Vietnam Journal of Science and Technology ***56*** *(4A) (2018) 171-181*

EXTRACTION OF POLYSACCHARIDES FROM LINGZHI BY ULTRASONIC-ASSISTED ENZYMATIC METHOD

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Received: 23 July 2018; Accepted for publication: 9 October 2018

ABSTRACT

The medicinal values of polysaccharides (PS) in Lingzhi have been shown to lie in many anti-cancer effects and good benefits for human health. Lingzhi, which is rich in healthy PS, has been used more and more commonly in Viet Nam in recent years. In the present work, ultrasonic-assisted enzymatic extraction (UAEE) was used for extraction of PS from Lingzhi. The experiments were conducted according to a Box-Behnken design (BBD), with four independent variables: extraction temperature, ultrasonic power, pH, and temperature time. The results showed that the best adequate extraction conditions were extraction time of 144 min, extraction temperature of 55 °C, ultrasonic power of 240 W, pH 7.9, and temperature time of 144 min. Under these conditions, the predicted optimal yield was 3.608 %. Whereas by following the optimized condition, the experimental yield of PS was 3.72 ± 0.14 %, which was in good agreement with that of the prediction. Compared to the hot water extraction (HWE) method, ultrasonic-assisted extraction (UAE) method and enzyme-assisted extraction (EAE) method, the yield of PS obtained by UAEE was favorable. The PS yield obtained by HWE, and EAE were 1.96 % and 3.10 %, respectively. These results demonstrated that UAEE was an appropriate and effective extraction of polysaccharides from Lingzhi.

*Keywords:*Lingzhi,ultrasonic-assisted enzymatic extraction, polysaccharides, Box-Behnken design.

1. INTRODUCTION

Lingzhi is a species in the genus of *Ganoderma* that can be found in temperate zones or regions. It has been known as one of the most popular medicinal mushrooms in Viet Nam, China, Japan, and other Asian countries. Recently, it has been found that Lingzhi has plenty of bioactive compounds including triterpenes, polysaccharides (PS), proteins, nucleotides, and metals. Among these components, PS has been identified as one of its major bioactive

components, showing multiple medicinal effects such as anti-proliferation, anti-angiogenesis, anti-HIV, anti-herpetic, and antiviral.

As far as regarded, numerous methods of extraction have been developed with the objective of obtaining extracts with higher yields and lower costs (e.g., enzyme-assisted extraction, ultrasound, microwave, maceration, mechanical rabbling, and heat reflux). Enzyme and ultrasound are the main and most conventional extraction methods for PS. Both enzymes and ultrasound have been shown to be promising and eco-friendly alternatives to the standard chemical systems, leading to an economically viable performance. However, enzyme-assisted extraction (EAE) requires long extraction time. Ultrasonic wave can improve the capability of enzyme. Thus, to achieve a higher polysaccharide yield in shorter extraction time, ultrasonic-assisted enzymatic extraction (UAEE) was used for extraction of PS from Lingzhi. The experiments were conducted according to a Box-Behnken design (BBD).

2. MATERIALS AND METHODS

2.1. Materials

Lingzhi (*Ganodermalucidum*) was provided by Genetic and medicinal plant conservation park, Research Center of Ginseng and Medicinal Materials, district 12, Ho Chi Minh City (Vietnam). The fruiting bodies were harvested and dried in maturity stage with mature spores (Fig. 1), and be stored in closed plastic bag. Pectinex ultra SP-L enzyme was purchased from Brenntag Co., Ltd (Viet Nam). D-glucose was purchased from Sigma-Aldrich Chemical Co., Ltd (USA). Ethanol, butanol, chloroform, phosphate citrate buffer (pH 6-8), and borate buffer (pH 9-10) were purchased from Xilong Scientific Co., Ltd (China).

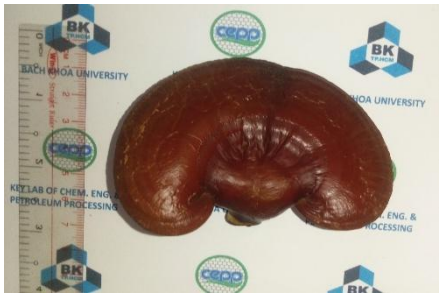

Figure 1. The fruiting body in maturity stage with mature spores.

