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Optimization of Hydroxyl Radical Scavenging Activity of Exopolysaccharides from *Inonotus obliquus* in Submerged Fermentation Using Response Surface Methodology

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The objectives of this study were to investigate the effect of fermentation medium on the hydroxyl radical scavenging activity of exopolysaccharides from *Inonotus obliquus* by response surface methodology (RSM). A two-level fractional factorial design was used to evaluate the effect of different components of the medium. Corn flour, peptone, and KH_2PO_4 were important factors significantly affecting hydroxyl radical scavenging activity. These selected variables were subsequently optimized using path of steepest ascent (descent), a central composite design, and response surface analysis. The optimal medium composition was (% w/v): corn flour 5.30, peptone 0.32, KH_2PO_4 0.26, $MgSO_4$ 0.02, and $CaCl_2$ 0.01. Under the optimal condition, the hydroxyl radical scavenging rate (49.4%) was much higher than that using either basal fermentation medium (10.2%) and single variable optimization of fermentation medium (35.5%). The main monosaccharides components of the RSM optimized polysaccharides are rhamnose, arabinose, xylose, mannose, glucose, and galactose with molar proportion at 1.45%, 3.63%, 2.17%, 15.94%, 50.00%, and 26.81%.

Keywords: *Inonotus obliquus*, exopolysaccharides, hydroxyl radical scavenging activity, medium optimization, response surface methodology, monosaccharides component

Edible and medicinal mushrooms have an established history of use in the human diet and traditional therapies [11, 24, 29]. Many mushroom-derived preparations are widely applied in modern clinical practice in China, Japan, Korea, and other Asian countries, either for preventive or curative purposes. The medicinal mushroom *Inonotus obliquus* (*I. obliquus*) belongs to the family Hymenochaetaceae, Basidiomycetes [22, 29], and has been a folk remedy for a long time in Russia and northern latitudes [11, 24, 29]. Many fungal triterpenoids, steroids, and phenolic compounds from *I. obliquus* have various biological activities [11, 18, 39]. In particular, polysaccharides from *I. obliquus* exhibit strong immunomodulating, antitumor, and antioxidant activities [22, 26, 31, 34].

The limited natural resource and difficult artificial cultivation of *I. obliquus* to obtain fruit body make it impossible to obtain a large quantity of polysaccharides. Submerged cultures offer a promising alternative. Many medicinal mushroom polysaccharides are being commercially produced by submerged culture [6, 14, 16] because of a high productivity compared with production from fruit bodies [20, 23]. In addition, submerged culture is fast, cost-effective, easy to control, and without heavy metal contamination. In submerged fermentation, production of polysaccharides is sensitive to medium components and fermentation process parameters. The optimization of medium and fermentation process parameters has focused on getting the maximum biomass or yield of polysaccharides for most medicinal mushrooms in previous studies [8, 13, 14, 16, 21, 35]. Little is known about the qualitative effect of fermentation condition on the bioactivity of polysaccharides from submerged culture. In submerged fermentation, the biological activities of polysaccharides are associated with nutritional condition [3]. Carbon source, nitrogen source, and inorganic salts affect both the yield and bioactivity of polysaccharides. Although previous works have been done on the accumulation of polysaccharides from the culture of many different mushrooms [13, 14, 16, 21, 35], the factors affecting the activity of polysaccharides of *I. obliquus* have not yet been reported. In order to obtain exopolysaccharides (EPSs) with the maximum activities, we optimized the medium composition to maximize the antioxidant activity of EPSs. It is well-known that reactive oxygen species

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(ROS), such as hydroxyl radicals, superoxide anion, and hydrogen peroxide, are related to the pathogenesis of various diseases [1, 4]. Hydroxyl radical is the most reactive among the oxygen radicals and induces severe damage to the adjacent biomolecules [5]. In this paper, the scavenging rate of hydroxyl radical was chosen as an indicator to evaluate the antioxidant activity of EPS from *I. obliquus*. We used response surface methodology (RSM) including the fractional factorial design, the steepest ascent (descent) method, and the central composite design (CCD) in the experimental design [9, 12, 17]. Response surface methodology is a well-known method applied in the optimization of medium constituents and other critical variables responsible for the production of biomolecules. Statistical experimental designs can be employed at various phases of the optimization process, such as for screening experiments and for finding optimum conditions for a desired response. Single variable optimization methods are tedious and can also lead to misinterpretation of results because they overlook the interaction between different factors involved [15]. This method had been successfully applied in the optimization of medium compositions [10, 21, 25, 33] and fermentation processes [32, 36, 38] to improve the production of polysaccharides. However, to find the most important factors among related factors is the most important thing firstly considered. The optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response followed by checking the adequacy of the model [7, 19, 21, 27]. Finally, gas chromatography (GC) was employed to investigate the monosaccharide components of exopolysaccharides from different media.

