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Ultrasound enhanced production and antioxidant activity of polysaccharides from mycelial fermentation of *Phellinus igniarius*

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ABSTRACT

In this study, a novel flat-plate ultrasound technology was developed to stimulate polysaccharides production from *Phellinus igniarius* mycelial fermentation. Three-factor-three-level Box–Behnken design was used to optimize ultrasonic treatment time, duty cycle time and culture time for a high yield of *P. igniarius* polysaccharides (PIPS). Optimal conditions were found to be ultrasound treatment time 65 min, duty cycle time 25 s, and culture time 3.8 d that gave a maximum PIPS yield of 1.8002 g/L, which increased ~22.64% compared with the control (without any ultrasound). PIPS mainly contained low-molecular weight (MW) polysaccharides (3.1 kDa, 80%) composed of glucose, rhamnose and mannose in a molar ratio of 11.0:14.0:1.0. PIPS with higher carbohydrate and uronic acid contents exhibited strong antioxidant activities *in vitro*. Laser scanning confocal microscope (LSCM) observations suggested that ultrasound could change the morphology and structure of *P. igniarius* mycelium, and accelerate the transfer of nutrients and metabolites.

1. Introduction

Phellinus igniarius (L.: Fr.) Quél *(P. igniarius)*, which is a basidiomycetous fungus belonging to the *Hymenochaetaceae basidiomycetes*, is one of the most well-known medicinal mushrooms in traditional Chinese medicine (Liu et al., 2006; Lung, Tsai, & Huang, 2010; Mo, Yang, & Shi, 2003; Shon & Nam, 2004; Song, Liu, Yang, & Hu, 2008). *P. igniarius* has been widely used in China, Japan, and Korea for many years. Nowadays the medicinal value of *P. igniarius* had gained worldwide attention and attracted great research effort towards the scientific rediscovery of this traditional remedy. Polysaccharides are regarded as the major bioactive constituents of *P. igniarius* and shown a broad spectrum of health effects and pharmacological activities, such as antitumor, immunomodulatory, antioxidant and other medicinal properties (He et al., 2012; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Zhang, Cui, Cheung, & Wang, 2007). Because of the limited and unstable supply of wild *P. igniarius*, cultivation of fungal mycelia or mushrooms (in fruit form) by solid and submerged fermentation has been the major source of fungal materials. Specifically, liquid or submerged fermentation is a more favorable and efficient process than solid-state fermentation for the production of mycelial biomass and bioactive compounds including polysaccharides (Seviour, McNeil, Fazenda, & Harvey, 2011; Zhong & Tang, 2004). Therefore, the development of novel, highly efficient strategies for polysaccharides production by a *P. igniarius* submerged fungal culture continues to a subject of research interest.

Recently, high-intensity ultrasound was often used as an inexpensive, reproducible, simple and efficient alternative method of industrial relevance to increase the productivity of biological processes involving live cells and bioactive enzymes (Chisti, 2003; Kwiatkowska, Bennett, Akunna, Walker, & Bremner, 2011). Most previous studies have demonstrated that ultrasonic treatment played an important role in stimulating the production of valuable products in bacteria, yeast, filamentous fungi, and plant cell cultures (Herran, Lopez, Perez, & Chisti, 2008; Liu, Li, Sun, & Liu, 2012; Runyan et al., 2006; Sulaiman, Ajit, Yunus, & Chisti, 2011). For example, high-intensity ultrasound (20 kHz) had a positive influence on carbohydrate metalism in milk fermentation and stimulated the production of major organic acids in the later stage of the milk fermentation by *Bifidobacterium lactis*, *Bifidobacterium breve* ATCC 15700, and *Bifidobacterium infantis* (Nguyen, Lee, & Zhou, 2012).

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Furthermore, an efficient intermittent high-intensity ultrasonic treatment was proven to significantly improve laccase production from *Trametes versicolor* mycelia cultures (Wang, Ma, Guo, Zhuang, & Liu, 2013). Herran et al. (2008) indicated that sonication at any power of ultrasound did not affect biomass growth profiles but medium and high intensity reduced lovastatin production growth morphology modification and fungus pellets disruption which caused the biomass grow mainly as dispersed hyphae. The overall conclusion was that ultrasound could be used to alter morphology and broth rheology without affecting growth of filamentous fungi. Sonication appeared to influence primary and secondary growth metabolism differently depending on the conditions. However, to the best of our knowledge, little or no efforts are available regarding the improvement of bioactive polysaccharides production from a submerged fungal culture by ultrasonic stimulation.

Therefore, in the present study, an efficient intermittent high-intensity ultrasound (68 kHz) strategy was employed to improve polysaccharides production from a *P. igniarius* mycelial fermentation. Ultrasonic treatment conditions for the high production of *P. igniarius* polysaccharides were designed and optimized using a statistical experimental design and response surface methodology (RSM). The effects of ultrasonic treatment on physicochemical properties and antioxidant activity of polysaccharides from cultured *P. igniarius* mycelia were investigated. In addition, the possible mechanism of ultrasonic stimulation on *P. igniarius* mycelial fermentation was also discussed in this paper.

2. Materials and methods

2.1. Materials and chemicals

P. igniarius NO.5.95 was purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China) and grown in solid slant medium, potato dextrose agar (PDA) slant was incubated at 26 °C. This strain was maintained at 4 °C after subculturing at 26 °C every 3–4 weeks on potato dextrose agar (PDA) slants. Monosaccharide standards, hydrogen peroxide (H_2O_2), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 2,4,6-tris (2-pyridyl)-*s*-triazine were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were of laboratory grade and used without further purification.

2.2. Culture media and conditions

The *P. igniarius* strain was initially incubated at 26 °C on PDA slants. After one week, five mycelia sections (~5 × 5 mm) were excised from the slant and used to inoculate the seed culture medium. The seed culture was grown in a 250 mL flask containing 100 mL of potato dextrose broth at 26 °C on a rotary shaker incubator (130 rev/min) for 8 d, and then the cultured broth was gently homogenized. The flask fermentation culture experiments were performed in a 250 mL flask containing 100 mL of medium inoculated with 10% (v/v) of the seed culture. Fermentation medium consisted of the following components (g/L): maize flour (50), bran (15), mulberry shoot powder (10), KH_2PO_4 (2), $MgSO_4$ (1). All the culture in flasks was carried out on a rotary shaker at 130 rev/min, 26 °C.

