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Deep Eutectic Solvents Based Ultrasonic Extraction of Polysaccharides from Edible Brown Seaweed *Sargassum horneri*

Jinggui Nie, Danting Chen and Yanbin Lu *

Institute of Seafood, Key Laboratory of Aquatic Products Processing of Zhejiang Province, Zhejiang Gongshang University, Hangzhou 310012, China; niejinggui@163.com (J.N.); cdt1144173162@163.com (D.C.)

* Correspondence: luyanbin@zjgsu.edu.cn; Tel.: +86-571-87103135

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Abstract: In this work, a method for ultrasonic extraction of polysaccharides from *Sargassum horneri* using deep eutectic solvents was proposed. The studied deep eutectic solvents were composed of choline chloride, 1,2-propanediol and water. Based on the single-factor experiment results, four experimental factors were systematically evaluated, giving the optimal extraction conditions as follows: molar ratio of choline chloride to 1,2-propanediol of 1:2, water content of 30% (v/v), solid-liquid ratio of 1:30 (g/mL), and the extraction temperature of 70 °C. Fourier transform infrared spectroscopy and X-ray diffraction were utilized to investigate changes in the chemical characteristic of extracted polysaccharides. The results indicated that deep eutectic solvents had stronger protein and calcium carbonate removal ability than that of a conventional hot water extraction method. Moreover, in vitro antioxidant activity tests exhibited that the obtained polysaccharides had significant inhibition effects on DPPH and ABTS radicals. The proposed deep eutectic solvents assisted ultrasonic extraction protocol was considered to be a green, fast and effective protocol for extracting polysaccharides from *Sargassum horneri*.

Keywords: *Sargassum horneri*; deep eutectic solvents; ultrasonic extraction; polysaccharides; antioxidant activity

1. Introduction

Sargassum horneri, an edibal brown seaweed, was one of the main components in the subtidal seaweed flora extensively distributed in East China Sea, which was rich in dihomo-gamma-linolenic acid and polysaccharides [1]. Among these compounds, polysaccharides had been recognised as the main active components which had many functions, including antivirus, antioxidant, and anti-aging activities [2,3]. Due to the potential bioactivity of polysaccharides, the development of efficient extraction and purification of polysaccharides from *S. horneri*, is of great importance. Generally, the traditional extraction protocol of polysaccharides was the water-boiling method. However, under high temperature, many components were dissolved and might lose their activity. Therefore, researchers had developed other auxiliary protocols, such as auxiliary water enzyme, ultrasonic-assisted extraction, microwave-assisted extraction, and vacuum extraction, to extract polysaccharides more quickly and efficiently in recent years [3]. However, in the process to extract polysaccharides from *S. horneri* it is necessary to use organic solvents which have several disadvantages, such as toxicity and unsafety [4]. In addition, scientists had also been committed to designing new environmentally friendly green extraction solvents that met technical and economic needs [5]. In recent years, there had been a significant increase in the use of ionic liquids (ILs) as green solvents for the extraction and

purification of natural bioactive components [6,7]. However, due to the potential toxicity and low biodegradability of ILs, there was a concern that it might have a potential impact on human health and the environment [8]. Moreover, the synthesis and purification of ILs required a higher cost than ordinary solvents [9,10]. To overcome these drawbacks of ILs, it was imperative to find alternative solvents to replace ILs.

Recently, it was found that the use of deep eutectic solvents (DESs) as a green alternative to replace traditional organic solvent had got great development [11]. Their physicochemical properties were very similar to the ILs [12]. DESs could be easily prepared from natural and available compounds in a very simple manner by mixing and heating. From the point of view of sustainable chemistry, these characteristics made it very attractive [13]. It had been successfully used in the extraction of flavonoids [14], carrageenan [15], phenolic acids [16], alkaloids [17] and other active components from traditional Chinese medicine. As we know, ultrasonic crushing could greatly speed up the extraction process, shorten the extraction time and improve the extraction yield [18]. Moreover, DESs had the advantages of biodegradability, low cost, and simple synthesis [19]. Therefore, the development of DESs-assisted ultrasonic extraction of polysaccharides was a promising method. To the best of our knowledge, there had been no detailed study of the use of DESs-assisted ultrasonic extraction of polysaccharides from *S. horneri*. Therefore, the purpose of this study was to establish an environmental friendly and efficient method for extracting polysaccharides from *S. horneri*. The extraction process was carefully optimized. At the same time, the chemical properties and antioxidant activity of extracted *S. horneri* polysaccharides were also studied.