Materials and Methods

Materials

Corn flour and soybean powder were purchased from a local market. Yeast extract, peptone, and malt extract were purchased from Hangzhou Microbiology Institute (Hangzhou, Zhejiang, China). Glucose and other chemical reagents were of analytical grade.

Microorganism

I. obliquus (CBS314.39) was from the Centraal Bureau voor Schimmelcultuur, Utrecht, The Netherlands. It was maintained on malt extract agar slants containing (% w/v) malt extract 3, peptone 0.3, and agar 1.5 at pH 5.6±0.2. The slants were cultivated at 25°C for about 2 weeks. When the mycelia overgrew the slants, they were stored at 4°C and subcultured every 3 months.

Seed Culture Preparation

The seed culture was prepared by adding mycelia on a malt extract agar slant into a 250-ml Erlenmeyer flask with 100 ml of medium. The medium contained (% w/v) glucose 2, peptone 0.3, yeast extract 0.2, KH_2PO_4 0.1, $MgSO_4$ 0.15, and $CaCl_2$ 0.01. Cultures were incubated for 4−5 days in a rotary shaker (150 rpm) at 28°C.

Shake-Flask Culture

The harvested seed culture was added into 250-ml Erlenmeyer flasks containing 100 ml of liquid fermentation medium to be optimized in the following procedures (described below). The inoculate rate was 10% (v/v). The culture was incubated at 28°C for 9 days on a rotary shaker maintained at 150 rpm to produce mycelia and exopolysaccharides.

Extraction and Purification of Exopolysaccharides

Harvested fermentation broth was filtered in a vacuum to separate mycelia from broth. The filtrate was concentrated under vacuum into one quarter of the original volume. The concentrate was mixed with four times volume of absolute ethanol, stirred vigorously, and left overnight at 4°C. The precipitated exopolysaccharides were repeatedly washed with 95% ethanol to remove adherent sugar residue and other small molecules, and then centrifuged (6,500 ×*g* for 10 min) and lyophilized. The EPS extract was obtained [16].

The Sevage method was employed to remove protein. The EPS extract water solution was mixed with Sevage reagent [chloroform: butanol=5:1 (v/v)] and oscillated intensively. The crude EPS was obtained after the treated water solution had been concentrated, precipitated, and lyophilized as stated above.

Assay of Crude EPS Hydroxyl Radical Scavenging Activity

The hydroxyl radical (·OH) scavenging activity of crude EPS was measured with the salicylic acid method as described by Smirnoff and Cumbes [28] with some modification. The reaction mixture (4 ml) contained 1 ml of H_2O_2 (8.8 mmol/l), 1 ml of $FeSO_4$ (9 mmol/l), 1 ml of salicylic and (9 mmol/l), and 1 ml of crude EPS solution (1 mg/ml). The H_2O_2 was added into the mixture to start up the reaction. The reaction mixture was incubated at 37°C for 60 min, and then centrifuged at 15,000 ×*g* for 6 min. The absorbance (A) of the reaction solutions at 510 nm was measured. The scavenging rate was calculated according to the equation:

$$\text{Scavening rate (\%)} = \frac{A_0 - (A_x - A_{x0})}{A_0} \times 100\% \quad (1)$$

where A_0 is the absorbance for control (double-distilled water), A_x is the absorbance for the reaction mixture with EPS sample solution, and A_{x0} is the absorbance for background (*i.e.*, the reaction mixture without H_2O_2).

