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Optimization of extraction process by response surface methodology and preliminary characterization of polysaccharides from *Phellinus igniarius*

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ABSTRACT

Polysaccharides of *Phellinus igniarius* have many bioactivities. In this study, response surface methodology along with Box–Behnken design based on the single-factor experiments was firstly applied to optimize the extraction conditions by its liquid-cultured mycelia. According to analysis, extraction temperature and ratio of mycelia to water significantly affected extraction yield. The optimal conditions were extraction temperature 70 °C, extraction time 1.5 h and the ratio of mycelia to water 1:6.2. Under these conditions, the maximal yield of crude intracellular polysaccharide (IPS) from mycelia was 50.39 ± 0.41 mg/g, which was agreed with model predictions. The preliminary characterization was mainly β-galactan by gas chromatograph and infrared spectrum analysis.

1. Introduction

Mushrooms have long attracted attentions as traditional food and medicines. *Phellinus igniarius*, one of the most well-known traditional medicinal mushrooms, is a medicinal basidiomycetous fungus belonging to the *Hymenochaetaceae basidiomycetes*, and widely used in Asia for many years. It is also called Sanghuang (yellow polyporus), *Phellinus linteus*, *Phellinus gilvus*, and *Phellinus rimosus* (Yang et al., 2007). The main compound polysaccharides from *P. igniarius* has attracted great attention for many years, due to their various biological functions such as inhibiting tumor growth and metastasis, and low toxicity (Kim et al., 2006; Li et al., 2004; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Sliva, Kawasaki, Stanley, Harvey, & Slivova, 2006; Zhang, Cui, Cheung, & Wang, 2007). Therefore, there is a great need to supply the market with high-quality polysaccharides. However, little attention was devoted to the extraction, purification and characterization of *P. igniarius* polysaccharides. Therefore, we reported the optimization of extracting parameters for the production and its preliminary characterization of intracellular polysaccharides (IPS).

According to previous study, the extraction yields of IPS were mainly affected by extraction time, temperature and ratio of mushroom materials to water (Qiao et al., 2009). In order to determine these optima, the general practice was varying one parameter while keeping others invariable. However, this single variable optimization did not depict the net effects of various parameters on the reaction rate. Response surface methodology (RSM) has been successfully used for optimizing complex process, extraction technology, conditions of enzyme reaction, and so on (Lee, Yusof, Hamid, & Baharin, 2006; Sun et al., 2009; Zou, Hang, Chen, Chu, & Zhuang, 2008). The advantage of RSM is that it can reduce the number of experimental trials and evaluate the interactions between multiple parameters. It is more effective and precise than many approaches. A literature survey indicated that there was no investigation on the extraction of polysaccharides from mycelia of liquid-cultured *P. igniarius*. Therefore, the objectives of this study were to optimize extraction conditions by RSM and evaluate preliminary properties of *P. igniarius* polysaccharides.

2. Materials and methods

2.1. Microorganism and culture conditions

P. igniarius N0.5.51 from China General Microbiological Culture Collection Center (CGMCC), was used in submerged culture. Agar

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slants containing potato–dextrose–agar were inoculated with mycelia and incubated at 25 °C for 10 days, and then used as inoculums for seed culture. The seed culture was grown in 500 ml shake flasks for 4 days at initial pH 6.0–6.1, 25 °C, and 200 rpm with a medium containing 30 g l^{-1} glucose, yeast power juice (put 15 g l^{-1} yeast power into the water, boiled for 15 min, and discarded the precipitation.), 1.0 g l^{-1} $MgSO_4$, 1.0 g l^{-1} KH_2PO_4. The flask culture experiments were performed in 250 ml flasks containing 50 ml of the medium inoculating with 10% (v/v) of the seed culture. Submerged fermentation was carried out in the medium of following composition (g l^{-1}): glucose 40.0, glutamic acid 4.0, $(NH_4)_2SO_4$ 4.0, KH_2PO_4 1.0, $MgSO_4$ 1.0. All media were sterilized at 115 °C for 30 min. The fermentation cultivation was inoculated at 10% (v/v) of the above seed culture medium and kept at 25 °C and 200 rpm in 250 ml shake flasks for 7 days (Guo, Zou, & Sun, 2009).