2. Results and Discussion

2.1. Optimization of the DESs-Assisted Ultrasonic Extraction of Polysaccharides

2.1.1. Effect of Molar Composition of Studied DESs

Generally, DESs are composed of a hydrogen bond acceptor with a donor in a certain proportion. Among them, the physicochemical properties of DESs, including viscosity, solubility, and most importantly, polarity, will have great influence on the extraction yield of the targeted components [9–11]. Considering the polarity and solubility of polysaccharides, the selected DESs were composed of hydrophilic choline chloride and 1,2-propanediol accroding to the literatures [13–18]. Thus, different choline chloride and 1,2-propanediol molar ratios could affect the viscosity and polarity of the DESs. In this work, the experiments of extracting polysaccharides with DESs by different molar ratios were carried out, and the results were shown in Figure 1A. It was obvious that the extraction yield of polysaccharides increased first with the increase in the molar ratio of choline chloride and 1,2-propanediol. When the molar ratio reached 1:2, the extraction yield was the largest. Because increasing the proportion of 1,2-propanediol in DESs not only increased its viscosity but also increased the surface tension. Further increasing the ratio of 1,2-propanediol in DESs might reduce the interaction of 1,2-propanediol with choline chloride and decrease the extraction yield [20]. Therefore, the molar ratio of 1:2 was selected as the best extraction condition for preparation of DESs.

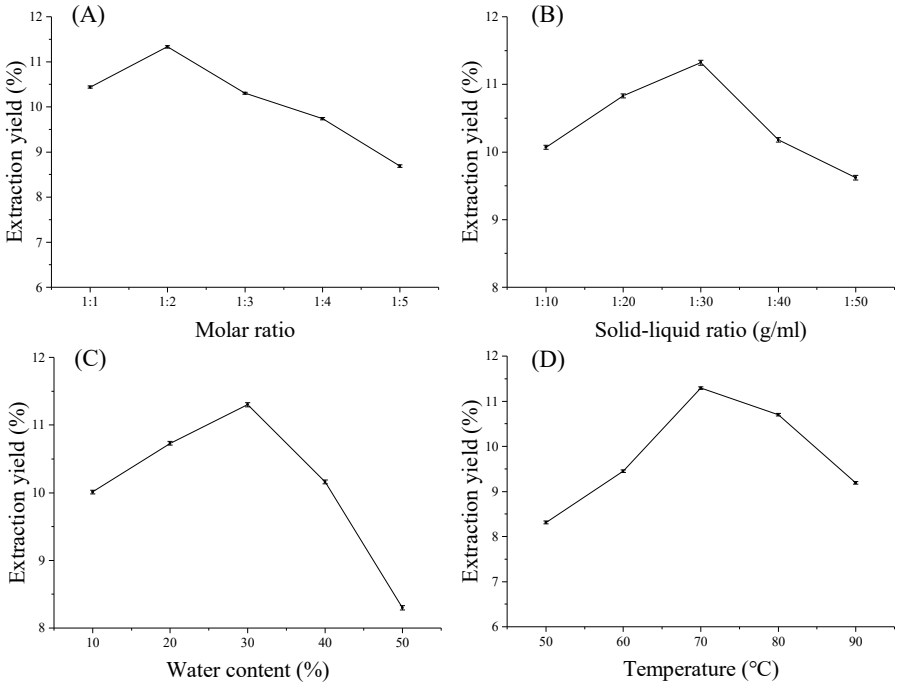

Figure 1. (**A**) Effect of different molar ratios on the extraction yield of crude polysaccharides. Expeimental conditions: water content of 30% (v/v), solid–liquid ratio of 1:30 (g/mL), extraction temperature of 70 °C, sonication time of 30 min, amplitude level of 40%; (**B**) Effect of different solid-liquid ratios on the extraction yield of crude polysaccharides. Expeimental conditions: molar ratio of 1:2, water content of 30% (v/v), extraction temperature of 70 °C, sonication time of 30 min, amplitude level of 40%; (**C**) Effect of different water content on the extraction yield of crude polysaccharides. Expeimental conditions: molar ratio of 1:2, solid–liquid ratio of 1:30 (g/mL), extraction temperature of 70 °C, sonication time of 30 min, amplitude level of 40%; (**D**) Effect of different extraction temperature on the extraction yield of crude polysaccharides. Experimental conditions: molar ratio of 1:2, solid-liquid ratio of 1:30 (g/mL), water content of 30% (v/v), sonication time of 30 min, amplitude level of 40%. Each experiment was carried out in triplicate.