2.2. Extraction and preparation of crude polysaccharide

2.2.1. Ultrasonic-assisted enzymatic extraction method

Two grams of mushroom powder was extracted with 100 mL Pectinex ultra SP-L enzyme solution (enzyme concentration 0.5 mg/mL). Extraction time, extraction temperature, ultrasonic power, and pH value were set according to the experimental design. Liquid phase (extract) was separated by filtration. After filtration, the solvent was partially removed by vacuum evaporation to one-fifth of the initial volume at 65 ^{o}C. The concentrate was precipitated with the addition of anhydrous ethanol to a final concentration of 80 % (v/v). The mixture was left for 24 h at room temperature. The mixture was then centrifuged (3000 rpm/min, 15 min) to separate the supernatant and the precipitate. The precipitate was

*Table 1.*Independent variables and their levels.

Independent variables	codes	levels		
		-1	0	+1
Time (min)	X_1	40	120	200
Power (W)	X_2	120	360	600
Temperature (^{o}C)	X_3	30	50	70
pH value	X_4	6	8	10

collected and deproteinized by the Sevage reagent (1-butanol/chloroform), and then dried to obtain the crude PS.

2.2.2.Hot water extraction method

Two grams of mushroom powder was wetted by hot water 100 ^{o}C, rate of water to raw material was 1:50 g/mL, with different extraction time (60, 120, 180, 240, and 300 min). Other steps were done similarly to the UAEE method.

2.2.3. Enzyme-assisted extraction method

Survey the PS extraction with extraction temperature and pH value set according to the experimental design, in extraction time of 60, 120, 180, 240, and 300 min. Other steps were done similarly to the UAEE method.

2.3. Experimental design

The single factor experiments were performed to determine the experimental variable ranges. The ranges included extraction time, 40–200 min; temperature, 30–70 °C; ultrasonic power, 120–600 W; pH value, 6–10. On the basis of the single factor experiments, a BBD with four factors and three levels was employed to optimize the parameters. Four independent variables including extraction time, extraction temperature, ultrasonic power, and pH value were designated as X_1, X_2, X_3, and X_4, respectively. The independent variables and their levels were given in Table 1. Twenty seven randomized experimental runs were carried out. The conditions of the variables used in each experimental assay were given in Table 2.

*Table 2.*Factors and levels for RSM, and Box-Behnkendesign with the independent variables.

run	X_1	X_2	X_3	X_4	Y %	
					Experimental	Predicted
1	40 (-1)	30 (-1)	360 (0)	8 (0)	2.26 ± 0.12	2.235
2	120 (0)	70 (+1)	360 (0)	10 (+1)	3.42 ± 0.10	3.472
3	200 (+1)	50 (0)	120 (-1)	8 (0)	3.52 ± 0.19	3.475
4	40 (-1)	50 (0)	600 (+1)	8 (0)	2.34 ± 0.22	2.393
5	40 (-1)	50 (0)	360 (0)	6 (-1)	2.24 ± 0.19	2.305
6	200 (+1)	50 (0)	360 (0)	10 (+1)	3.32 ± 0.15	3.349
7	120 (0)	50 (0)	360 (0)	8 (0)	3.58 ± 0.10	3.556
8	200 (+1)	30 (-1)	360 (0)	8 (0)	2.62 ± 0.15	2.625
9	120 (0)	70 (+1)	360 (0)	6 (-1)	2.83 ± 0.20	2.818
10	120 (0)	50 (0)	360 (0)	8 (0)	3.56 ± 0.10	3.556
11	120 (0)	50 (0)	120 (-1)	10 (+1)	3.48 ± 0.16	3.539
12	120 (0)	30 (-1)	600 (+1)	8 (0)	1.97 ± 0.15	1.983
13	120 (0)	30 (-1)	360 (0)	10 (+1)	2.82 ± 0.13	2.748
14	120 (0)	50 (0)	120 (-1)	6 (-1)	2.95 ± 0.14	2.885
15	120 (0)	30 (-1)	360 (0)	6 (-1)	2.06 ± 0.17	2.094
16	120 (0)	70 (+1)	600 (+1)	8 (0)	3.03 ± 0.15	2.987
17	120 (0)	50 (0)	360 (0)	8 (0)	3.60 ± 0.11	3.556
18	120 (0)	30 (-1)	120 (-1)	8 (0)	2.91 ± 0.12	2.955
19	40 (-1)	70 (+1)	360 (0)	8 (0)	2.99 ± 0.19	2.959
20	120 (0)	70 (+1)	120 (-1)	8 (0)	3.41 ± 0.14	3.399
21	200 (+1)	50 (0)	360 (0)	6 (-1)	2.71 ± 0.11	2.695
22	200 (+1)	50 (0)	600 (+1)	8 (0)	2.67 ± 0.20	2.665
23	40 (-1)	50 (0)	360 (0)	10 (+1)	3.03 ± 0.16	2.959
24	200 (+1)	70 (+1)	360 (0)	8 (0)	3.31 ± 0.13	3.349
25	120 (0)	50 (0)	600 (+1)	10 (+1)	2.84 ± 0.12	2.847
26	120 (0)	50 (0)	600 (+1)	6 (-1)	2.21 ± 0.16	2.193
27	40 (-1)	50 (0)	120 (-1)	8 (0)	2.96 ± 0.18	2.967