2.2. Extraction of crude IPS

Mycelia by submerged culture were washed by distilled water and ground in a disintegrator. The homogenates were diluted and extracted under extracting time (1–5 h), temperature (50–100 °C) and ratio of mycelia to water (1:210). Then, the mixtures were centrifuged at 5000 r/min for 15 min, and precipitated with ethanol (Qiao et al., 2009).

2.3. Determination of IPS

The crude IPS was precipitated by 95% (v/v) ethanol which were four times of volume to, and then separated by centrifugation at 10,000*g*. The precipitation was dissolved in the water and determined by phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.4. Experiment design and statistical analysis

Single-factor-test was employed to determine the preliminary range of the extraction variables including extraction temperature, extraction time and ratio of mycelia to water. Then, a three-level-three-factor BBD was used in this study, requiring 17 experiments shown in Table 1. All trials were performed in triplicate. The extraction yields were treated as responses. An SAS Software Version 9 (SAS Institute Inc., NC, USA) was used to generate the experimental designs, statistical analysis and regression model. Experiment data were fitted to a second-order polynomial model and regression coefficients obtained which was as follows:

$$Y = \beta_0 + \sum_{i=3}^{3} \beta_i X_i + \sum_{i=3}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ii} X_i X_j$$

where β_0, β_i, β_{ii}, and β_{ij} were the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j were the independent variables. The fitness of the second-order model was expressed by the regression coefficient R^2 and its statistical significance was determined by F test. t-Test was used for evaluating regression significance.

2.5. Analysis of monosaccharides

The crude IPS was dialyzed for 48 h and lyophilized. Then the sample was hydrolyzed by 2 M trifluoro-acetic acid (TFA) at 100 °C for 3 h and dried overnight at 60 °C in vacuum drying oven. The treated sample and monosaccharides including glucose (Glc), mannose (Man), xylose (Xly), arabinose (Ara) and galactose (Gal) were acetylated respectively by adding of mixture of methanol, pyridine and acetic anhydride. Acetylated alditols of samples and sugars were separated by gas chromatography (Sichuan Instrument Co. Model: SC-6000, Chongqing, China) with a fused silica capillary column (Agilent Co. Model: HP-5, 30 m × 0.32 mm × 0.25 μm, USA) and a flame ionization detector. The oven temperature was maintained at 180 °C and then gradually increased to 220 °C at a rate of 8 °C/min. The temperature of detector and injector was set at 270 °C and 240 °C, respectively (Kim, Park, Park, & Kim, 1994).

2.6. FT-IR spectroscopy

One milligram of IPS was milled with 300 mg of KBr, and pressed into a pellet for transmission infrared spectroscopy (Ignjatović, Savić, Najman, Plavšić, & Uskoković, 2001). FT-IR was analyzed for detecting functional groups and recorded on an infrared spectrometer (Perkinelmer Co., USA).

3. Results and discussion

3.1. Effect of temperature on extraction yield of IPS

As shown in Fig. 1(A), the effect of temperature on extraction yield was investigated. The temperature was changed from 50 °C to 100 °C, while other extraction variables were set as follows: ratio of mycelia to water 1:4 and extraction time 2 h. The yield of IPS increased with temperature until 70 °C and began to decrease, and the maximum extraction yield was 31.89 ± 1.51 mg/g at 70 °C. This

Table 1
Box–Behnken design matrix (in coded level of three variables) and response values for the yield of IPS.

Run	Time (X_1/h)	Ratio of mycelia to water (X_2)	Temperature (X_3/°C)	Polysaccharide yield (mg/g)
1	1	1:4	70	39.5447
2	3	1:4	70	48.0393
3	1	1:8	70	34.5765
4	3	1:8	70	42.7793
5	1	1:6	60	35.5574
6	3	1:6	60	25.4052
7	1	1:6	80	30.2361
8	3	1:6	80	34.2332
9	2	1:4	60	37.8625
10	2	1:8	60	27.8329
11	2	1:4	80	36.3912
12	2	1:8	80	36.9307
13	2	1:6	70	50.0256
14	2	1:6	70	49.6578
15	2	1:6	70	51.9016
16	2	1:6	70	50.1973
17	2	1:6	70	49.8049