2.1.2. Effect of Solid–Liquid Ratio

Generally, the solid to liquid ratio (sample-solvent ratio) is a key factor in extraction yield. In this study, different solid to liquid ratios were tested, including 10, 20, 30, 40 and 50 g/mL, and the results were shown in Figure 1B. The extraction yield of polysaccharides increased with the increase in solid–liquid ratio. When the solid–liquid ratio reached 1:30 (g/mL), the extraction yield of polysaccharides began to decline. This may be because the extraction equilibrium can be reached quickly with a small amount of extraction solvent. Although a large amount of extraction solvent increased the leaching rate of the target compound, it also caused the waste of the extraction solvent and complicated the extraction process [21]. Therefore, the solid–liquid ratio of 1:30 was selected for the extraction of polysaccharides from *S. horneri*.

2.1.3. Effect of Water Content

The water content (volume ratio, %) of the DESs aqueous solution affects the viscosity and increases its polarity. Therefore, the extraction yield seems to depend largely on the water content [5]. In this study, experiments were carried out to extract polysaccharides with DESs at different water contents, and the results were shown in Figure 1C. The extraction yield of polysaccharides increased with the increase in water content. When the water content reached 30% (v/v), the extraction yield reached the maximum. Further increasing the water content, the extraction yield of polysaccharides began to decrease. This might be attributed to the fact that low water content was difficult to penetrate into

plant cells to achieve high extraction yields. However, high water content would increase the polarity of the mixture and reduce the interaction between molecules [22]. Therefore, in this work, the water content 30% (v/v) was selected as the best extraction condition for the extraction of polysaccharides from *S. horneri*.

2.1.4. Effect of Temperature

The extraction temperature is a crucial factor on extraction yield. In this study, the effects of different extraction temperatures on the polysaccharides yield were tested, including 50, 60, 70, 80 and 90 °C, and the results were shown in Figure 1D. When the temperature was set at 70 °C, the extraction yield reached the maximum value. This might be due to the fact that increase in temperature could produce a higher mass transfer rate and solvent diffusion rate, thus improving the extraction yield. However, higher temperature might also cause the thermal degradation of target compounds [23]. Hence, the temperature 70 °C was optimized in this work.

From the above evaluation, the optimal extraction condition for the studied DESs-assisted ultrasonic extraction of polysaccharides from *S. horneri* could be as follows: a molar ratio of 1:2, a solid–liquid ratio of 1:30 (g/mL), a water content of 30% (v/v), and an extraction temperature of 70 °C. Thus, we performed three parallel experiments under these conditions. The results showed that the relative extraction yield of crude polysaccharides from *S. horneri* was 11.31%.

2.1.5. Comparison with the Conventional Hot Water Extraction

In this work, the polysaccharides from *S. horneri* were also extracted by the conventional hot water method, producing the relative yield of 13.52%. Although the relative yield of crude polysaccharides by hot water extraction was a little bit higher than that of DESs-assisted ultrasonic extraction (11.31%), the proposed DESs extraction method only cost 30 min for each ultrasonic process, saving 75% of time consumption and greatly enhancing the productivity. Moreover, the temperature of ultrasonic extraction was optimized at 70 °C, which was milder than that of water extraction method, and might not damage the structure of polysaccharides present in *S. horneri*. Therefore, DESs exhibited as a promising solvent alternative for the extraction of polysaccharides from brown seaweeds.