The Design expert (Version 11, Stat-Ease Inc., Minneapolis, MN, USA) software was used for the experimental design, data analysis,and model building. All data were expressed as the mean ± standard deviation of three separate experiments.

2.4. Analytical methods

2.4.1. Determination of the PS yield

PS content was estimated using the phenol-sulfuric acid colorimetric method with D-glucose as standard [1] and calculated based on the following formula: $Y\ \% = \frac{C}{W} \times 100$ where *Y* is the yield of PS, *C* is the weight of PS (g) and *W* is the weight of raw material (g).

2.4.2. Scanning electron microscopy (SEM)

The microstructures of Lingzhi were imaged before and after undergoing HWE, EAE, and UAEE methods using environmental scanning electron microscopy at Institute of Applied Materials Science. The images were taken and collected at a magnification of 30x.

2.4.3. FTIR (Fourier-transform infrared spectroscopy) analysis

The PS were ground with KBr powder and pressed into pellets for FT-IR measurement. The frequency range used was 4000 cm^{-1}-400 cm^{-1} to detect functional groups.

3. RESULTS AND DISCUSSION

3.1. Effect of independent variables on extraction yield

3.1.1. Effect of the extraction time on PS yield

Extraction time can significantly affect extract yield. It was reported that a longer extraction time condition had a positive effect on the production of PS [2]. In this study, the extraction time were set at 40, 80, 120, 160, and 200 min to investigate its effect on PS yield when other parameters were set as follows: extraction temperature of 50 °C, ultrasonic power of 360 W, and pH 9. The effect of extraction time on extraction yield was shown in Fig. 2a. The yield of PS increased considerably during the first 120 min while extraction time was longer than 120 min, PS yield decreased. These results could be explained that extended extraction would result in the degradation of the PS [3]. Thus, an extraction time condition of 120min was favorable for the PS extraction. This extraction time was selected as the middle point for response surface model (RSM) experiments.

3.1.2. Effect of the extraction temperature on PS yield

The extraction temperature is an important factor influencing the extraction yield. Liquid temperature affected cavitation behavior, solubility of PS, diffusion coefficient, and the enzyme activity. To investigate the effect of extracting temperature on PS yield, the extraction process was carried out at different extraction temperatures of 30, 40, 50, 60, and 70 ^{o}C, when the other extraction variables were set as follows: extraction time of 120 min, ultrasonic power of 360 W, and pH 9. As shown in Fig. 2b, there was an increasing trend in the yield of PS when the

extraction temperature increased from 30 to 70 °C, reaching a maximum at 50 °C, and then declined when extraction temperature continued to rise. These results were in agreement with the results as reported by Sisak et al. [4], Duvetter et al. [5] for commercial pectinase samples. Therefore, the variable temperature range used in the RSM experiments was 30–70 °C.