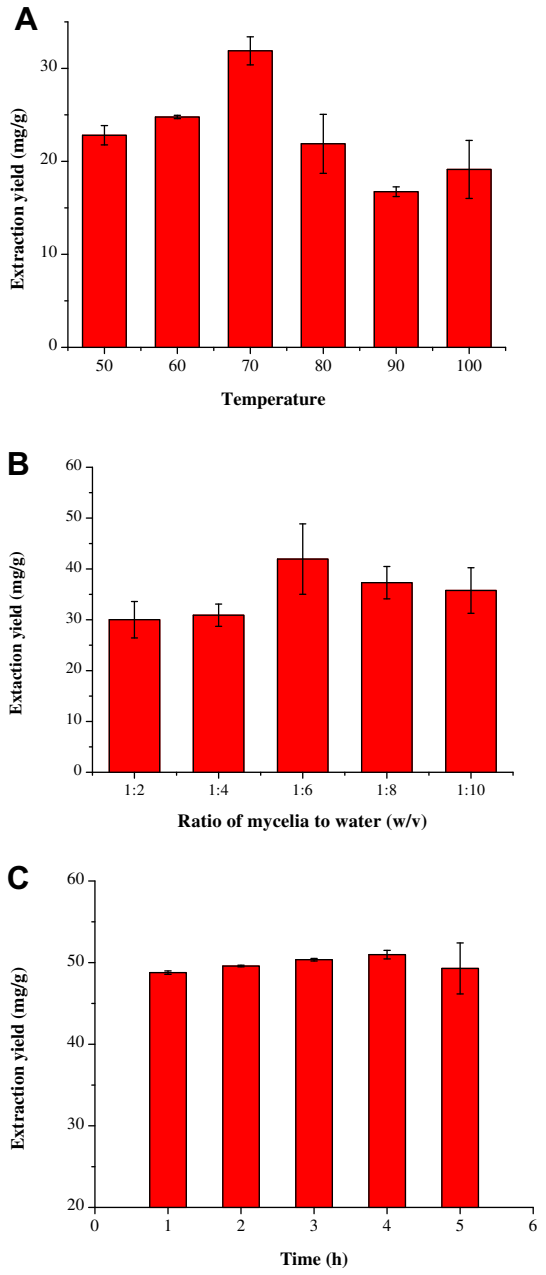

Fig. 1. Effects of extracting temperature (A), ratio of mycelia to water (B) and extracting time (C) on the yield of IPS.

result indicated that temperature enhanced the polysaccharides extraction from the mushroom particles into the water to a certain level followed by their possible loss, due to decomposition at a higher temperature.

3.2. Effect of ratio of mycelia to water on extraction yield of IPS

The yield of IPS extracted by different ratio of mycelia to water from 1:2 to 1:10 was shown in Fig. 1(B). The extraction temperature and extraction time was fixed at 70 °C and 2 h, respectively. The extraction yields of the polysaccharides increased from 30.02 ± 3.59 mg/g to 41.93 ± 6.92 mg/g with the ratio increasing from 1:2 to 1:6. Then, it tended to go steady. The more water used, the more ethanol was exploited to precipitate IPS. Therefore, extraction ratio 1:6 was favorable for polysaccharides production.

3.3. Effect of extraction time on extraction yield of IPS

Extraction time was another factor that would influence the extraction yield. When the extraction time increased from 1 h to 5 h, other experimental conditions were as follows: ratio of mycelia to water 1:4 and extraction temperature 70 °C. The results showed that the extraction yield began to increase from 48.78 ± 0.21 to 50.36 ± 0.52 mg/g, as seen in Fig. 1(C). A longer extraction time represented a positive effect on the yield of polysaccharides, but the yield increased slightly. Therefore, time rang (1–3 h) was selected as the optimal in the present experiment with saving cost taken into consideration.

According to the single-parameter study, we adopted extraction temperature 60–80 °C; ratio of mycelia to water 1:4–1:8; extraction time 1–3 h for RSM experiments.

