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Technology optimization for polysaccharides (POP) extraction from the fruiting bodies of *Pleurotus ostreatus* by Box–Behnken statistical design

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ABSTRACT

Response surface methodology (RSM), based on A Box–Behnken design (BBD), was used to optimize the extraction conditions of polysaccharides (POP) from the fruiting bodies of *Pleurotus ostreatus*. Four independent variables such as extraction temperature (°C), ratio of water to raw material, number of extraction and extraction time (h) were investigated. The experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis and also analyzed by appropriate statistical methods. The optimum extraction conditions, determined by the 3-D response surface and contour plots derived from the mathematical models, were as follows: extraction temperature 94.9 °C, ratio of water to raw material 22, number of extraction 4, and extraction time 2.7 h. Under these conditions, the experimental value was 64.1 ± 1.42, which is well matched with value predicted by the model.
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1. Introduction

In Asia, mushrooms have long been used as traditional foods and medicines on account of their immunomodulatory and antitumor properties (Sun & Liu 2009a, 2009b; Sun et al., 2008; Tong et al., 2009). Edible mushrooms contain an abundance of resources that possess a multitude of biological activities. Especially the polysaccharides extracted from edible mushrooms exhibit lower toxicity and fewer side effects than chemical drugs. Therefore, mushrooms represent a potential valuable resource for natural drugs.

Pleurotus ostreatus is a traditional Chinese medicinal and edible fungus distributed in Heilongjiang Province of China, which is a Tricholomataceae fungus belonging to the Basidiomycetes. People also called it "Pinggu" in China. The medicinally beneficial functions of *P. ostreatus*, such as their antioxidant, immunoloregulation, antitumor, antiviral, antiinflammatory, antibiotic and cholesterol-lowering activities, are well known worldwide (Jayakumar, Ramesh, & Geraldine, 2006; Jayakumar, Thomas, & Geraldine, 2007; Regina, Elisabeth, Jamile, Jorge, & Sandra, 2008; Sun & Liu, 2009a; Wang, Gao, & Ng, 2000).

When many factors and interactions affect desired response, response surface methodology (RSM) is an effective tool for optimizing the process, which was originally described by Box and Wilson (Box & Wilson, 1951). RSM is a collection of statistical and mathematical techniques that has been successfully used to determine the effects of several variables and optimize processes (Atkinson & Donev, 1992). RSM has been successfully applied for optimizing conditions in food and pharmaceutical research (Batistuti, Barros, & Areas, 1991; Ibanoglu & Ainsworth, 2004; Shieh, Koehler, & Akoh, 1996; Varnalis, Brennan, MacDougall, & Gilmour, 2004; Vega, Balaban, Sims, O'Keefe, & Cornell, 1996). The main advantage of RSM is to reduce number of experimental trials needed to evaluate multiple variables and their interactions. Therefore, it is less laborious and time consuming than other approaches required optimizing a process (Giovanni, 1983). Usually, it applies an experimental design such as Box–Behnken (BBD), central composite (CCD) and Doehlert designs (DDD) to fit a second-order polynomial by a least squares technique. An equation is used to describe how the test variables affect the response and determines the interrelationship among the variables (Liu, Miao, Wen, & Sun, 2009).

The objective of this work was to optimize the production process of POP from the fruiting bodies of *P. ostreatus* using RSM. Employing a BBD (4 factors and 3 levels) to study the effects of extraction temperature, ratio of water to raw material, number of extraction and extraction time on the purity of POP.

2. Materials and methods

2.1. Materials

The fruiting bodies of *P. ostreatus* was purchased from Heilongjiang Tianjin Fungi Co., Ltd. Phenol was from Beijing Dingguo

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Biotechnology Co., Ltd. D-glucose was form Amresco Inc. All other reagents were of analytical grade.

2.2. Extraction of crude polysaccharides and determination of polysaccharides yield

The fruiting bodies of *P. ostreatus* (2000 g) was ground in a blender to obtain a fine powder (Particle diameter size: 400–500 μm) and then was extracted 3× with 80% EtOH at 75 °C for 6 h to defat and remove some colored materials, oligosaccharides, and some small molecule materials under reflux. The pretreated samples were separated form the organic solvent through the nylon cloth (Pore diameter: 38 μm). Each dried pretreated sample (20 g) was extracted by water in a designed temperature, water to raw material ratio, number and time. The water extraction solutions were separated form insoluble residue by centrifugation (2000 *g* for 10 min, at 20 °C), and then precipitated by the addition of dehydrated alcohol to a final concentration of 80% (v/v). The precipitates (POP) collected by centrifugation (2000 *g* for 10 min, at 20 °C) were washed by dehydrated alcohol for three times and dried under reduced pressure. The sugar content was measured by phenol–sulfuric method using D-glucose as a standard (Dubois et al., 1956). The purity (%) of POP is calculated as the sugar content of extraction/dried crude polysaccharide weight.