3.1.3. Effect of the ultrasonic power on PS yield

Figure 2c showed the effect of different ultrasonic conditions (120, 240, 360, 480, 600 W) on PS yield while the other extraction parameters were set as follows: extraction time of 120 min, extraction temperature of 50 °C, and pH 9. As ultrasonic power increased from 120 W to 240 W, PS yield increased, reaching a maximum value at 240 W. When ultrasonic powers were higher than 240 W, PS yield declined. An explanation for this phenomenon was that suitable ultrasonic wave could improve mass transfer, which potentiated an increasing delivery of the substrate to the active sites of the enzyme [6-8], also could facilitate the cell wall destruction of target sample. However, higher ultrasonic power could inactivate enzyme and weaken the cavitation effect [9, 10]. Therefore, the variable range of power used in the RSM experiments was 120–600 W.

3.1.4. Effect of the pH on PS yield

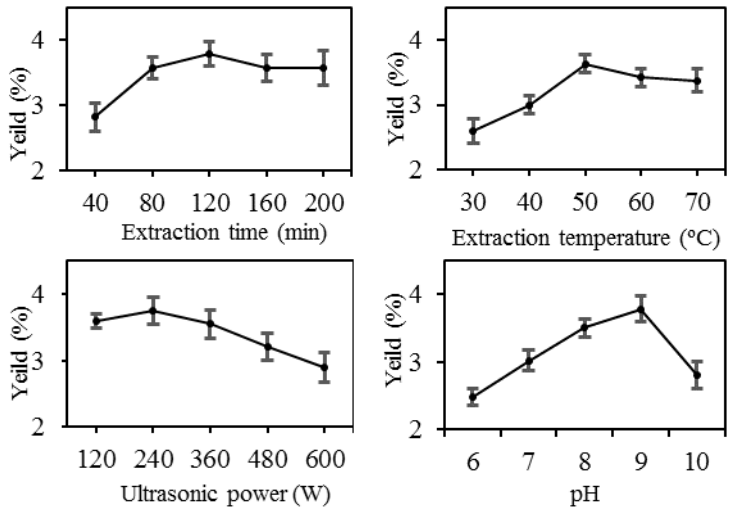

Figure 2. Effects of extraction time (a), extraction temperature (b), ultrasonic power (c), and pH (d) on the yield of PS (%).

The pH of solutions may have great influences on the activities of different enzymes and their conformations [11]. When pH of a particular medium changes, it leads to an alteration in the shape of the enzyme. Besides enzymes, the pH level may also affect the charge and shape of the substrate as well. To investigate the effect of different pH conditions on the yield of PS, the extraction process was performed in 120 min under different pH conditions from 6 to 10 at 50 °C, and ultrasonic power of 240 W. Figure 2d revealed that the extraction yield rose together with the increase of pH values from 6 to 9 and peaked at 9. This finding wasin agreement with the outcomes of Ghazi et al. [12]. The extraction yield significantly decreased when the pH was further increased. Thus, the variable pH range used in the RSM experiments was 6–10.

3.2. Optimization of extraction conditions by BBD

3.2.1. Predicted model and statistical analysis

On the basis of the single factor experiments results, a total of 27 runs was performed for optimizing these three variables in the current BBD. Via multiple regression analysis on the experimental data, the predicted response on PS yield and the test variables were related by the second-order polynomial equation given by the following expression:

$$Y = 3.556 + 0.195X_1 + 0.362X_2 - 0.346X_3 + 0.327X_4 - 0.059X_1X_3 + 0.140X_2X_3 - 0.360X_1^2 - 0.404X_2^2 - 0.321X_3^2 - 0.369X_4^2$$