3.4. Predicted model and statistical analysis

The design matrix and the corresponding results of RSM experiments to determine the effects of the three independent variables including extraction time (X_1); ratio of mycelia to water (X_2) and extraction temperature (X_3) were shown in Table 1. By employing multiple regression analysis on the experiment data, the predicted model was obtained by the following second-order polynomial function:

$$Y_1 = 50.31744 - 2.46479X_1 + 2.525511X_2 + 0.183918X_3 - 2.843076X_1X_1 - 0.072982X_1X_2 + 2.6423X_1X_3 - 6.239423X_2X_2 + 3.537351X_2X_3 - 12.72004X_3X_3$$

The fit statistics of extraction yield (Y_1) for the selected quadratic predictive model was shown in Table 2. The coefficient of the variation (C.V.) and value of adjusted determination coefficient R^2_{Adj} was 6.596 and 0.9048, respectively, which indicated a high degree of precision of reliability of the experimental values and a high degree of correlation between the observed and predicted values. The ANOVA analysis was shown in Table 3. The *P*-values were used as a tool to check the significance of each coefficient. The smaller the *P*-value was, the more significant the corresponding coefficient was (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). It can be seen that the variable with the largest effect was the interaction effects of ratio of mycelia to water and extraction temperature ($X_2 \times X_3$), followed by the linear terms of extraction

Table 2
Fit statistics of *Y*.

	Master model	Predictive model
RMSE	2.6421	2.6421
R-square	95.84%	95.84%
Adjusted *R*-square	90.48%	90.48%
Coefficient of variation	6.5958	6.5958

Table 3
Test of significance for regression coefficients.

Effect	Estimate	Stand error	*t* Ratio	*P*-value
X_1	−2.4648	0.93413	−2.6386	.0335
X_2	2.5255	0.93413	2.7036	.0305
X_3	0.18392	0.93414	0.19689	.8495
$X_1 \times X_1$	−2.8431	1.2876	−2.208	.063
$X_1 \times X_2$	−0.073	1.3211	−0.05525	.9575
$X_1 \times X_3$	2.6423	1.3211	−0.0552	.9575
$X_2 \times X_2$	−6.2394	1.2876	−4.8457	.0019
$X_2 \times X_3$	3.5374	1.2876	−4.8457	.0019
$X_3 \times X_3$	−12.72	1.2876	−9.8788	<.001