Selection of Carbon Source and Nitrogen Source

The basal medium designed by Kim *et al.* [22] with some modification was employed. The basal medium contained (% w/v) glucose 3, peptone 0.4, $MgSO_4$ 0.05, KH_2PO_4 0.15, and $CaCl_2$ 0.01. For screening carbon source, glucose was replaced by sucrose, fructose, maltose, corn flour, or soluble starch while keeping other components in the medium constant. For screening the nitrogen source, soybean powder, yeast extract, KNO_3, or $(NH_4)_2SO_4$ were used while keeping other components constant. The best carbon and nitrogen sources were determined according to the hydroxyl radical scavenging rate of crude EPS.

Two-Level Fractional Factorial Design

The purpose of this optimization step was to identify which ingredients of the medium have significant effects on the hydroxyl radical scavenging rate of crude EPS. The important variables of the fermentation medium were investigated using a two-level fractional

Table 1. The code of different experimental variables.

Variable	Medium component	High level (+1)	Low level (−1)	Zero level (0)
X_1	Corn flour	5%	2%	3.5%
X_2	Peptone	0.5%	0.3%	0.4%
X_3	KH_2PO_4	0.25%	0.1%	0.175%
X_4	$MgSO_4$	0.1%	0.02%	0.06%
X_5	$CaCl_2$	0.02%	0.01 %	0.015%

factorial design, which allows for screening of five variables with only eight experiments. We used FF0508 as abbreviation of this two-level fractional factorial design, where 5 was the number of variables, and 8 was the number of experiments to be done. Each variable had two levels, high and low, denoted by (+1) and (−1). The center points (0) of all the five variables were determined according to the basal medium. They were the same as the counterparts of the basal medium. Details are given in Table 1. The Statistics Package for Social Science (SPSS) software 13.0 (SPSS, Chicago, IL, U.S.A.) was used for analyzing the experimental data.

For statistical calculations, the variable X_i was coded as x_i according to the equation

$$x_i = \frac{(X_i - X_{0i})}{\Delta X_i} \quad i=1,2,3,4,5,6,\cdots\cdots k \tag{2}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_{0i} is the real value of the independent variable at the center point, and ΔX_i is the step change.

The fit quality of the first-order model equation was expressed by the coefficient of determination R^2, its statistical significance was determined by an F-test, and the significance of the regression coefficients were tested by t-test.

Path of Steepest Ascent (Descent)

The first-order model equation was obtained by fractional factorial design:

$$Y = \beta_0 + \sum_{i-1}^{n} \beta_i x_i \tag{3}$$

where Y is the predicted response, β_0 and β_i are constant coefficients, and x_i is the coded independent variable.

The factors screened by fractional factorial design were further optimized using the steepest ascent (descent) method to lead the experimenter closely towards the vicinity of the optimum of the surface. The direction of steepest ascent (descent) is the direction in which Y increases (decreases) most rapidly. This direction is parallel to the normal to the fitted response surface. One usually takes as the path of steepest ascent (descent) the line through the center of the region of interest and normal to the fitted surface. Thus, the steps along the path are proportional to the regression coefficient β_i. The path of steepest ascent (descent) started from the center of the first design, to move away from the first design center along the path of steepest ascent (descent). Once the path of steepest ascent no longer led to increase, this procedure should be discontinued in favor of a more elaborate experiment.

We moved 2%, −0.035%, and 0.025% in corn powder, peptone, and KH_2PO_4, respectively. These new units were determined according to the concentration range of unity level from the first design and estimated coefficient ratio from the first-order model.

Central Composite Design (CCD)

The CCD is one of the RSM. CCD was conducted in the optimum vicinity, which was determined by the path of steepest ascent (descent) experiment, to locate the true optimum concentrations of corn powder, peptone, and KH_2PO_4. The full CCD, based on three basic principles of an ideal experimental design, primarily consists of a complete 2^n factorial design, where n is the number of test variables, n_0 center points ($n_0 \geq 1$) and two axial points on the axis of each design variable at a distance of r from the design center. Hence, the total number of design points is $N=2^n+2n+n_0$ and two axial points on the axis of each design variable at a distance of r from the design center. The experimental data of CCD were fitted with a second-order polynomial equation by the multiple regression method as

$$Y = \beta_0 + \sum_{i-1}^{k} \beta_i x_i + \sum_{i-1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j \tag{4}$$

where Y is the predicted response, β_0, β_i, β_{ii}, β_{ij} are constant coefficients, and x_i, x_j are the coded independent variables. The variables $x_i x_j$ represent the first-order interaction between x_i and x_j ($i<j$).