2.3. Ultrasonic treatment

A flat plate ultrasonic reactor equipment (Fig. 1) designed by our laboratory was employed in this study. The major units of the equipment were made up of a flat plate ultrasonic generator put in a water-tank and a frequency and power controller. When ultrasonic

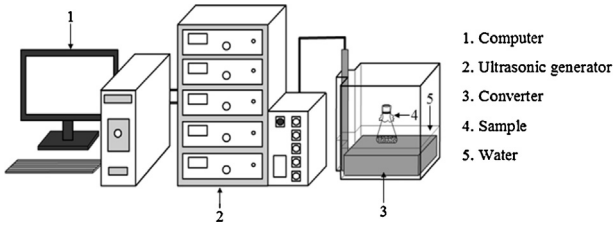

Fig. 1. Schematic diagram of ultrasonic treatment during *P. igniarius* NO.5.95 mycelium fermentation.

treatment was conducted, the flasks were set on the center of flat plate ultrasonic reactor, the water in water-tank and the medium in the flask in the same horizontal plane. The water temperature in the ultrasonic bath was controlled at 26 °C. The ultrasonic irradiation frequency and power were set at 68 kHz and 600 W, respectively. The ultrasonic stimulation was supplied using the duty cycle time, which consisted of working time and fixed resting time (5 s). To test effect of ultrasonic treatment, the *P. igniarius* mycelial pellets in flasks were exposed to different ultrasonic treatment time after they had been cultured for different growth stages (day 1–6). After ultrasonic treatments, the flasks with the mycelial pellets were returned to the shaker for continued cultivation. Samples without any ultrasonic treatment were used as the control. All experiments were conducted in triplicate.

2.4. Extraction and isolation of polysaccharides from P. igniarius mycelia

After fermentation under ultrasonic stimulation, the mycelia broth of *P. igniarius* was separated from the liquid medium (supernatant) and the mycelia (precipitate) by centrifugation. The mycelia were washed by distilled water for thrice and then freeze-dried. The dried mycelia were grinded into powder, weighed and packaged in plastic zipper bags before use. For extraction of polysaccharides, in brief, the pretreated mycelium powder was extracted with distilled water in a solid to solvent ratio of 1:10 to reflux at 95 °C thrice for 8 h. The supernatant was collected by centrifugation (5000 rpm, 10 min), was concentrated by evaporation under reduced pressure, and was precipitated with 4 volumes of 95% ethanol at 4 °C overnight. Most of separated proteins were removed by using a Sevag method (Staub, 1956), and were dialyzed (MWCO 8000–12,000 Da, USA) against distilled water for 48 h, and were freeze dried to yield partially purified polysaccharides, designated as PIPS. PIPS yield was represented by the dry mass of PIPS per volume of the cultured broth in g/L. On the other hand, polysaccharides isolated from *P. igniarius* mycelial fermentation without ultrasonic stimulation was performed as control experiment based on above method, namely as C-PIPS.

2.5. Experimental design and data analysis

Three conditions were selected as the experimental variables based on our preliminary tests, ultrasonic treatment time (X_1, min), duty cycle time (X_2, s) and culture time (X_3, d). These conditions were initially analyzed by a single-factor test of their individual effects on the PIPS yield. Results (data not shown) showed that the optimal conditions were selected in the range of 30–90 min, 20–30 s, and 2–6 d, respectively, for the following optimization experiments by statistical design. The optimization experiments were performed with a 3^3 (three-factor-three-level) factorial Box–Behnken design (BBD) and PIPS yield as the response value. The 3^3 BBD was composed of 17 experimental trials including 12 factorial points and 5 axial points, and obtained results are summarized in Table 1. Five replicates at the center point were

Table 1
Box–Behnken design matrix and the response values for PIPS yield.

Run numbers	Coded variable levels			PIPS yield (g/L)
	X_1 ultrasonic treatment time (min)	X_2 duty cycle time (s)	X_3 culture time (d)	
1	30	20	4	1.6206
2	90	30	4	1.6867
3	30	30	4	1.6006
4	90	20	4	1.6304
5	30	25	2	1.5215
6	90	25	2	1.5833
7	30	25	6	1.4948
8	90	25	6	1.5048
9	60	20	2	1.5733
10	60	30	2	1.5243
11	60	20	6	1.4501
12	60	30	6	1.4709
13	60	25	4	1.7925
14	60	25	4	1.7812
15	60	25	4	1.8116
16	60	25	4	1.8008
17	60	25	4	1.7913

included to estimate the pure error sum of squares. The response value in each trial was the average of triplicates ($n = 3$). Regression analysis was performed of the experimental data from the BBD to find the correlation of the response function to the variables by the following 2nd order polynomial model,

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=1}^{3} A_{i,j} X_i X_j \quad (1)$$

where Y is the response value (PIPS yield, g/L), A_0, A_i, A_{ii}, and A_{ij} the coefficients of variables for the intercept, linear, quadratic and interactive terms, respectively ($i \neq j$) (Box, Hunter & Hunter, 1978; Dean & Voss, 1999; Henriques, Jessouroun, Lima, & Alves, 2006).

The actual experimental variables (X_i) were converted to coded variables (x_i) by $x_i = (X_i - X_0)/\Delta X_i$, where X_0 is the actual value of X_i at the center point and ΔX_i is the incremental step. The model coefficients were derived by multiple nonlinear regressions of experimental data. The analysis of variance (ANOVA) was performed to examine the goodness of model fit to the experimental data and the statistical significance of all terms in the model equation. The optimal values of variables and response surface contour plots were computed from the model equation. All regression and statistical parameters were calculated according to software package Design-Expert (Stat-Ease, Inc., Minneapolis, USA).

2.6. Physicochemical properties and compositions of polysaccharides

All analytical methods mentioned in this section have been described detailedly in our previous work (Wang, Pei, Ma, Cai, & Yan, 2014). The total carbohydrate content, protein content and uronic acid content of polysaccharides were determined by phenol – sulfuric acid method using D-glucose as a reference, Bradford method using bovine serum albumin (BSA) as a reference, and sulfuric acid – carbazole method using glucuronic acid as a reference, respectively. Molecular weight (MW) and molecular weight distribution (MWD) were investigated by high pressure gel permeation chromatography (HPGPC) with the detailed instruments and conditions as reported in Wang et al. (2014). Dextran MW standards ranging from 5.2 kDa to 1482 kDa (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were used for calibration. Data were collected and analyzed by the online Breeze software package (Waters Corp., Milford, MA, USA). The monosaccharide compositions of

Table 2
Regression coefficient estimation and their significant test for the quadratic polynomial model of the yield of the polysaccharides.