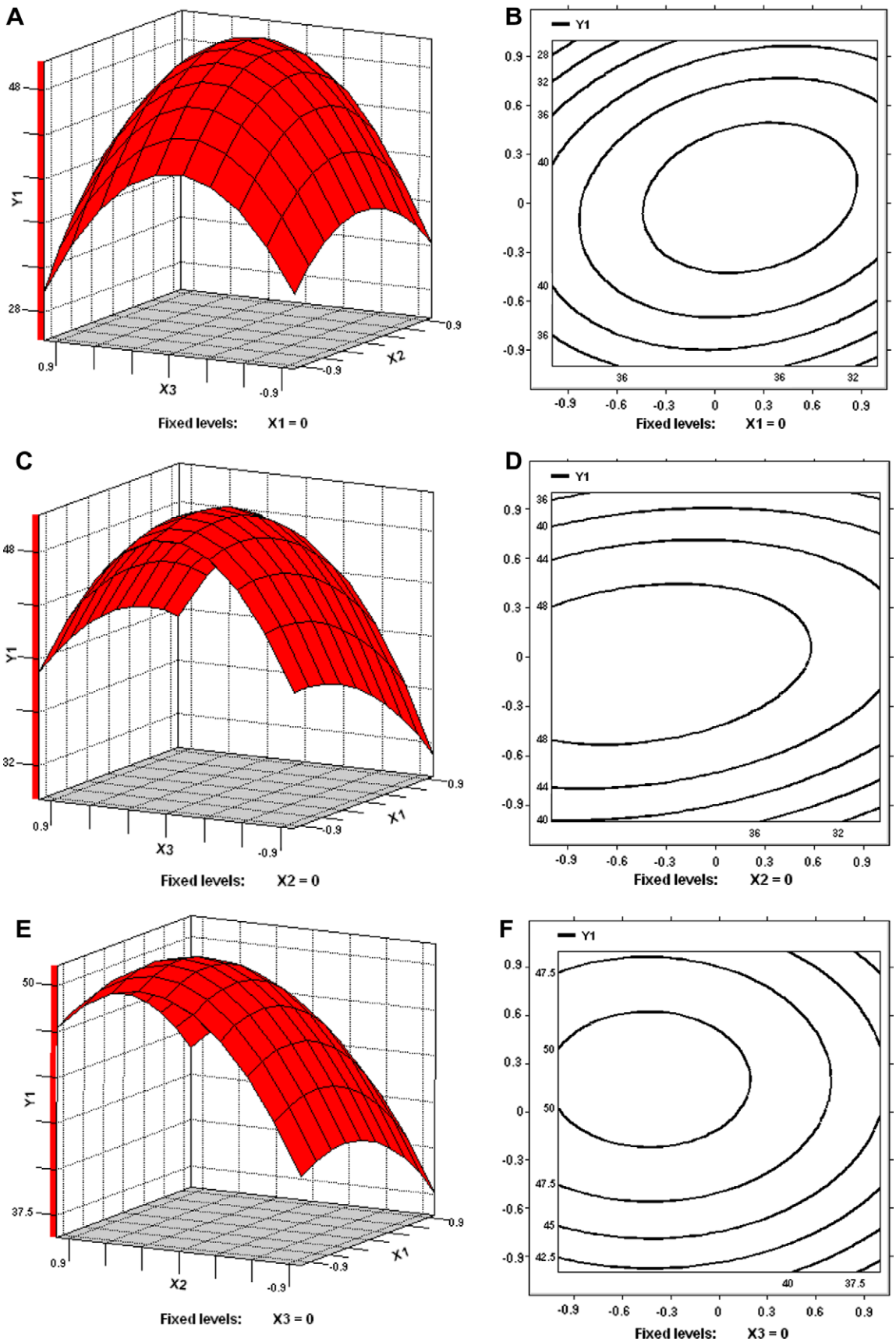

Fig. 2. Response surface plots (A, C, and E) and contour plots (B, D and F) showing the effect of extracting time (X_1), ratio of mycelia to water (X_2) and extracting temperature (X_3) on the yield of IPS.

time (X_1) and ratio (X_2). The other term coefficients (X_3, $X_1 \times X_1$, $X_1 \times X_2$, $X_1 \times X_3$) were not influential ($P > .05$). According to previous reports, the effect of linear terms and interaction terms were always different among different specials and different material treatment. It might have two reasons. Firstly, polysaccharides from different specials have different solubility. Secondly, dried materials needed a higher extraction temperature, ratio of material to water, and a longer extraction time compared with fresh materials (Qiao et al., 2009; Sun et al., 2009; Wang, Luo, & Ena, 2007). It might be because the cells of fresh materials were expanded and the polysaccharides were easy to be separated out.

3.5. Response surface plot and contour plot

3D response surface and 2D contour plots were the graphical representations of regression function. They showed the type of interactions between two tested variables and the relationship between responses and experiment levels of each variable. Different shapes of the contour plots indicated different interactions between the variables. Circular contour plot indicated that the interactions between the corresponding variables were negligible, while elliptical contour plot indicated otherwise (Muralidhar et al., 2001). In present study, the response surface and contour plots were obtained by using SAS version 8.0 and were presented in Fig. 2. As shown in Fig. 2(A), when extraction time (X_1) was fixed at 0 level, ratio of mycelia to water (X_2) and extraction temperature (X_3) demonstrated quadratic effects on the extraction yields. The elliptical contour plot shown in Fig. 2(B) indicated the mutual interactions between ratio of mycelia to water and extraction time were significant. Shown in Fig. 2(C) and (D), when ratio of mycelia to water was fixed at 0 level, extraction time (X_1) displayed a quadratic effect on the response yield. When extraction time kept at lower level, the yield increased at first and then decreased with the increase of temperature (X_3). From Fig. 2(E) and (F), it showed that when extract temperature (X_3) was fixed at 0 level, the variations of yields was negligible with extraction time increase. Ratio of mycelia to water demonstrated quadratic effects on the response, when extraction time was at lower level. By analyzing the plots, the predicted values (50.314 mg/g) of the tested variables for polysaccharides, lied in the following condition: extracting time 1.5 h, ratio of mycelia to water 1:6.2 and extracting temperature 70 °C. In the optimal conditions, the experiment yield of crude IPS was 50.39 ± 0.41 mg/g, which agreed with the predicted value. Therefore, the results indicated suitability of the model employed and the success of RSM in optimizing the extraction conditions.

