệ 2 là 574,851 $\pm$ 5,569 µg/g_{dm}. Tiếp đó, khi sử dụng vi sóng như một phương pháp hỗ trợ trích ly, hàm lượng fucoidan thu nhận là 986,968 $\pm$ 3,514 µg/g_{dm}, trong khi đó, dưới sự hỗ trợ của enzyme, fucoidan được trích ly từ loại rong *C. demersum* này đạt 1049,046 $\pm$ 9,838 µg/g_{dm} ở nhiệt độ 50 °C trong 3 giờ với tỷ lệ enzyme/nguyên liệu là 2/20 (v/w). Đặc biệt hơn, tính mới của nghiên cứu thể hiện ở sự kết hợp giữa 2 yếu tố enzyme - vi sóng để xử lý bổ trợ cho quá trình trích ly cho thấy một hàm lượng fucoidan thu nhận được là 1246,871 $\pm$ 8,352 µg/g_{dm} ở trình tự vi sóng - enzyme, cao hơn đáng kể so với trình tự ngược lại được khảo sát trong nghiên cứu này.

Từ khóa: Ceratophyllum demersum, enzyme, fucoidan, vi sóng.