Source	Sum of squares	DF[a]	Mean square	F-value	p-Value
X_1	0.003515	1	0.003515	16.22	0.0050
X_2	0.001365	1	0.001365	6.30	0.0404
X_3	0.009926	1	0.009926	45.79	0.0003
X_1^2	0.020	1	0.020	94.43	<0.0001
X_2^2	0.035	1	0.035	161.47	<0.0001
X_3^2	0.17	1	0.17	774.22	<0.0001
X_1X_2	0.0003294	1	0.0003294	1.52	0.2575
X_1X_3	0.0006708	1	0.0006708	3.09	0.1220
X_2X_3	0.001218	1	0.001218	5.62	0.0496
Model	0.26	9	0.029	133.14	<0.0001
Residual	0.001517	7	0.000217		
Lack of fit	0.000999	3	0.000333	2.57	0.1921
Pure error	0.000518	4	0.00013		
Cor. total	0.26	16			
			$R^2 = 0.9942$	$R^2_{adj} = 0.9867$	CV = 0.91

[a] DF, degree of freedom.

polysaccharides were analyzed by combination of acid hydrolysis and gas chromatography (GC) on an Agilent 7890A instrument (HP-5 fused-silica capillary column, 30 m × 0.25 mm × 1 μm) with the conditions as reported before (Yan, Li, Wang, & Wu, 2010). D-Arabinose (D-Ara), D-glucose (D-Glc), D-galactose (D-Gal), D-mannose (D-Man), L-rhamnose (L-Rha), and D-xylose (D-Xyl) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) were used as monosaccharide standards. FTIR spectrum was determined using a Nexus 670 FTIR spectrometer (Thermo Nicolet Co., USA) in the wavenumber range from 500 to 4000 cm^{-1} with KBr pellets and referenced against air.

2.7. In vitro antioxidant activity

In this work, the hydroxyl radical scavenging activity, Trolox equivalent antioxidant capacity (TEAC) assay, and ferric reducing ability of plasma (FRAP) assay were used to evaluate the *in vitro* antioxidant activities of polysaccharides. All treatments and activity assays were performed in triplicate and the results were represented by their mean ± standard deviation (SD).

For the adapted hydroxyl radical method, the polysaccharides were previously dissolved at different concentrations (0.05–1.5 mg/mL) in distilled water. A volume of 0.2 mL of the tested solution (sample or control) was added into an equal volume of an aqueous solution of 5 mM $FeSO_4$. A volume of 0.2 mL of 1% (v/v) H_2O_2 was then added into the mixture under continuous stirring. After stirring and then incubation at room temperature for 60 min, the absorbance of the resulting solution was measured at 510 nm using a UV-1600 Spectrophotometer (Beijing Ruili Analytical Instrument Co., Ltd., China). The hydroxyl radical scavenging activity (%) was determined using the following formula: scavenging activity (%) = $(1 - A/A_0) \times 100\%$, where A, and A_0 are the absorbance values of the sample and blank control (distilled water), respectively. Vitamin C (V_c) was used as a positive antioxidant reference.

Concerning the TEAC assay, the TEAC of polysaccharides was tested against $ABTS^{\cdot+}$ radical, which was generated from the oxidation of ABTS by potassium persulphate. The TEAC values of the samples were derived from the calibration curve generated with Trolox as a reference. On the other hand, in the FRAP assay, ferric to ferrous ion reduction at low pH led to the formation of a colored ferrous-tripyridyltriazine complex, and the FRAP values of the samples were obtained by calibration with ferrous sulphate. Details of the operating conditions and methods were described in details before (Leung, Zhao, Ho, & Wu, 2009).

2.8. Laser scanning confocal microscope (LSCM) observation

For the morphological observations, *P. igniarius* mycelium was initially cultured in 250 mL flask for 4 d and the detailed method was described in Section 2.2. Then three samples were prepared as follows: one flask was as the control without ultrasonic treatment; the other two flasks were treated with ultrasonic stimulation (68 kHz, 600 W, 25 s, and 26 °C) for 60 min and 120 min, respectively. After fermentation, mycelia were separated from the culture broth by centrifugation, and washed three times with sterile distilled water at room temperature. The morphology of the mycelia was observed using a laser scanning confocal microscope (LSCM, FV1000, Olympus, Tokoy, Japan).

3. Results and discussion

3.1. Optimization of ultrasonic conditions by RSM

3.1.1. Statistical analysis and the model fitting

In this work, a 3^3 BBD was used to set up the optimization experiments on the three major conditions including ultrasonic treatment time (X_1, min), duty cycle time (X_2, s) and culture time (X_3, d). Table 1 shows the experimental conditions by BBD and the experimental results, with the PIPS yields 1.4501 g/L to 1.8116 g/L. The maximum PIPS yield was attained at ultrasonic treatment time 60 min, duty cycle time 25 s, and culture time 4 d. The following second-order polynomial model was derived by regression of the experimental data with the model Eq. (2),

$$Y = 1.80 + 0.021X_1 - 0.013X_2 - 0.035X_3 - 0.0091X_1X_2 - 0.013X_1X_3 + 0.017X_2X_3 - 0.070X_1^2 - 0.091X_2^2 - 0.20X_3^2 \quad (2)$$

As shown by the ANOVA data for the regression model (Table 2), the close-to-unit R^2 value 0.9942 and the high F-value 133.14 at $p < 0.0001$ the after of model are both indicative of the high correlation of the model to the experimental results. The F-value 2.57 and p-value 0.1921 for lack of fit implies that it is not significant relative to the pure error, which indicates that the model equation is adequate for predicting the yield of PIPS under any combination of values of the variables. Moreover, the low coefficient of variation (C.V.) 0.91 suggests a high reliability of the model for predication of experimental values. Table 2 also shows the statistical parameters for the coefficients of model variables in Eq. (2) above. A smaller p-value means a higher significance level of the corresponding coefficient (Box et al., 1978; Dean & Voss, 1999). According to this rule, the coefficients for the linear terms (X_1, X_2 and X_3), the quadratic terms (X_1^2, X_2^2 and X_3^2) and the interactive term ($X_2 \times X_3$) of the polynomial model are significant with a small $p < 0.05$, but the interactive term coefficients ($X_1 \times X_2$ and $X_1 \times X_3$) are insignificant ($p > 0.05$). The results indicated that ultrasonic treatment time, duty cycle time and culture time were all significantly correlated with the PIPS yield. Moreover, the interaction between duty cycle time and culture time was significant, while interaction between any other two conditions was insignificant.