Analysis of Monosaccharides by GC

The exopolysaccharides obtained from basal, single variable optimized, and RSM optimized medium were hydrolyzed using a method based on Benhura and Chidewe [2]. Ten mg of crude EPS was added into a 10-ml screw-capped tube with 4 M trifluoroacetic acid. The mixture was reacted in a 110°C oven for 12 h and then evaporated to dryness under a stream of nitrogen. Monosaccharides were derivatized based on a method by Ye *et al.* [37] with some modification. Ten mg of hydroxylamine hydrochloride and 0.5 ml of pyridine were added to the former three tubes separately, followed by oximization in a 90°C water bath for 30 min and cooled down to room temperature. Then, 0.5 ml of acetic anhydride was added and the mixture was shaken homogeneously. The solution was subjected to acetylation in a 90°C water bath for 30 min and cooled down to room temperature for formation of aldonitrile peracetylated derivatives. The derivatives solution was evaporated to dryness under a stream of nitrogen and 1 ml of chloroform was added to dissolve the separated substance. The derivatization of six different kinds of standard monosaccharides was carried out likewise. Each 0.5-μl chloroform solution was injected into a VARIAN CP-3800 (Varian, Inc, U.S.A.) equipped with a VARIAN CP-Sil 5 CB capillary chromatography column (25m× 0.53 mm, 0.25 μm film thickness) and a flame-ionization detector for analysis.

The GC operation was performed under the following conditions: injection temperature, 270°C; detector temperature, 250°C. The temperature in the oven was programmed as follows: 110°C in the beginning, maintained for 5 min, increased to 180°C at 3°C/min and maintained for 5 min, and increased to 220°C at 5°C/min and maintained for 10 min. The monosaccharide components were identified by matching the GC retention time with the six standard compounds. The relative amount of each monosaccharide was calculated as the proportion of the compound peak area to the internal standard (myo-inositol) peak area.

Table 2. Effects of different carbon source and nitrogen source on hydroxyl radical scavenging rate of *I. obliquus* exopolysaccharides.

Carbon source	Scavenging rate (%)	Nitrogen source	Scavenging rate (%)
Glucose	10.20±0.68d	Peptone	35.52±1.00a
Fructose	24.48±0.98c	KNO_3	9.42±0.34e
Sucrose	9.94±0.46d	$(NH_4)_2SO_4$	17.44±0.98d
Maltose	31.38±1.32b	Soybean flour	31.88±0.88b
Corn flour	34.20±1.36a	Yeast extract	28.12±1.72c
Soluble starch	23.52±1.32c		

Data based on three trials. Values are the mean ± SD, and alphabet letters indicate the same letters in the same column are not statistically significantly different according to Turkey–HSD ($p<0.05$).

RESULTS

Selection of Carbon Source and Nitrogen Source

The effects of different carbon source and nitrogen source on the hydroxyl radical scavenging rate of *I. obliquus* EPS are given in Table 2. Corn flour was the best carbon source with a scavenging rate of 34.20%, followed by maltose.

Among the nitrogen sources, peptone was the best for hydroxyl radical scavenging activity with the scavenging rate of 35.52%, while corn flour was employed as the carbon source. Thus, corn flour and peptone were chosen as the sources of carbon and nitrogen for further optimization.

Fractional Factorial Design

A total of five medium components were screened through eight experimental runs, and the corresponding fractional factorial experimental design matrix for screening of important variables for hydroxyl radical scavenging rate is shown in Table 3. The RSM analysis for the optimization of medium constituents by SPSS 13.0 showed that the hydroxyl radical scavenging rate (Y1) was a function of the concentration of corn flour (x_1), peptone (x_2), KH_2PO_4 (x_3), $MgSO_4$(x_4), and $CaCl_2$ (x_5). The following second-order polynomial equation was found to represent the scavenging activity adequately:

Table 3. The matrix of fractional factorial design for screening the important variables for the hydroxyl radical scavenging rate of exopolysaccharides (FF0508).