Statistical analysis of each coefficient was checked by F-test and p-value, and the analysis of variance (ANOVA) for the RSM. The ANOVA of quadratic regression model demonstrated that the model was highly significant and had a good fit of the model, evident from the Fisher's F-test with a very high model F-value (209.13) but a very low p-value ($p < 0.0001$). The goodness-of-fit of the model was also evaluated by the determination coefficient ($R^2 = 0.9959$), indicated that 99.59 % of the variations could be explained by the fitted and adjusted determination coefficient (Adj-$R^2 = 0.9912$). This figure suggested that the total variation of 99.12 % for PS yield was attributed to the independent variables and only about 0.88 % of the total variation could not be explained by the model. In addition, Pre-R_2 is 0.979, which was smaller and very close to Adj-R^2 (Adj-R^2 – Pre-$R^2 < 0.2$), indicating a high rate of correlation between the observed and predicted data from the regression model [13]. A fairly low coefficient variation value (C.V. %) of 1.59 % (< 5.00 %) for the extraction yield represented the dispersion degree between predicted and observed values, which indicated that the model was reproducible. The linear coefficient X_1, X_2, X_3, X_4; the quadratic coefficients X_1^2, X_2^2, X_3^2, X_4^2; and the products of the coefficients X_2X_3, X_1X_3 had significant effects on PS yield ($p < 0.05$). The effects of the other coefficients on PS yield were not significant ($p > 0.05$).

3.2.2. The interaction between the variables

To visualize the combined effects of two operational parameters on the extraction yield, the response was generated as a function of two independent variables: triaxial response surfaces and planar contour plots, and was determined using Design Expert software. The response surfaces for the effect of independent variables on average extraction efficiency of PS were showed in Fig. 3. Each figure showed the simultaneous effects of two factors on the PS yield while all other factors were kept at zero level. Different shapes of the contour plots indicated different interactions between the variables. A circular contour plot indicated that the interaction among related variables was insignificant, while elliptical contour suggested the interaction among related variables was significant. Figure 3a showed the PS yield (Y) increased rapidly when rate of extraction time (X_1) to extraction temperature (X_2) increased in the range of 40–120 min and 30–50 °C, respectively. But beyond 120min and 50 °C, extraction yield (Y) decreased slightly. The circular contour plot (Fig. 3A) indicated that the mutual interactions between extraction time (X_1) and extraction temperature (X_2) were insignificant. Similar trends were observed when investigating the effects of extraction time and ultrasonic power (Fig. 3b, Fig. 3B), of extraction time and pH (Fig. 3c, Fig. 3C), of ultrasonicpower and extraction temperature (Fig. 3d, Fig. 3D), of pH and extraction temperature (Fig. 3e, Fig. 3E), and of pH and ultrasonic power (Fig. 3f, Fig. 3F).

3.2.3. Optimum conditions for extraction of PS

By employing the software Design-Expert, the predicted optimum extraction conditions included extraction time of 143.94 min, extraction temperature of 54.64 °C, ultrasonic powerof 248.01 W, and pH 7.9. Under the optimal conditions, the predicted maximum yield of PS was 3.608 %. However, to meet the operability of the actual conditions, we modified predicted optimum extraction conditions as follows: extraction time of 144 min, extraction temperature of 55 °C, ultrasonic powerof 240 W, and pH 7.9. Under these conditions, the experimental yield of PS was 3.72 ± 0.14 %, which was in good agreement with the prediction of 3.608 %.