3.1.2. Response surface and contour plots

Fig. 2 presents the 3D response surface and 2D contour plots with data computed from the model equation (Eq. (2)). At a fixed culture time (3D plot in Fig. 2a), the PIPS yield increased with the increase of ultrasonic treatment time from 30 to 65 min, and then dropped with a longer treatment time than 65 min. At a fixed duty cycle time (3D plot in Fig. 2b), the PIPS yield increased with culture time changing from 2 to 3.8 d, and then decreased with culture time from 3.8 to 6 d. At a fixed ultrasonic treatment time (3D plot in Fig. 2c), the PIPS yield increased gradually with the duty cycle time till 24.5 s, but decreased slightly with a longer duty cycle time. It was concluded that the optimal conditions for the PIPS production under high-intensity ultrasound (68 kHz) were 65 min ultrasonic treatment time, 24.5 s duty cycle time, and 3.8 d culture time. Culture time was the most significant variable affecting the PIPS yield, followed by ultrasonic treatment time and then duty cycle time. The interactive effect between two independent variables (with the 3rd variable fixed at zero level or the center point) can be observed from the 2D-contour plots. The duty cycle time (X_2) and culture time (X_3) had a significant interaction as indicated by the elliptical contours (Fig. 2c) and a relative insignificant interaction as indicated by the nearly circular contours, which is in agreement with the above model analysis.

3.1.3. Verification of model and optimal conditions

In order to validate the adequacy of model equation, Eq. (2), and obtain the maximum yield of PIPS from *P. igniarius* mycelial fermentation under ultrasonic treatment, a verification experiment was carried out under the optimal conditions, *i.e.* ultrasound treatment time, 64.95 min, duty cycle time 24.55 s, and culture time 3.80 d. Under the conditions, the yield of PIPS was 1.7995 g/L. However, taking account of the operating convenience, the optimal conditions can be modified as follows: ultrasound treatment time 65 min, duty cycle time 25 s, and culture time 3.8 d. Under the modified conditions, the experimental yield of 1.8002 g/L ($N = 3$), which was close to the predicted value. The close agreement between model-predicted and experimental PIPS yield is a satisfactory verification of the response model and the optimal conditions for the precipitation process. The result also showed the yield of PIPS increased about 22.64% compared with the control C-PIPS (1.4679 g, $N = 3$).

3.2. Effects of ultrasonic treatment on physicochemical properties of polysaccharides

Under ultrasonic treatment at the given frequency (68 kHz) and power (600 W), the preliminary properties and chemical composition of PIPS was different from that of the control, C-PIPS, and the obtained results were summarized in Table 3. It has been reported that ultrasonic treatments can improve metabolite production by increasing the release of intracellular products (Chu, Zhang, Zhuang, Chen, & Li, 2002; Dai, Wang, Duan, & Sakanishi, 2003). In this study, the carbohydrate content of PIPS (82.77%) was slightly higher than C-PIPS's (79.58%), which verified that ultrasonic treatment was beneficial for the production of polysaccharides during fermentation process. Similarly, PIPS contained the higher content of uronic acid (14.76%), while C-PIPS contained the lower content of uronic acid (13.44%). In our previous studies, it was found that there was a direct relationship between the uronic acid content with the free-radical scavenging capacities of *P. linteus* polysaccharides (Wang et al., 2014). The higher uronic acid content may indicate the higher antioxidant activities of polysaccharide PIPS. In addition, both PIPS and C-PIPS contained the smaller amount of protein.

The monosaccharide compositions of PIPS and C-PIPS were analyzed by GC and the results were listed in Table 3. According to the monosaccharide standards, PIPS mainly comprised D-Glc, L-Rha and D-Man with a molar ratio of 11.0:14.0:1.0, and C-PIPS comprised D-Glc, L-Rha and D-Man with a molar ratio of 9.0:3.0:1.0. The result indicated that D-Glc and L-Rha are two predominant monosaccharides in PIPS structure and their contents showed an increase under ultrasonic irradiation. More specifically, the L-Rha content in PIPS was much higher than that of the control polysaccharide C-PIPS. As reported in literature and our results, ultrasonic treatment on microorganism fermentation could not only accelerate mycelium growth, but also promoted the secondary metabolite biosynthesize. After ultrasonic irradiation, the cells may be hurt