Run	x_1	x_2	x_3	x_4	x_5	Scavenging rate (%)
1	1	1	1	1	1	39.22
2	1	1	−1	−1	−1	33.98
3	1	−1	1	−1	−1	44.96
4	1	−1	−1	1	1	41.18
5	−1	1	1	1	−1	19.60
6	−1	1	−1	−1	1	17.04
7	−1	−1	1	−1	1	27.50
8	−1	−1	−1	1	−1	24.82

Data based on three trials. Values are the mean.

Table 4. Analysis of the two-level fractional factorial design (FF0508) for screening important variables.

Variable	Regression coefficient	t-value	Sig.
X_1	8.798	21.333	0.002
X_2	−3.578	−8.677	0.013
X_3	1.782	4.325	0.050
X_4	0.168	0.409	0.722
X_5	0.198	0.476	0.681

$$Y_1(\%)=44.83+8.798x_1-3.578x_2+1.782x_3+0.168x_4+0.198x_5 \quad (5)$$

The effects of X_1, X_2, X_3, X_4, and X_5 were calculated to be 4.399, −1.789, 0.891, 0.084, and 0.099, respectively.

Regression coefficients of the variables on the responses, the associated F-values, and significant levels are shown in Table 4. The fit quality of first-order model was evaluated by the determination coefficient R^2, which was calculated to be 0.996. It indicated that 99.6% of the variability in the response could be explained by the model.

The corresponding analysis of variance (ANOVA) indicated that the model was highly significant. It was evident from the calculated F value (109.747) and a very low probability value (P>F=0.009). The calculated F value (109.747) was also greater than the tabulated F value ($F_{(5,2)}$=19.3) at 0.05 level, which indicated that the first-order was highly significant.

In Table 4, a p-value less than 0.10 was obtained for the three variables: corn flour (X_1), peptone (X_2), and KH_2PO_4 (X_3). This indicated that they were significant; but $MgSO_4$ and $CaCl_2$ were not significant. It also can be seen that the increase in concentration of corn flour, KH_2PO_4, $MgSO_4$, and $CaCl_2$ had a positive effect on hydroxyl radical scavenging activity but peptone had a negative effect. As a result, corn flour, peptone, and KH_2PO_4 were selected for further optimization. Other nutrients concentrations were set at their low level tested in the fractional factorial design.

Path of Steepest Ascent (Descent)

The path of ascent (descent) was determined to find the proper direction of changing variables, and then the concentration was increased or decreased according to the sign of the main effect to improve the hydroxyl radical scavenging rate. The path of steepest ascent (descent) started from the zero level of fractional factorial design and moved along the path in which corn flour and KH_2PO_4 increased, while peptone decreased. The design and data obtained from the experiment are shown in the Table 5.

The highest scavenging rate was 45.72% when the concentrations of corn flour, peptone, and KH_2PO_4 were 5.5%, 0.365%, and 0.2% respectively. It suggested that the point was near the region of maximum scavenging rate response. Further optimization with CCD and RSM was around this point.

Table 5. Experimental results of the path of steepest ascent (descent).

	Corn flour (%)	Peptone (%)	KH_2PO_4 (%)	Scavenging rate (%)
Step length	2	0.035	0.025	
1	3.5	0.4	0.175	36.78
2	5.5	0.365	0.2	45.72
3	7.5	0.33	0.225	35.12
4	9.5	0.295	0.25	26.80
5	11.5	0.26	0.275	8.48

Data based on three trials.
Values are the mean.

CCD and RSM Analysis

CCD is a very useful tool to determine the optimal level of medium constituents and their interaction. Based on the fractional factorial design, corn flour, peptone, and KH_2PO_4 were selected for their significant effects on hydroxyl radical scavenging rate. The levels of the variables for the CCD experiment were selected according to the result of the path of steepest ascent (descent) experiment. The concentrations of these major nutrients tested are presented in Table 6. The CCD design matrix is presented in Table 7.

The RSM analysis for the optimization of medium constituents by SPSS 13.0 showed that the hydroxyl radical scavenging rate of crude EPS (Y2) was a function of the concentration of corn flour (x_1), peptone (x_2), and KH_2PO_4 (x_3). The experimental results of the CCD design were fitted with the second-order polynomial equation as

$$Y_2=48.91-1.944x_1+1.028x_2+0.360x_3+0.900x_1x_2 +0.720x_1x_3-0.085x_2x_3-4.239x_1^2-2.153x_2^2 -1.927x_3^2 \quad (6)$$

The regression equation was evaluated by the coefficient of correlation (R) and the determination coefficient (R^2). Here, the value of R (0.985) indicates a high agreement between the experimental and predicted values. The value of determination R^2 (0.970) indicates that the response model can explain 97% of the total variations. The value of adjusted determination coefficient (R^2=0.942) was also high enough to indicate the significance of the model.