2.2. Characterization of Extracted Polysaccharides

2.2.1. Infrared Spectrometry Analysis

Fourier transform infrared (FT-IR) spectrum of polysaccharides extracted by DESs and hot water are shown in Figure 2A. In both spectra, the absorption peaks at 3331, 2934, 1747, 1411, and 1036 cm^{-1} are characteristic peaks in polysaccharides [24–27]. The broad and strong absorption peak at 3331 cm^{-1} is the stretching vibration peak of O-H. The small peak at 2934 cm^{-1} is the C-H stretching vibration peak on the sugar ring or branch. The peak at 1747 cm^{-1} should be a C=O stretching vibration in the amide group. The peak at 1411 cm^{-1} should be a C-H stretching vibration peak. The peak at 1036 cm^{-1} may be a C-O-C stretching vibration peak.

However, it can be clearly seen from Figure 2A that the polysaccharides extracted by DESs has no obvious absorption peak around 1310 cm^{-1}, while the polysaccharides extracted by hot water has a sharp peak. Generally, the absorption peak at 1310 cm^{-1} could be attributed to the C-N telescopic vibration absorption peak [28]. The polysaccharides extracted by DESs are less contamination from protein than those extracted by hot water. The high purity of the *S. horneri* polysaccharides extracted by the DESs method can be attributed to the hydrogen bonding interaction between the DESs and the polysaccharides components [29]. The competitive hydrogen bond formation between DESs and carbohydrate leads to the destruction of the intramolecular hydrogen bond network, thereby weakening the hydrogen bond interactions in the *S. horneri*. As a result, the polysaccharides dissolved in DESs were separated from the protein. Therefore, we can infer that the DESs' extracted *S. horneri* polysaccharides have obvious deproteinization effect compared with the hot water extracted polysaccharides.

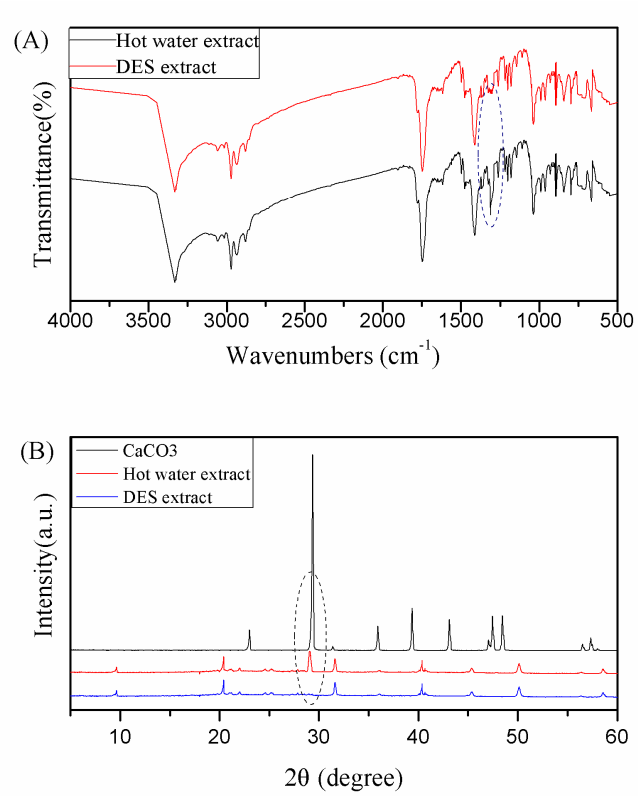

Figure 2. (**A**) The FT-IR spectrum of hot water extracted *S. horneri* polysaccharides and DESs extracted *S. horneri* polysaccharides; (**B**) X-ray curve of $CaCO_3$, hot water extracted *S. horneri* polysaccharides and DESs extracted *S. horneri* polysaccharides.