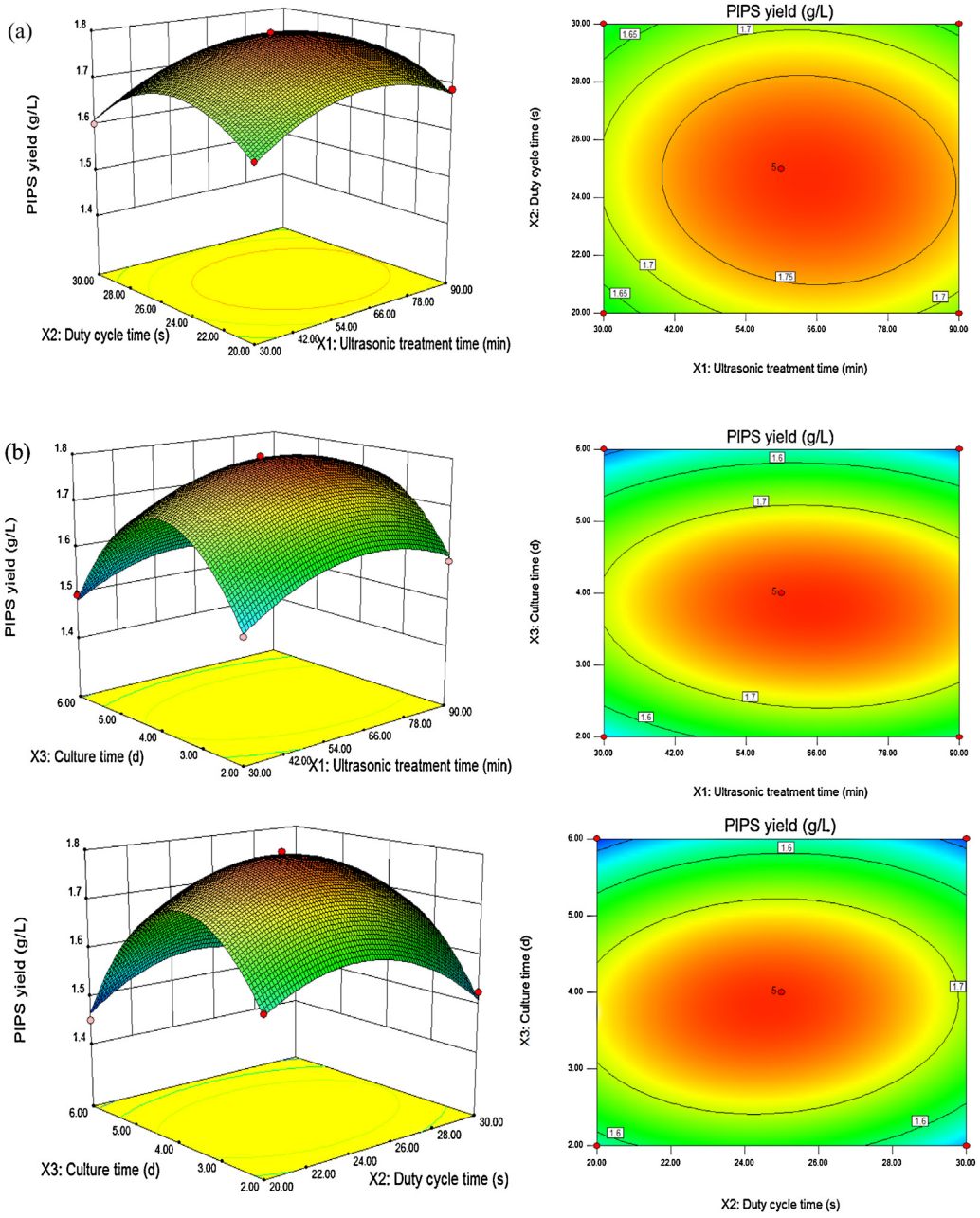

Fig. 2. Response surface plots and contour plots showing (a) the effects of ultrasonic treatment time (X_1, min) and duty cycle time (X_2, s) on the yield of PIPS (Y, g/L); (b) the effects of ultrasonic treatment time (X_1, min) and culture time (X_3, d) on the yield of PIPS (Y, g/L); and (c) the effects of duty cycle time (X_2, s) and culture time (X_3, d) on the yield of PIPS (Y, g/L).

in some ways and make cell membrane flaw, which accelerated all sorts of nutrition or molecule transport between cell membrane (Dai et al., 2003; Matsuura, Hirotsune, & Nunokawa, 1994). As a result, ultrasonic treatment strengthened the ability of cell metabolism in the fermentation process, and improved the contents of glucose and rhamnose.

HPGPC was employed to determine MWs and MWDs of PIPS and C-PISPS and the results were displayed in Table 3 and Fig. 3a.

Table 3
Preliminary properties and chemical compositions of polysaccharides extracted from cultured *P. igniarius* mycelia.

Sample	Carbohydrate (wt%)	Protein (wt%)	Uronic acid (wt%)	Sugar composition (mol%)			MW (kDa)	Area (%)
				D-Glc	D-Man	L-Rha		
C-PIPS	79.58 ± 0.39	2.76 ± 0.64	13.44 ± 0.18	9.0	1.0	3.0	≥25,400	68
							3.8	32
PIPS	82.77 ± 0.73	1.69 ± 0.49	14.76 ± 0.63	11.0	1.0	14.0	≥21,100	20
							3.1	80

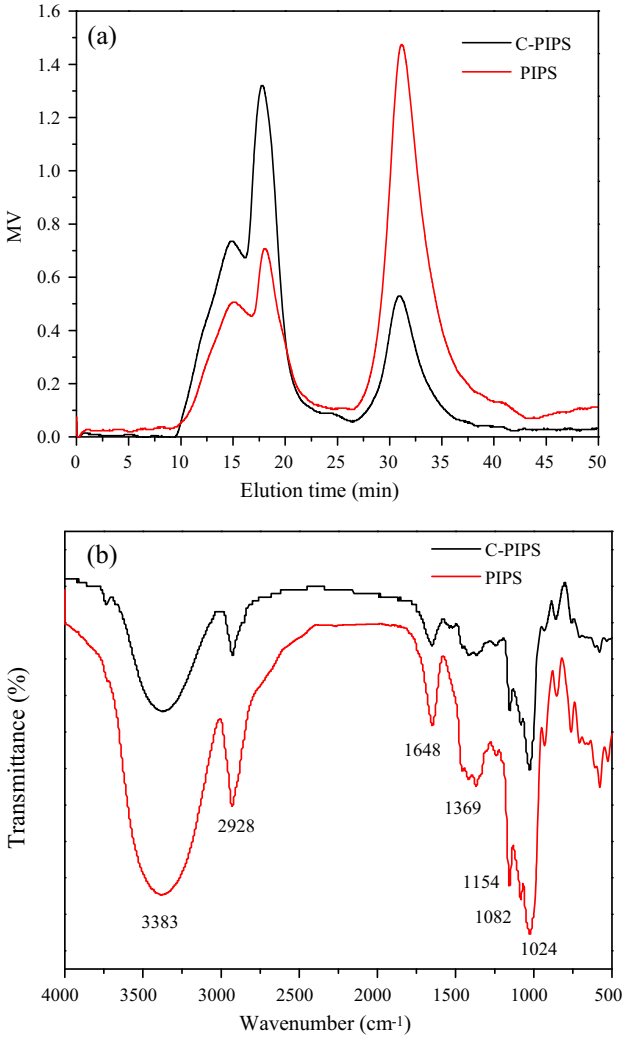

Fig. 3. (a) Gel permeation chromatograms and (b) FTIR spectra of PIPS and C-PIPS.