The corresponding analysis of variance (ANOVA) is given in Table 8. It is evident that the calculated F value (35.324) is also greater than the tabulated F value [$F_{(9,10)}$= 4.94] at 0.01 level, which indicates that the second-order is highly significant. The significance of the regression coefficients of the model is shown in Table 9.

A t-test and P-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength between each independent variable. It can be seen that the linear effect of corn flour and peptone are significant (P<0.01). The quadratic effect of corn flour, peptone, and KH_2PO_4 (P<0.01) and the interaction effect of corn flour and peptone (P<0.05) are also significant. The interaction effect of corn flour×KH_2PO_4 and peptone×KH_2PO_4 are not significant.

The 3D response surface and the 2D contour plots of the regression model are used to explain the effects of the

Table 6. Variables range, code values, and levels of independent variables in CCD.

Variable	Parameter	Rang levels and code				
		1.682	1	0	−1	−1.682
x_1 (%)	Corn flour	7.18	6.5	5.5	4.5	3.82
x_2 (%)	Peptone	0.47	0.4	0.3	0.2	0.13
x_3 (%)	KH_2PO_4	0.42	0.35	0.25	0.15	0.08

Table 7. The matrix of the CCD experiment, and the corresponding experimental data.

Run	x_1	x_2	x_3	Scavenging rate (%)
1	1	1	1	40.52
2	1	1	−1	39.88
3	1	−1	1	37.24
4	1	−1	−1	35.30
5	−1	1	1	42.48
6	−1	1	−1	43.78
7	−1	−1	1	41.84
8	−1	−1	−1	43.74
9	1.682	0	0	34.64
10	−1.682	0	0	39.20
11	0	1.682	0	44.46
12	0	−1.682	0	41.18
13	0	0	1.682	45.10
14	0	0	−1.682	41.82
15	0	0	0	47.72
16	0	0	0	49.02
17	0	0	0	50.32
18	0	0	0	47.72
19	0	0	0	49.06
20	0	0	0	49.60

Data based on three trials.
Values are the mean.

Table 8. Analysis of variance (ANOVA) and model summary for the quadratic model of the CCD experiments.

Model		Sum of squares	df	Mean square	F	Sig.
1	Regression	407.458	9	45.273	35.324	0.000[a]
	Residual	12.817	10	1.282		
	Total	420.275	19			

R=coefficient of correlation=0.985; R^2=coefficient of determination=0.970; adjusted R^2=0.942.
[a]Significant at p<0.01.

Table 9. Model coefficients estimated by multiples linear regression (significance of regression coefficients).

Variable	Regression coefficient	t-Value	Sig.
x_1	−1.944	−6.332	0.000
x_2	1.028	3.348	0.007
x_3	0.360	1.173	0.268
x_1x_2	0.900	2.244	0.049
x_1x_3	0.720	1.795	0.103
x_2x_3	−0.085	−0.212	0.836
x_1^2	−4.239	−14.185	0.000
x_2^2	−2.153	−7.206	0.000
x_3^2	−1.927	−6.449	0.000

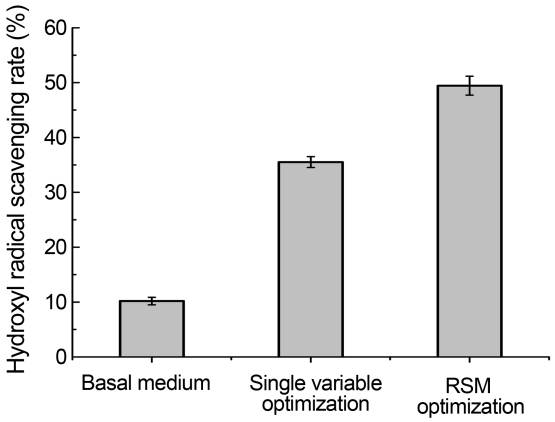

Fig. 2. The hydroxyl radical scavenging rate of EPS produced in basal medium, single variable optimization medium, and RSM optimization medium ($p<0.01$).

independent variables and interactive effects of independent variables on the response. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. From the 3D response surface plots and the 2D corresponding contour plots (Fig. 1), the optimal values of the independent variables and the corresponding response could be predicted, and the interaction between each independent variable pair could be understood.