2.2.2. X-Ray Diffraction Analysis

The X-ray has a wavelength in the range of 0.01 to 10 nm, which is equivalent to the atomic distance in the crystal, so that when X-ray passes through the crystal, it can cause electron vibration in the crystal. Thus it can be used to determine the configuration of the crystal [30]. In this study, X-ray diffraction (XRD) analysis was performed to determine the crystal structure and relative crystallinity of the polysaccharides obtained from *S. horneri* by both DESs-based extraction and conventional hot water extraction method. The XRD patterns of the polysaccharides extracted by DESs, the polysaccharides extracted by hot water, and $CaCO_3$ are shown in Figure 2B. It can be seen that the DESs-extracted sample exhibits typical diffraction peaks at $2\theta = 9.64°$, 20.42°, 29.06°, 31.62°, 40.38° and 50.12°, which is the lattice type of the general polysaccharides. The XRD pattern of the polysaccharides extracted by DESs is almost the same as the polysaccharides obtained by the hot extraction method. However, the diffraction peak of $CaCO_3$ at about $2\theta = 29.36°$ is not observed in the polysaccharides extracted from DESs. This may be because the polysaccharides extracted by DESs do not contain $CaCO_3$. Further, the crystallinity index of the sample was calculated according to the Segal method. The CrI of the *S. horneri* polysaccharides was calculated by the DESs method to be 65.87%, which was calculated to be 54.14% by the conventional hot water extraction method. The increase in CrI after the DESs treatment was due to the removal of $CaCO_3$ in the polysaccharides. This may be because choline chloride in DESs can directly invade the internal structure of *S. horneri*, and then directly contact calcium carbonate and protein to have a reaction, leading to both deproteinization and demineralization. Therefore, we can infer that the *S. horneri* polysaccharides extracted by the proposed DESs method have a more significant mineral-removing effect than that of conventional hot water extraction method.

2.3. Antioxidant Activity of Extracted Polysaccharides

The DPPH free radical is a stable free radical that is widely used as a tool for estimating the free-radical-scavenging activities of antioxidants [31]. The antioxidant mechanism of DPPH radical scavenging is related to the acceptability of DPPH radical to hydrogen. DPPH is converted to DPPH-H, a non-free radical form, from hydrogen provided by antioxidants [32]. Figure 3A exhibited DPPH radical scavenging activity of polysaccharides extracted by DESs and compared with BHT as positive control. Under the experimental conditions, with the increase in concentration, the DPPH free-radical-scavenging effect of *S. horneri* polysaccharides was gradually enhanced. When the polysaccharides concentration increased from 0.5 to 3 mg/mL, the DPPH radical scavenging yield increased from 32.36% to 67.82%. The results showed that the *S. horneri* has significant DPPH removal efficiency. This may be because the *S. horneri* polysaccharides can eliminate excess oxygen radicals produced in vitro by blocking free radical reaction chains, so the polysaccharides have an obvious scavenging effect on DPPH free radicals.

The ABTS assay is a well-established method for measuring the antioxidant capacity of potential antioxidants. This is because ABTS free radicals can be oxidized to free radical cations ($ABTS^+$) for measuring the antioxidant capacity of water-soluble and fat-soluble food samples [33]. Figure 3B exhibited ABTS radical scavenging activity of polysaccharides extracted by DESs and compared with ascorbic acid which was used as positive control. When the mass concentration was 0.5–3.0 mg/mL, with the increase of concentration, the removal yield of *S. horneri* polysaccharides showed an obvious upward trend. When the mass concentration of the polysaccharides was 3 mg/mL, the ABTS free radical clearance yield reached 71.28%. However, the scavenging yield of ascorbic acid on ABTS radical was stable at about 90%. The removal yield was better than the *S. horneri*, and did not change with the increase in concentration. Therefore, we can only say the *S. horneri* polysaccharides have a certain ABTS free-radical scavenging ability and can be used as an antioxidant product for future research.