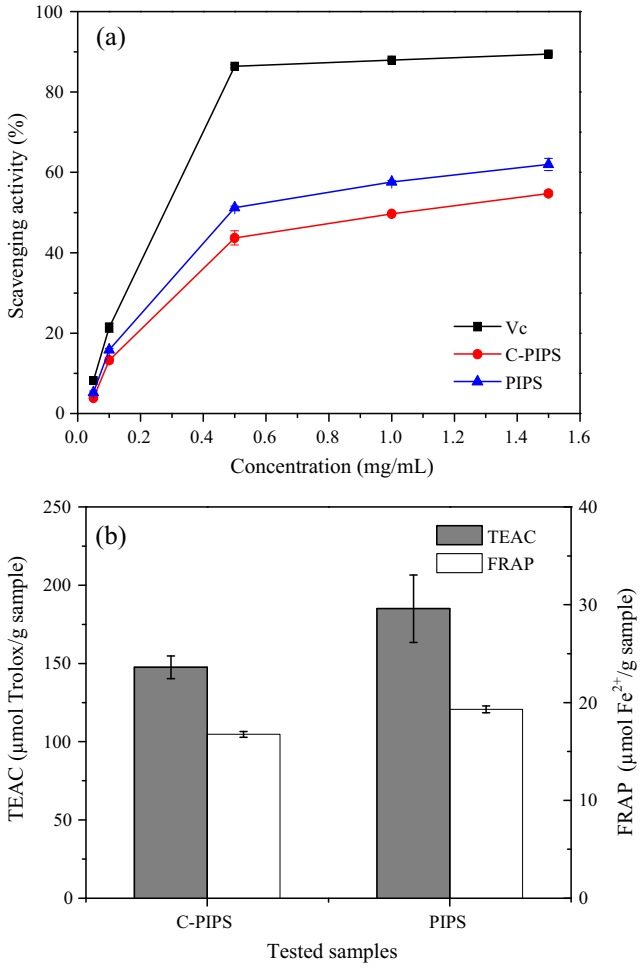

Fig. 4. *In vitro* antioxidant activities of PIPS and C-PIPS by (a) scavenging capacity on hydroxyl radicals; and (b) TEAC and FRAP assays. Each value is expressed as means ± SD ($n = 3$).

As for C-PIPS, there are two main peaks with the MWs of around 25,400 kDa and 3.8 kDa, respectively, and the high-MW fraction of ~68% was the dominant according to the peak area (Table 3). However, PIPS showed two main peaks with the MWs of about 21,100 kDa and 3.1 kDa, respectively, and the low-MW fraction of 80% was the main composition (Table 3). Under ultrasonic irradiation, the polysaccharide could be easily caused by degradation and transferred the high-MW fractions into the low-MW fractions, resulting to more amount of low-MW fractions in PIPS compared with that in the control C-PIPS. The decreased PIPS MW may arise from the disaggregation of polymer clusters or cleavage of polymer main chain by the ultrasound energy. From our experimental results, high-intensity ultrasound cleaved the covalent glycosidic bonds to lower MW molecules. In addition, it has been reported that high-MW polysaccharides from *P. cruentum* had no obvious antioxidant activities, but low-MW polysaccharides after degradation exhibited an inhibitory effect on oxidative damage (Sun, Wang, Shi, & Ma, 2009). Thus, PIPS may show stronger antioxidant activities and radical-scavenging effects than that of C-PIPS.

Fig. 3b illustrates the FTIR spectra of PIPS and C-PIPS. Two characteristic absorptions of polysaccharides were observed; a strong and wide stretching peak at approximately 3383 cm^{-1} for the O—H stretching vibration, and a weak absorption peak of approximately 2928 cm^{-1} for the C—H stretching vibration. The two peaks corresponded to the characteristic absorption values of these polysaccharides. More specifically, the intensities of the two absorption peaks in PISP were much stronger than that in C-PIPS, which suggested that ultrasonic irradiation resulted in the cleavage of glycosidic bonds. Furthermore, three absorption bands at 1200–1000 cm^{-1} corresponded to the stretching vibrations of C—O—H groups significantly enhanced, due perhaps to the cleavage of C—O—C bonds. These spectral data suggest that ultrasonic treatment mainly caused the breakage of the glycosidic linkages but no dramatic change in the primary chemical structure of PIPS molecules.

3.3. Effects of ultrasonic treatment on antioxidant activities of polysaccharides

3.3.1. Hydroxyl radical scavenging activity

Hydroxyl radicals, which are well known as the most reactive free radicals, can react with almost all the biomacromolecules functioning in living cells, and induce severe damage to the adjacent biomolecules (Rollet-Labelle et al., 1998). Therefore, it is important that hydroxyl radicals are effectively scavenged for antioxidant defense in the cell or food systems. In our study, the hydroxyl radical scavenging effect of PIPS was evaluated by comparison with C-PIPS and V_c. As shown in Fig. 4a, all the tested samples were found to have the ability to scavenge hydroxyl radicals in a dose-dependent manner. The scavenging capacity of PIPS was stronger than C-PIPS at the tested concentration range (0.05–1.5 mg/mL).

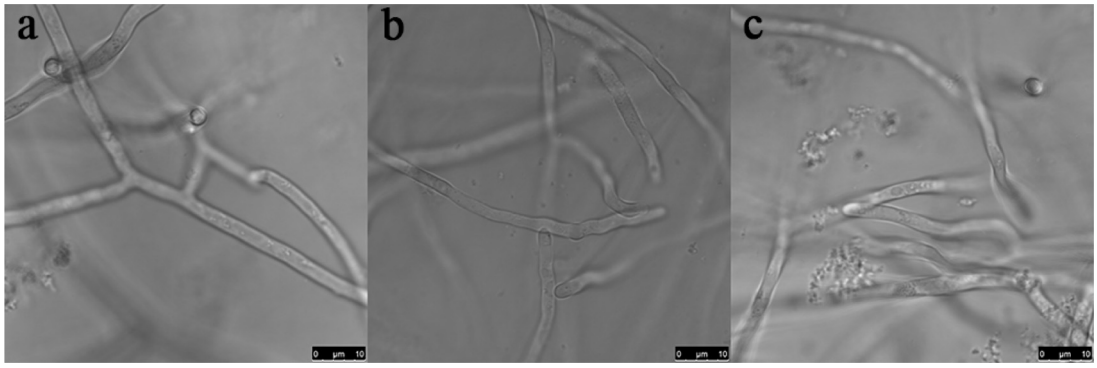

Fig. 5. Changes in laser scanning confocal microscopic images of *P. igniarius* mycelium. (a) Control treatment; Ultrasonic treatment at the optimal conditions (duty cycle time, 25 s, culture time, 3.8 d) for 60 min (b), and 120 min (c), respectively.