The predicted optimal levels of corn flour, peptone, and KH_2PO_4 were obtained by applying the regression analysis to Eq. (6): x_1=−0.2046, x_2=0.1956, x_3=−0.05, and Y=49.23%, corresponding to X_1=5.300%, X_2=0.320%, and X_3=0.255%.

To confirm the result, a fermentation experiment was conducted according to the optimal fermentation medium. The practical hydroxyl radical scavenging rate of crude EPS at 1 mg/ml from submerged cultures of *I. obliquus* reached 49.44%, corroborating the validity and the effectiveness of this model. The antioxidation activity of the crude EPS from the optimal fermentation medium increased by 385% and 39% compared with those from the basal fermentation medium and the single variable optimization medium, respectively (Fig. 2).

Monosaccharide Compositions in The EPS

As shown in Table 10, the EPS from the basal medium contained mannose, glucose, and galactose with molar proportion at 23.33%, 36.67%, and 40.00%. The EPS from the single variable optimized medium was composed of rhamnose, xylose, mannose, glucose, and galactose with molar proportion at 1.61%, 3.23%, 29.84%, 20.97%, and 44.35%. The EPS from the RSM optimized medium was composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose with molar proportion at 1.45%, 3.63%, 2.17%, 15.94%, 50.00%, and 26.81%. The results clearly demonstrated that mannose, glucose, and galactose were the dominant monosaccharides in the three EPS samples,

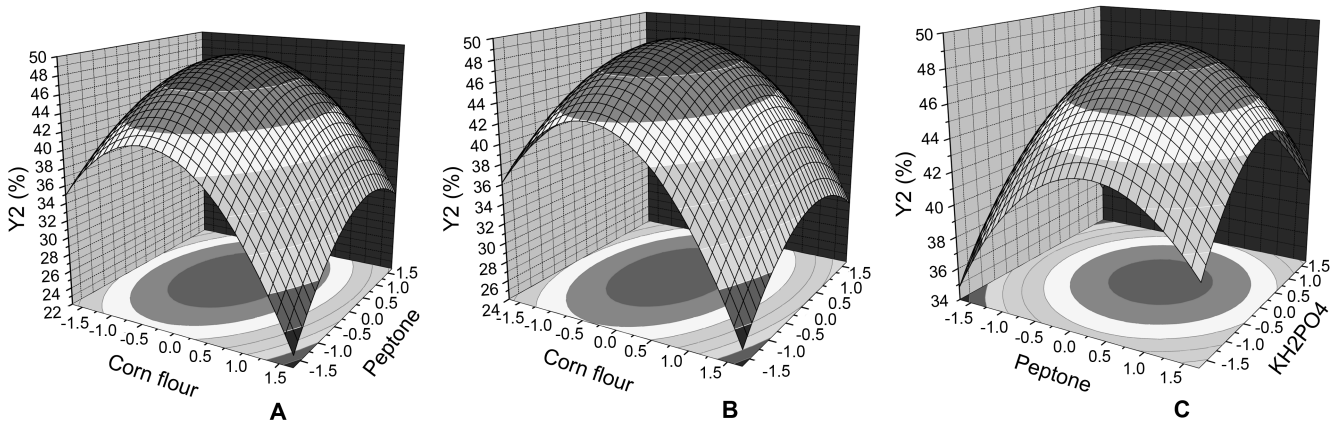

Fig. 1. Three-dimensional mesh plot and 2D contour plot of hydroxyl radical scavenging rate of *I. obliquus* crude exopolysaccharides. **A**. The effect of corn flour (X_1) and peptone (X_2) on hydroxyl radical scavenging rate (Y) with other components set at center level. **B**. the effect of corn flour (X_1) and KH_2PO_4 (X_3) on hydroxyl radical scavenging rate (Y) with other components set at center level. **C**. The effect of peptone (X_2) and KH_2PO_4 (X_3) on hydroxyl radical scavenging rate (Y) with other components set at center level.