2.3. Experimental design and statistical analysis

After determining the preliminary range of the extraction variables through a single-factor test, a BBD with four independent variables (*X*1, extraction temperature; *X*2, ratio of water to raw material; *X*3, number of extraction; *X*4, extraction time) at three levels was performed (Box & Behnken, 1960). For statistical calculation, the variables were coded according to

$$\chi_i = (X_i - X_0)/\Delta X_i \quad (1)$$

where χ_i is a coded value of the variable; X_i the actual value of variable; X_0 the actual value of the X_i on the center point; and ΔX_i the step change value. The range of independent variables and their levels are presented in Table 1, which was based on the results of preliminary experiments. The purity of POP was the dependent variables. As seen from Table 2, the complete design consisted of 27 experimental points, and the experiment was carried out in a random order.

Data from the BBD were analyzed by multiple regressions to fit the following quadratic polynomial model.

$$Y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki}\chi_i + \sum_{i=1}^{4} \beta_{kii}\chi_i^2 + \sum_{i<j=2}^{4} \beta_{kij}\chi_i\chi_j \quad (2)$$

Y represents the response function. β_{k0} is an intercept. Where β_{ki}, β_{kii}, and β_{kij} are the coefficients of the linear, quadratic, and interactive terms, respectively. And accordingly χ_i, χ_{ii}, and χ_{ij} represent the coded independent variables, respectively. The fitted polynomial equation is expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions (Lu, Engelmann, Lila, & Erdman, 2008). According to the analysis of variance, the regression coefficients of individual linear, quadratic, and interaction terms were determined. The regression coefficients were then used to make statistical calculation to generate dimensional and contour maps from the regression models. SAS (version 8.0, USA) software package was used to analyze the experimental

Table 1
Independent variables and their levels used in the response surface design.

Independent variables	Factor level		
	−1	0	1
*X*1: Extraction temperature (°C)	90	95	100
*X*2: Ratio of water to raw material	16	19	22
*X*3: Number of extraction	2	3	4
*X*4: Extraction time (h)	2	2.5	3

Table 2
Response surface Box–Behnken design and results for the purity of POP.

Run	X_1/extraction temperature (°C)	X_2/ratio of water to raw material	X_3/number of extraction	X_4/extraction time (h)	Polysaccharides purity (%)
1	−1(90)	−1(16)	0(3)	0(2.5)	37.2
2	−1(90)	1(22)	0(3)	0(2.5)	59.1
3	1(100)	−1(16)	0(3)	0(2.5)	41.2
4	1(100)	1(22)	0(3)	0(2.5)	56.1
5	0(95)	0(19)	−1(2)	−1(2)	43.1
6	0(95)	0(19)	−1(2)	1(3)	51.6
7	0(95)	0(19)	1(4)	−1(2)	55.1
8	0(95)	0(19)	1(4)	1(3)	59
9	−1(90)	0(19)	0(3)	−1(2)	47.1
10	−1(90)	0(19)	0(3)	1(3)	53.2
11	1(100)	0(19)	0(3)	−1(2)	49.3
12	1(100)	0(19)	0(3)	1(3)	58.9
13	0(95)	−1(16)	−1(2)	0(2.5)	30.9
14	0(95)	−1(16)	1(4)	0(2.5)	47
15	0(95)	1(22)	−1(2)	0(2.5)	57.2
16	0(95)	1(22)	1(4)	0(2.5)	61.2
17	−1(90)	0(19)	−1(2)	0(2.5)	47.2
18	−1(90)	0(19)	1(4)	0(2.5)	58.1
19	1(100)	0(19)	−1(2)	0(2.5)	52.1
20	1(100)	0(19)	1(4)	0(2.5)	58.4
21	0(95)	−1(16)	0(3)	−1(2)	37.3
22	0(95)	−1(16)	0(3)	1(3)	43.2
23	0(95)	1(22)	0(3)	−1(2)	58.4
24	0(95)	1(22)	0(3)	1(3)	61.2
25	0(95)	0(19)	0(3)	0(2.5)	58.6
26	0(95)	0(19)	0(3)	0(2.5)	58.6
27	0(95)	0(19)	0(3)	0(2.5)	58.6

data. The *P*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. The effect of different extraction temperature on the polysaccharides yields

Different extraction temperature was set at 70, 75, 80, 85, 90, 95, and 100 °C, respectively, to investigate the influence of extraction temperature on the purity of POP when the other reaction conditions were set as follows: ratio of water to raw material 19, number of extraction 3, and extraction time 2.5 h. Fig. 1a indicated that the maximum purity of POP was reached when extraction temperature increase from 90 to 95 °C. And then there was no increase when extraction temperature continued to rise. Therefore, the range of 90–100 °C was adopted to be optimal extraction temperature in this work.