Note to mention, that the hydroxyl radical scavenging effect of PIPS was much lower than V_C. Xing, Liu, Guo, and Yu (2005) reported that the hydroxyl radical scavenging activity of lower MW chitosan fractions (9 kDa) was much higher than that of high-MW fractions (760 kDa). Furthermore, Xie, Xu, and Liu (2001) reported that the hydroxyl group in the polysaccharide unit can react with hydroxyl radical by the typical hydrogen-abstraction reaction to scavenge the free radical. In the present study, PIPS with higher amounts of low-MW fractions and hydroxyl groups showed a stronger hydroxyl radical scavenging activity than C-PIPS. This was in accordance with our previous report (Yan et al., 2009). In addition, the uronic acid consisted in polysaccharide structure also played an important role in the scavenging of hydroxyl radicals. Therefore, ultrasonic treatment could not only improve the production of polysaccharides during *P. igniarius* mycelium fermentation, but also enhance the *in vitro* antioxidant activity and radical-scavenging capacity of the polysaccharides.

3.3.2. TEAC and FRAP assays

The *in vitro* antioxidant activities of PIPS and C-PIPS by TEAC assay (on $ABTS^{\cdot+}$ radical scavenging activity) and FRAP assay (on ferric reducing ability) were presented in Fig. 4b. It was obvious that PIPS obtained from *P. igniarius* mycelial fermentation by ultrasonic irradiation showed the higher antioxidant activities with TEAC value of 185.02 μmol Trolox/g sample and FRAP value of 19.31 μmol Fe^{2+}/g sample. The trend was similar with the result of hydroxyl radical scavenging activity. In general, the antioxidant effects were attributed to their hydrogen-donating abilities (Wang et al., 2010). The presence of uronic acid groups in the polysaccharides could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group implies a stronger hydrogen atom-donating capacity. PIPS with higher uronic acid content showed the stronger antioxidant activity. Moreover, PIPS contained higher amounts of glucose and rhamanose exhibited better antioxidant capacities than C-PIPS, suggesting that monosaccharide compositions in PIPS molecules contributed to the antioxidant activities. It is well-known that most polysaccharides isolated from fungi or mushrooms are relatively nontoxic and do not cause significant side effects. Therefore, on the basis of the above data, PIPS may be developed as an effective natural antioxidant for the food and pharmaceutical industry.

3.4. Possible ultrasonic stimulation mechanism

To study possible mechanism of *P. igniarius* mycelial fermentation by ultrasonic treatment, the changes in cell morphology were observed by LCSM. Fig. 5 shows the LCSM photographs of the mycelium without any ultrasound (Fig. 5a), mycelium after ultrasonic treatment for 60 min (Fig. 5b) and 120 min (Fig. 5c), respectively. It was clear that the mycelium without ultrasound showed more smooth, while the mycelium with ultrasonic treatment (60 min) had more projections in the surface. When ultrasonic treatment time was increased to 120 min, the mycelium appeared a part of breakage and resulted in the release of contents. In addition, cavitation generated at high-intensity ultrasound frequency can also cause the breakage. These results indicated that ultrasonic treatment was beneficial in that it allows for an increased transfer of nutrients and metabolites by changing the morphology and structure of *P. igniarius* myclium, which may contribute to the promotion of growth and metabolism of *P. igniarius*. For example, the mycelia biomass production of *P. igniarius* increased and then slightly decreased with increasing the ultrasonic treatment time from 0 to 150 min (Supplementary data). The reduced biomass growth at the longer sonication time was clearly reflected in a lower yield of PIPS. This result indicated that ultrasound treatment can stimulate the growth of *P. igniarius* mycelium and improve the production of mycelia biomass during submerged fermentation. However, too much exposure to ultrasound, the mycelium may be damaged to some extent, which resulted in hindering the fermentation process.

4. Conclusions

In summary, a novel and efficient high-intensity ultrasound method was employed to improve polysaccharides production from *P. igniarius* mycelial fermentation. RSM was used to optimize the ultrasonic conditions for a high yield of PIPS. The optimal ultrasonic conditions for the PIPS were as follows: ultrasound treatment time 65 min, duty cycle time 25 s, and culture time 3.8 d. Under these conditions, the yield of PIPS was 1.8002 g/L, which increased about 22.64% compared with C-PIPS. Under ultrasonic irradiation, PIPS had higher carbohydrate and uronic acid contents, and it comprised glucose, rhamanose and mannose in molar ratio of 11.0:14.0:1.0 with a large amount of low-MW fractions (3.1 kDa, 80%). PIPS showed stronger radical scavenging capacities and antioxidant activities than the control C-PIPS. LCSM observations suggested that ultrasonic treatment can enhance diffusion of nutrients and metabolites and accelerate the growth and metabolism of *P. igniarius*. Thus, PIPS could be explored as a novel natural antioxidant for application in functional foods or medicine. Further investigations on purification and structure of PIPS are currently underway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.07.027.

References

Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*. Hoboken, NJ: John Wiley & Sons, Inc.

Chisti, Y. (2003). Sonobioreactors: using ultrasound for enhanced microbial prodcutivity. *Trends in Biotechnology*, *21*, 89–93.

Chu, J., Zhang, S. L., Zhuang, Y. P., Chen, J., & Li, Y. R. (2002). Factors affectting the biosynthesis and secretion of gentamicin. *Process Biochemistry*, *38*, 815–820.

Dai, C. Y., Wang, B. C., Duan, C. R., & Sakanishi, A. (2003). Low ultrasonic stimulates fermentation of riboflavin producing strain *Ecemothecium ashbyii*. *Colloids and Surfaces B: Biointerfaces*, *30*, 37–41.

Dean, A. M., & Voss, D. T. (1999). *Design and analysis of experiments*. New York, NY: Springer-Verlag, Inc.

He, P., Geng, L., Wang, J., Wang, Z., Mao, D., & Xu, C. (2012). Purification, characterization and bioactivity of an extracellular polysaccharide produced from *Phellinus igniarius*. *Annals Microbiology*, *62*, 1697–1707.

Henriques, A. W. S., Jessouroun, E., Lima, E. L., & Alves, T. L. M. (2006). Capsular polysaccharide production by *Neisseria meningitidis* serogroup C: Optimization of process variables using response surface methodology. *Process Biochemistry*, *41*, 1822–1828.