Table 10. The monosaccharides components of EPSs obtained from three different kinds of fermentation media.

Medium	Constitutive sugar (unit, mol%)					
	Rha	Ara	Xyl	Man	Glu	Gal
Basal medium	0	0	0	23.33	36.67	40.00
Single variable optimized medium	1.61	3.23	0	29.84	20.97	44.35
RSM optimized medium	1.45	3.63	2.17	15.94	50.00	26.81

Rha: rhamnose; Ara: arabinose; Gal: galactose; Glu: glucose; Xyl: xylose; Man: mannose.

but the sorts of monosaccharides and molar proportion were different for those from the three kinds of medium

DISCUSSION

The effects of various carbon and nitrogen sources on the hydroxyl radical scavenging rate of *I. obliquus* crude EPS was studied based on the basic medium with one-factor design (Table 2). According to the result, when corn flour was used as the carbon source, the crude EPS had the maximum hydroxyl radical scavenging rate (34.20%). Corn flour is a kind of natural carbon source. It contains all kinds of nutrients such as biotin and thiamine that are important vitamins for *I. obliquus* growth. Therefore, corn flour not only serves as a carbon source but also provides rich and balanced nutrients to promote *I. obliquus* growth and metabolism. In the fractional factorial design experiment, the increase in the concentration of carbon source had positive effects on hydroxyl radical scavenging rate. The positive effects of the carbon source may be caused by the requirement of a large quantity of substrate to synthesize polysaccharides. The result is in agreement with previous works that selected EPS production as an indicator [21, 30, 35].

It is known that bioactivities of polysaccharides are dependent on the sorts of monosaccharides, glycoside bond types, molecular weight, microstructure, and configuration, *etc*. [34]. The biochemical properties of the polysaccharides with the high hydroxyl radical scavenging activity produced by the selected carbon and nitrogen sources in this study deserve further investigations.

In our preliminary study of the biochemical property–activity relationship of the EPS, the results from GC analysis evidently demonstrated the effect of the sorts of monosaccharides on the antioxidant activity of polysaccharides. The EPS obtained from the basal medium, which was only composed of mannose, glucose, and galactose without rhamnose, xylose, and arabinose, had a low antioxidant activity. The EPS obtained from the single variable optimized medium increased its activity, when rhamnose and arabinose appeared. High activities of the EPS from the RSM optimized medium were observed when sugars were composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose. The hydroxyl radical scavenging activity was enhanced with the increase of the sorts of monosaccharide components. Rhamnose, arabinose, and xylose may have an important role in hydroxyl radical scavenging activity, although they have small molar proportion in the EPS. The results are consistent with previous works [22, 34]. The carbon source of the single variable optimized and RSM optimized media was corn flour, whereas the basal medium contained only glucose. The results indicate that adding corn flour is useful for increasing the sorts of monosaccharide components of EPS and enhancing the hydroxyl radical scavenging activity. The EPSs from single variable and RSM optimized media almost have the same monosaccharide components but with one sugar compound different. In addition, the molar proportion of glucose of the RSM optimized EPS is higher, and the proportion of mannose and galactose are lower, than that of the single variable optimized EPS. These differences may contribute to the stronger hydroxyl radical scavenging activity of the RSM optimized EPS.

It should be noted that the RSM is a local optimum method and the evolutionary operation is a good candidate for global optimization. The optimum value is effective in a certain domain with RSM. The merit of RSM is its simpleness and it can be mastered easily compared with evolutionary methods. The RSM can combine the experimental design and the mathematical model, and get the accurate experimental result through the local experimental correlate fit and the global function. Although we cannot guarantee the calculated condition is the best condition in the global domain, it is the best one in the selected domain. To confirm the result, a fermentation experiment was conducted according to the optimal fermentation medium. The result corroborated the validity and the effectiveness of this model.

In conclusion, the bioactivity of fungal exopolysaccharides is strongly influenced by fermentation medium composition. The optimal concentrations of medium components (w/v) made up corn flour 5.30%, peptone 0.32%, KH_2PO_4 0.255%, $MgSO_4$ 0.02%, and $CaCl_2$ 0.01%. With this optimized medium, the antioxidant activity per unit of EPS from *I. obliquus* was significantly enhanced.

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