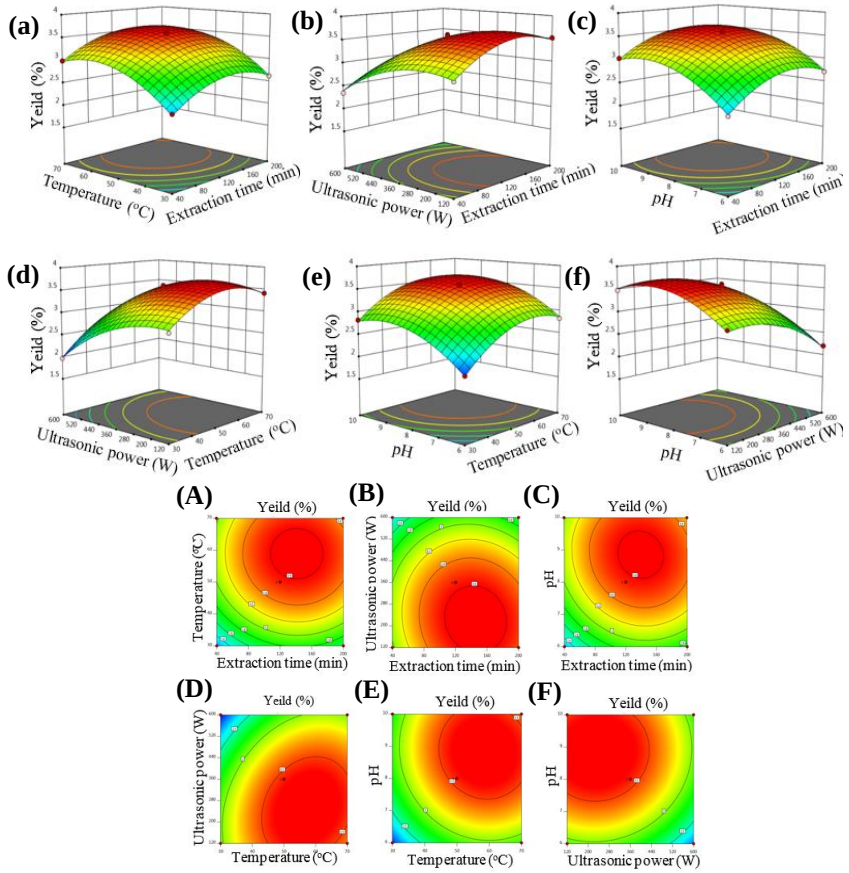

Figure 3. Response surface plots showing the effects of variables on average extraction efficiency of target compounds. (a) time and temperature; (b) time and ultrasonic power; (c) time and pH; (d) temperature and ultrasonic power; (e) temperature and pH; (f) pH and ultrasonic power.

3.2.4. Comparison of UAEE extraction with HWE, EAE under the optimal conditions

The efficiency of the PS yield by UAEE and other extraction methods were compared. The results illustrated in Fig. 4 revealed that the maximum yield of PS obtained by HWE was 1.96 ± 0.14 % at 100 ^{o}C, 180 min. The EAE gave 3.10 ± 0.13 % PS extraction when the enzyme concentration of 0.5 mg/mL was used at 50 ^{o}C in 240 min. Compared with EAE and HWE, the application of UAEE affected positively on the yield of PS obtained (3.72 ± 0.14 %). Not only an increase in the yield wasachieved, but also a significant reduction in the extraction time and temperature was recorded (50 ^{o}C, 144 min). This result proved that UAEE could be an appropriate and effective technique to extract PS from Lingzhi.

3.3. Preliminary characterization of PS

3.3.1. Scanning electron microscopy image analysis

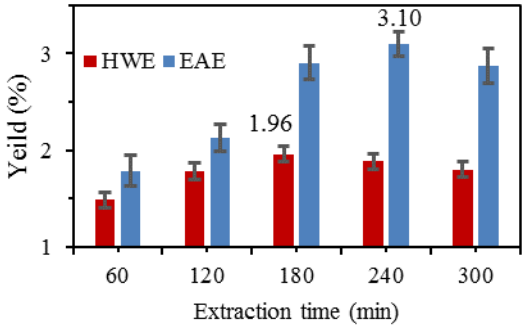

Figure 4. Effect of extraction time on the extraction yield of PS.

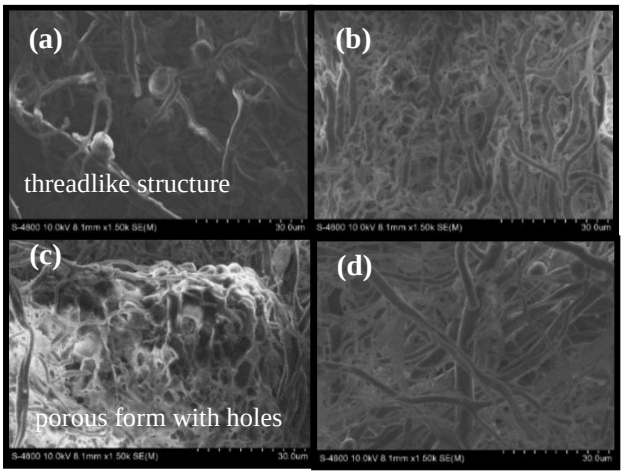

Figure 5. SEM micrographs showed the structure of mushroom before treatment (a), after treatment with HWE (b), EAE (c) and UAEE (d).