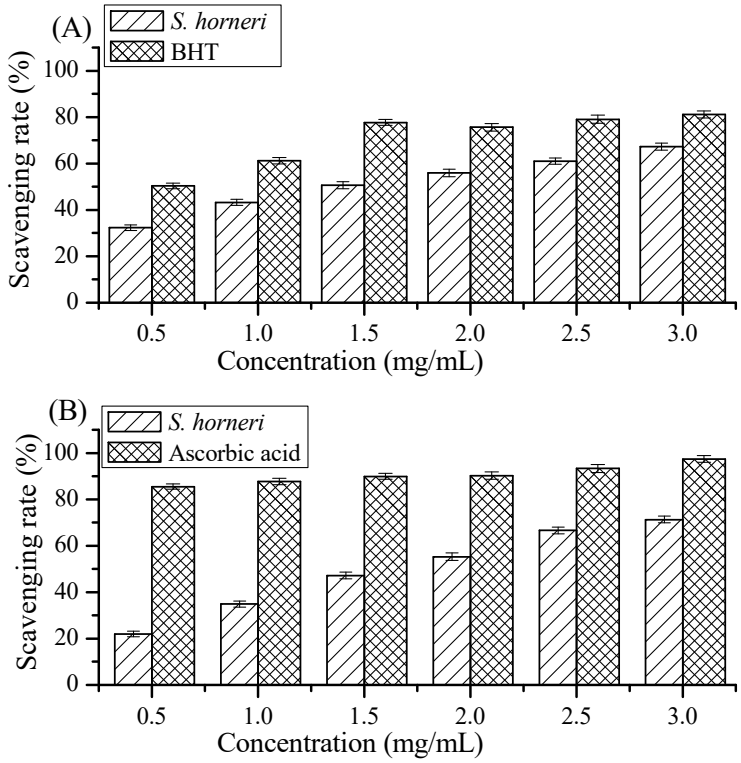

Figure 3. (**A**) Scavenging of DPPH free radicals by *S. horneri* polysaccharides; (**B**) Scavenging of ABTS free radicals by *S. horneri* polysaccharides.

3. Materials and Methods

3.1. Materials

The materials of *S. horneri* were purchased from Dong Tou county (Zhejiang, China) and identified by Prof. Juanjuan Chen from College of Marine Sciences, Ningbo University. The choline chloride (dried before use), ethanol and 1,2-propanediol were analytically pure and purchased from Xi Long Chemical Co. (Guangdong, China). The reagents used for antioxidant activity tests, including ascorbic acid (Vc), 2,6-ditert-butyl-4-methyl phenol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2-2-azino-bis-(3-ethyl-benzthia-zoline-6-sulfonic acid) (ABTS), were purchased from Sigma-Aldrich (Shanghai, China).

3.2. Ultrasonic-Assisted Extraction of Polysaccharides Using DESs

3.2.1. Preparation of DESs

DESs was prepared by mixing choline chloride and 1,2-propanediol in different ratios accordingly to the literature [10,11]. First, the mixture was prepared according to their ratio (1:1 to 1:5) and stored in a reaction flask. Then, it was continuously stirred for 60 min under the temperature of 80 °C until a clear homogeneous solution was obtained.

3.2.2. Ultrasonic-Assisted Extraction of Polysaccharides by DESs

A Sonics Vibra-Cell VCX-500 sonicator (Sonics and Materials Inc., CT, USA) equipped with a Ti-Al-V probe (1.3 cm diameter) was employed for ultrasound-assisted extraction. The maximum of 500 W output power and 20 kHz of frequency could be provided by the sonicator. The amplitude control unit allowed the probe to be set at any desired level in the 10%–100% range of the nominal power.

In this work, 10 g of *S. horneri* powder was weighed and added with different extraction solvents accordingly for all the ultrasonic experiments. Consequently, the effects of choline chloride and 1,2-propanediol molar ratio (1:1–1:5), water content of the studied DESs (10%–50%), solid–liquid ratio (sample-solvent ratio, 1:10–1:50), and extraction temperature (50–90 °C) on the yield of polysaccharides were carefully studied and evaluated by a single-factor experiment. The amplitude level of 40% and ultrasonic time of 30 min were optimized and selected for all the ultrasonic extractions. Afterwards, the obtained extract was precipitated by adding of absolute ethanol. After centrifugation at 8000 r/min for 10 min, the precipitate was taken out and finally washed twice with absolute ethanol to remove colored substances and other impurities. The precipitate was dried in a vacuum desiccator for 24 h. Three repeated extraction tests were performed.

In this work, the relative yield of crude polysaccharides was determined by phenol-sulfuric acid method using *D*-glucose as the standard substance [34,35]. The extraction yield (%) of polysaccharides was calculated as follows:

$$\text{Yield}(\%) = \frac{\text{the crude polysaccharides content (g)}}{\text{weight of sample (g)}} \times 100\% \quad (1)$$

3.3. Conventional Hot Water Extraction Method

The dried *S. horneri* was crushed, screened 100 mesh, and treated by water extraction [36]. Briefly, 30 g crude materials of *S. horneri* powder was extracted with distilled water (the mass/volume ratio was 1:65) for 2 h under the temperature of 95 °C. The obtained extract was cooled, filtered and centrifuged at 8000 rpm for 10 min. The supernatant was concentrated to 180 mL by rotary evaporator. Then, the crude polysaccharides were obtained by precipitation using 80% ethanol and subsequent lyophylization. Three repeated extraction tests were performed. The relative yield of crude polysaccharides was also determined by the phenol-sulfuric acid method which described in Section 3.2.2.