3.2. The effect of different ratio of water to raw material on the polysaccharides yields

The effect of different ratio of water to raw material (10, 13, 16, 19, 22, 25, and 28) on the purity of POP was seen in Fig. 1b, when the other three factors (extraction temperature, number of extraction, and extraction time) were fixed at 0 level (95 °C, 3 times, and 2.5 h). The result implied the purity of POP was suddenly enhanced to the critical value (58.6 ± 0.58%) at the ratio of 19, and thereafter there was a little rise when the ratio of water to raw material continued to increase.

3.3. The effect of different number of extraction on the polysaccharides yields

The purity of POP affected by different number of extraction (1–7 times) was seen in Fig. 1c, when other three factors (extraction temperature, ratio of water to raw material, and extraction time) were fixed at 95 °C, 19, and 2.5 h. The results showed that the purity of POP had obvious increase within the number of extraction (2–4). The purity of POP unexpectedly get the value (58.0 ± 0.99%) when the samples were extracted for 3 times, and then the purity of POP no longer obviously changed, when the number of extraction increasing.

3.4. The effect of different extraction time on the polysaccharides yields

Extraction time is not constant during the extraction stages. Here, extraction time was, respectively, set at 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 h to examine the influence of extraction time on the purity of the POP when other reaction conditions were as follows: extraction temperature 95 °C, ratio of water to raw material 19, and number of extraction 3. As shown in Fig. 1d, the purity of POP reached a maximum percentage of 58.9 ± 1.13 when the extraction time was 2.5 h. After this point, the purity of POP started to maintain a descending dynamic equilibrium with increasing the extraction time. Therefore, extraction time range of 2–3 h was considered in the present work.

3.5. Optimization of the procedure

3.5.1. Statistical analysis and the model fitting

RSM optimization is more advantageous than the traditional single parameter optimization in that it saves time, space, and

Table 3
Fit statistics for *Y*.

	Master model	Predictive model
Mean	51.81	51.81
R-square	98.49%	98.49%
Adjusted *R*-square	96.72%	96.72%
Coefficient of variation	2.950056	2.950056