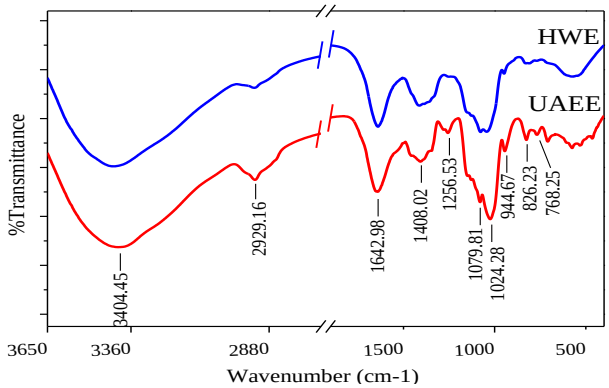

Figure 6. FTIR spectra of PS obtained using HWE and UAEE.

Surface features of the samples in different extraction methods were analyzed with SEM in Fig. 5. In Fig. 5a, the sample was observed before subjecting to any treatment. The cell wall had threadlike structure, composed of proteins, glucans, chitin fibers arranged in a certain order and connected together [14]. After treatment with HWE, as shown in Fig. 5b and compared with the intact structure, the sample structure was drastically changed due to the hydrolysis of hemicelloluse [15]. Meanwhile Fig. 5c of EAE treatment showed the slight deformation in the structure that had porous form. A possible explanation for this phenomenon was that oligosaccharides, phospholipids, proteins in the cell wall were susceptible to degradation by fructosyltransferase and protease activity in *Aspergillus*-derived enzymes [12, 16]. However, structure of fiber order and spaces that were not observed in HWE and UAEE treatment. Fig. 5d showed the structures in UAEE treatment changed similar to HWE treatment, the presence of fiber significantly decreased in that ultrasonic wave could improve mass transfer [8-10], also could destroy the structure of mushroom [17]. Thus, UAEE treatment enhanced the mass transfer rate of active ingredients.

3.3.2. FTIR analysis

The FTIR absorption spectra of the products wereshown in Fig.6. The FT-IR spectra of these PS fractions from HWE and UAEE had a strong and wide absorption band of approximately 3100–3700 cm^{-1} for O–H [18,19] stretching vibrations. The existence of a weak stretching vibration at 2929 cm^{-1} was attributed to the presence of saturated bonds of C–H [18-20]. These two absorption bands were characteristic absorptions of PS [21]. The absorption at 1642 cm^{-1} of C=O stretching vibration indicated that carbonyl groups were present in protein or PS-protein complex. The relatively weak absorption peaks at 1408 cm^{-1} might represent the deforming vibrations of C–H bond [20, 22]. The peak at 1256 cm^{-1} was unsymmetrical carbonyl stretching in the spectrum of UAEE exclusively [23]. The absorption peak at 1024 cm^{-1} was typical of glycogen [24]. The other bands obtained at 1079 and 944 cm^{-1} were attributed to galactose [19], and glucose [25], respectively. The absorption peak at 826 and 768 cm^{-1} might be related to the existence of fructose [25].

4. CONCLUSIONS

In this study, all the statistical indicators supported that response surface model was a successful tool to describe the ultrasonic process in extracting polysaccharides from Lingzhi. The optimal parameters of the polysaccharide extraction by ultrasonic-assisted enzymatic method were: extraction time of 144min, extraction temperature of 55 °C, ultrasonic power of 240 W, and pH 7.9. Under these conditions, the maximum experimental yield of 3.72 ± 0.14 % was achieved, which was consistent with the predicted value of 3.608 % and indicated the adequacy of response surface model to reflect the optimized extraction conditions. Compared with hot water extraction and enzyme-assisted extraction, ultrasonic-assisted enzymatic method gave higher polysaccharides yields in shorter extraction time. Furthermore, the polysaccharides obtained by ultrasonic-assisted enzymatic extraction method contained glucose, galactose, fructose, and protein groups in different proportions. These results demonstrated that ultrasonic-assisted enzymatic extraction method was an appropriate and effective extraction technique for polysaccharides from Lingzhi.

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