3.4. Characterization of Extracted Polysaccharides

The *S. horneri* polysaccharides obtained under the optimum conditions was characterized by the following methods.

Fourier transform infrared (FT-IR) spectra were recorded in the range of 4000–500 cm^{-1} using an FT-IR spectrometer (Thermo Scientific, Massachusetts, USA).

XRD patterns were recorded using a D/max-2500 X-ray diffractometer (Rigaku Denki, Tokyo, Japan) with Cu Kα_1 radiation (λ = 0.154 nm) in a range from 5° to 60° at a scanning yield of 5 °/min with a step interval of 0.02°. The relative crystallinity index (CrI) was calculated by the Segal method [37]

$$\text{CrI } (\%) = \frac{I_{110} - I_{am}}{I_{am}} \times 100 \quad (2)$$

where I_{110} is the peak intensity of the diffraction for the (110) plane at 2$\theta \approx 20°$ and I_{am} is the intensity of the amorphous diffraction at 2$\theta \approx 18°$.

3.5. Determination of Antioxidant Activity of Extracted Polysaccharides

3.5.1. DPPH Free Radical Clearance Yield Determination

A total of 0.5 mL of samples (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/mL) was added to 3.0 mL of a 0.01% (v/v) ethanol solution of DPPH. Absorbance at 517 nm was measured after 30 min. The scavenging activity of DPPH radical was calculated according to the following equation [32]

$$\text{Scavenging effect } (\%) = \frac{A_{517}(\text{blank}) - A_{517}(\text{sample})}{A_{517}(\text{blank})} \times 100 \quad (3)$$

where A_{517} (blank) was the absorbance of the control (deionizedwater, instead of sample) and A_{517} (sample) was the absorbance of the test sample mixed with reaction solution.

3.5.2. ABTS Free Radical Scavenging Yield Determination

The radical cation was prepared by mixing a 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for 4–8 h until the reaction was completed and the absorbance was stable. The $ABTS^+$ solution was diluted in ethanol to an absorbance of 0.700 ± 0.05 at 734 nm for measurements. The photo metric assay was conducted on 0.9 mL of the $ABTS^+$ solution and 0.1 mL of isolated polysaccharides dissolved in a MeOH solution and mixed for 45 s. Measurements were taken immediately at 734 nm after 1 min. The antioxidative activity of the polysaccharides was calculated by determining the decrease in absorbance at different concentrations by using the following equation [38]:

$$\text{Scavenging effect } (\%) = \frac{A_C - A_T}{A_C} \times 100 \quad (4)$$

Here, A_T and A_C are the respective absorbance of samples with and without polysaccharides.

4. Conclusions

In this study, a green and convenient method for extraction of polysaccharides from *S. horneri* based on DESs was proposed. The studied DESs were composed of choline chloride and 1,2-propanediol. Based on the single-factor experiment results, four experimental factors were systematically evaluated, giving the optimal extraction conditions as follows: molar ratio of choline chloride to 1,2-propanediol of 1:2, water content of 30% (v/v), solid–liquid ratio of 1:30 (g/mL), and the extraction temperature of 70 °C. The extraction yield of *S. horneri* polysaccharides reached 11.31% through the optimal extraction conditions. Additionally, FT-IR and X-ray diffraction were employed to analyze the characteristics of polysaccharides extracted from DESs and compared with the conventional hot water extraction. The polysaccharides extracted from *S. horneri* by DESs had obvious functions of removing proteins and minerals compared with hot water extraction. In vitro antioxidant experiments showed that the

polysaccharides extracted from *S. horneri* had obvious antioxidant activity, and the scavenging ability of DPPH and ABTS radical reached 67.82% and 71.28%, respectively. Therefore, *S. horneri* polysaccharides could be used as a potential natural antioxidant. This study provided a green and simple method for the extraction of polysaccharides in *S. horneri*, and revealed the great potential of DESs in the extraction of biopolymer from natural products.

Author Contributions: Y.L. conceived and designed the experiments, J.N., D.C. performed the experiments, J.N., D.C., Y.L. analyzed the data, J.N., D.C. wrote the paper, Y.L. reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

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