Herran, N. S., Lopez, J. L. Z., Perez, J. A. S., & Chisti, Y. (2008). Effects of ultrasound on culture of *Aspergillus terreus*. *Journal of Chemical Technology and Biotechnology*, *83*, 593–600.

Kwiatkowska, B., Bennett, J., Akunna, J., Walker, G. M., & Bremner, D. H. (2011). Stimulation of bioprocess by ultrasound. *Biotechnology Advances*, *29*, 768–780.

Leung, P. H., Zhao, S. N., Ho, K. P., & Wu, J. Y. (2009). Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of *Cordyceps sinensis* fungus Cs-HK1. *Food Chemistry*, *114*, 1251–1256.

Liu, R., Li, W., Sun, L. Y., & Liu, C. Z. (2012). Improving root growth and cichoric acid derivatives production in hairy root culture of *Echinacea purpurea* by ultrasound treatment. *Biochemical Engineering Journal*, *60*, 62–66.

Liu, Y. F., Yang, Y., Jia, W., Zhang, J. S., Tang, Q. J., & Tang, C. H. (2006). Determination of total flavones in the medicinal mushroom *Phellinus*. *Acta Edulis Fungi*, *13*, 49–52.

Lung, M. Y., Tsai, J. C., & Huang, P. C. (2010). Antioxidant properties of edible basidiomycete *Phellinus igniarius* in submerged cultures. *Journal of Food Science*, *75*, E18–E24.

Matsuura, K., Hirotsune, M., & Nunokawa, Y. (1994). Acceleration of cell growth and ester formation by ultrasonic wave irridation. *Journal of Fermentation and Bioengineering*, *77*(1), 36–40.

Mo, S. Y., Yang, Y. C., & Shi, J. G. (2003). Studies on chemical constitutes of *Phellinus igniarius*. *Zhongguo Zhongyao Za Zhi*, *28*, 339–341.

Moradali, M. F., Mostafavi, H., Ghods, S., & Hedjaroude, G. A. (2007). Immunonodulating and anticancer agents in the realm of macromycetes fungi (macrofungi). *International Immunopharmacology*, *7*, 701–724.

Nguyen, T. M. P., Lee, Y. K., & Zhou, W. (2012). Effect of high intensity ultrasound on carbohydrate metabolism of bifidobacteria in milk fermentation. *Food Chemistry*, *130*, 866–874.

Rollet-Labelle, E., Grange, M. J., Elbim, C., Marquetty, C., Gougerot-Pocidalo, M. A., & Pasquier, C. (1998). Hydroxyl radical as a potential intracellular mediator of polymorphonuclear neutrophil apopotosis. *Free Radical Biology and Medicine*, *24*, 563–572.

Runyan, C. M., Carmen, J. C., Beckstead, B. L., Nelson, J. L., Robinson, R. A., & Pitt, W. G. (2006). Low-frequency ultrasound increases outer permeability of *Pseudomonas aeruginosa*. *Journal of General and Applied Microbiology*, *52*, 295–301.

Seviour, R., McNeil, B., Fazenda, M. L., & Harvey, L. M. (2011). Operating bioreactors for microbial exopolysaccharide production. *Critical Reviews in Biotechnology*, *31*, 170–185.

Shon, Y. H., & Nam, K. S. (2004). Inhibition of cytochrome P450 isozymes and ornithine decarboxylase activities by polysaccharides from soybeans fermented with *Phellinus igniarius* or *Agrocybe cylindracea*. *Biotechnology Letters*, *26*, 159–163.

Song, T. Y., Lin, H. C., Yang, N. C., & Hu, M. L. (2008). Antiproliferative and antimetastatic effects of the ethanolic extract of *Phellinus igniarius* (Linnearus: Fries) Quelet. *Journal of Ethnopharmacology*, *115*, 50–56.

Staub, A. M. (1956). Removal of protein-Sevag method. *Methods in Carbohydrate Chemistry*, *5*, 5–6.

Sulaiman, A. Z., Ajit, A., Yunus, R. M., & Chisti, Y. (2011). Ultrasound-assisted fermentation enhances bioethanol productivity. *Biochemical Engineering Journal*, *54*, 141–150.

Sun, L. Q., Wang, C. H., Shi, Q. J., & Ma, C. H. (2009). Preparation of different molecular weight polysaccharides from *Porphyridium cruentum* and their antioxidant activities. *International Journal of Biological Macromolecules*, *45*, 42–47.

Wang, J., Guo, H., Zhang, J., Wang, X., Zhao, B., Yao, J., & Wang, Y. (2010). Sulfated modification, characterization and structure-antioxidant relationships of *Artemisia sphaerocephala* polysaccharides. *Carbohydrate Polymers*, *81*, 897–905.

Wang, F., Ma, A. Z., Guo, C., Zhuang, G. Q., & Liu, C. Z. (2013). Ultrasound-intensified laccase production from *Trametes versicolor*. *Ultrasonics Sonochemistry*, *20*, 118–124.

Wang, Z. B., Pei, J. J., Ma, H. L., Cai, P. F., & Yan, J. K. (2014). Effect of extraction media on preliminary characterizations and antioxidant activities of *Phellinus linteus* polysaccharides. *Carbohydrate Polymers*, *109*, 49–55.

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic and Medicinal Chemistry Letters*, *11*, 1699–1701.

Xing, R. G., Liu, S., Guo, Z. Y., & Yu, H. H. (2005). Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities *in vitro*. *Bioorganic and Medicinal Chemistry*, *13*, 1573–1577.

Yan, J. K., Li, L., Wang, Z. M., Leung, P. O., Wang, W. Q., & Wu, J. Y. (2009). Acidic degradation and enhanced antioxidant activities of exopolysaccharides from *Cordyceps sinensis* mycelial culture. *Food Chemistry*, *117*, 641–646.

Yan, J. K., Li, L., Wang, Z. M., & Wu, J. Y. (2010). Structural elucidation of an exopolysaccharide from mycelial fermentation of a *Tolypocladium* sp. fungus isolated from wild *Cordyceps sinensis*. *Carbohydrate Polymers*, *79*, 125–130.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science and Technology*, *18*, 4–19.

Zhong, J. J., & Tang, Y. J. (2004). Submerged cultivation of medicinal mushrooms for production of valuable bioactive metabolites. *Advances in Biochemical Engineering/Biotechnology*, *87*, 25–59.