3.6. Preliminary characterization of IPS

As shown in Fig. 3, the monosaccharides of IPS were mainly composed of Gal. However, previous study showed that IPS from *P. igniarius* included not only Gal but also Man and Glc (Yang et al., 2007). This result indicated that the sugar compositions were different between mycelia and fruiting body. The FT-IR spectra shown in Fig. 4 existed the characteristic absorption of —OH at the ranges of 3600–3200 cm^{-1} and 1350–1260 cm^{-1}, resonance absorption of —CH and —CH_2 at 2923 cm^{-1}. A hydration peak of polysaccharide at 890 cm^{-1} indicated that IPS contained the β-glycosidic linkage (Wu, Jiang, Liu, & Zhang, 2006). Therefore, the IPS was mainly β-galactan.

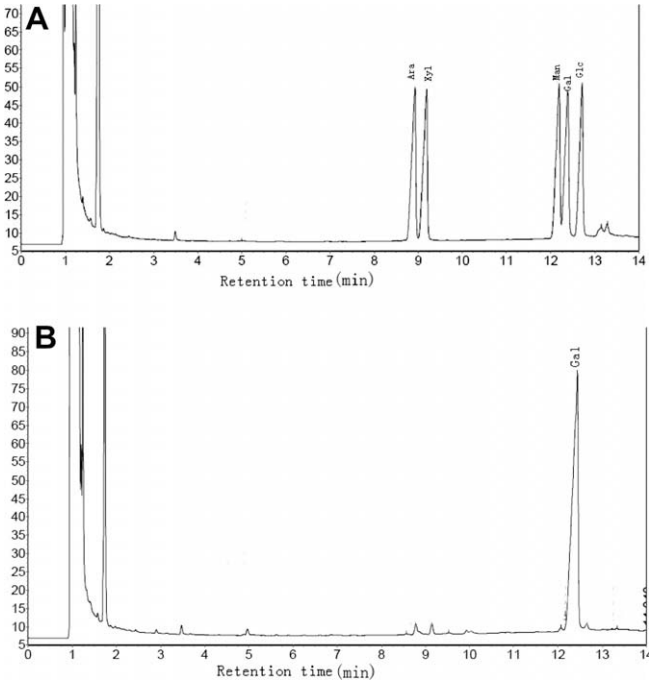

Fig. 3. GC analysis of alditotol acetate derivatives of IPS from *P. igniarius*. The standards were separated under the conditions described in Section 2 (A). The derivatives of hydrolysate were separated, and a number of galactose was included in the hydrolysate (B).

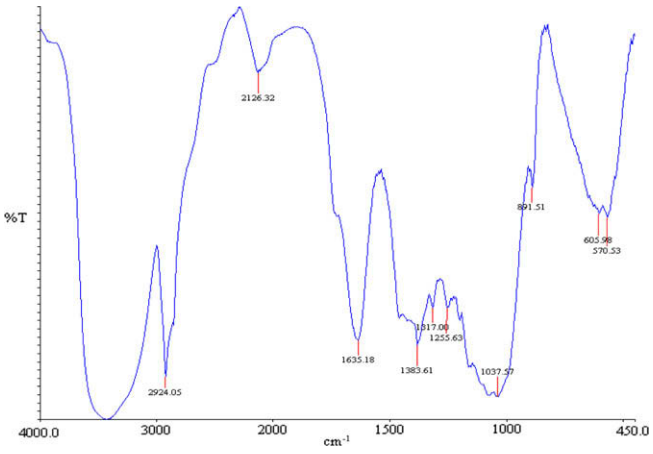

Fig. 4. FT-IR spectra of IPS from *P. igniarius*.

4. Conclusion

The single-factor experiments and BBD along with RSM were applied for optimizing extraction parameters in this study. The optimal conditions for the production of polysaccharide were as the following: extraction time 1.5 h, the ratio of mycelia to water 1:6.2 and extraction temperature 70 °C. In the optimal conditions, the experiment yield of IPS was 50.39 ± 0.41 mg/g, which was agreed with the predicted value. The preliminary characterization of IPS was mainly β-galactan by GC and IR analysis. The extraction information on mycelia of liquid-cultured *P. igniarius* obtained in this work should also be helpful in other species. The experimental conditions allow a fast and cost-saving process in extraction of polysaccharide from mycelia. Further studies on the precise chemical structures and biological functions of the new polysaccharide are in process.

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