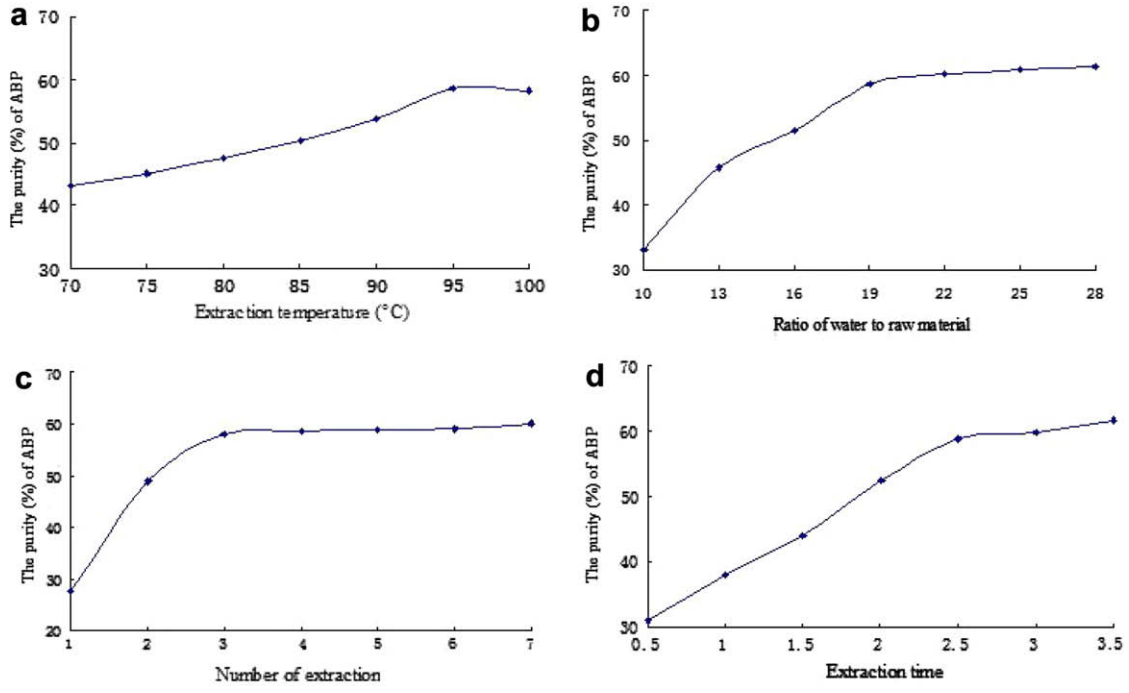

Fig. 1. Effect of different extraction parameters on the purity of POP (extraction temperature, °C; ratio of water to raw material; number of extraction; and extraction time, h).

raw material. There were a total of 27 runs for optimizing the four individual parameters in the BBD. Table 2 showed the experimental conditions and the results of purity of POP according to the factorial design. By applying multiple regression analysis on the experimental data, the response variable and the test variables were related by the following second-order polynomial equation:

$$Y = 58.6 + 1.175 * X1 + 9.7 * X2 + 4.725X3 + 3.066667X4 - 3.025X1 * X1 - 1.75X1 * X2 - 1.15X1 * X3 + 0.875X1 * X4 - 6.5125X2 * X2 - 3.025X2 * X3 - 0.775X2 * X4 - 2.65X3 * X3 - 1.15X3 * X4 - 3.0875X4 * X4 \quad (3)$$

The results of the analysis of variance, goodness-of-fit and the adequacy of the models were summarized in Table 3. The determination coefficient (R^2 = .9849) was showed by ANOVA of the quadratic regression model, indicating that only 1.51% of the total variations was not explained by the model. The value of the adjusted determination coefficient (Adj. R^2 = .9672) also confirmed that the model was highly significant. At the same time, a very low value 2.95 of coefficient of the variation (C.V.) clearly indicated a very high degree of precision and a good deal of reliability of the experimental values. The model was found to be adequate for prediction within the range of experimental variables. The regression coefficient values of Eq. (3) were listed in Table 4. The *P*-values were used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller was the value of *P*, the more significant was the corresponding coefficient. It can be seen from this table that the linear coefficients ($X1$, $X2$, $X3$, $X4$), a quadratic term coefficient ($X1^2$, $X2^2$, $X3^2$, $X4^2$) and cross product coefficients ($X1 * X2$, $X2 * X3$) were significant, with very small *P* values ($P < 0.05$). The other term coefficients were not significant ($P > 0.05$). The full model filled Eq. (3) was made three-dimensional and contour plots to predict the relationships between the independent and dependent variables.

3.5.2. Optimization of extraction conditions of polysaccharides

The graphical representations of the regression Eq. (3), called the response surfaces and the contour plots were obtained using SAS version 8.0, and the results of purity of POP affected by extraction temperature, ratio of water to raw material, number of extraction, and extraction time were presented in Figs. 2 and 3. RSM plays a key role in identifying the optimum values of the independent variables efficiently, under which dependent variable could arrive the maximum response. In the 3-D response surface plot and contour plot, the purity of POP was obtained along with two continuous variables, while the other two variables were fixed constant at their respective zero level (center value of the testing ranges). In the two figures, the maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when there is a perfect interaction between the independent variables (Muralidhar, Chirumamil, Marchant, & Nigam, 2001). The independent variables and maximum predicted values from the figures corresponded with the optimum values of the dependent variables obtained by the equations.

In Figs. 2a and 3a, when the 3-D response surface plot and the contour plot were developed for the purity of POP with varying extraction temperature and ratio of water to raw material at fixed number of extraction (0 level) and extraction time (0 level), the purity of POP increased with the increasing ratio of water to raw material, and reached the peak value rapidly at extraction temperature 94.9 °C, then dropped from 94.9 to 100 °C. The Figs. 2b and 3b showed the 3-D response surface plot and the contour plot at varying extraction temperature and number of extraction at fixed

Table 4
Regression coefficients of the predicted quadratic polynomial model.

Parameter	Estimate	Standard error	*t*-Ratio	*P*-value
$X1$	1.175	0.441228	2.663025	0.020676
$X2$	9.7	0.441228	21.98412	0.0001
$X3$	4.725	0.441228	10.70876	0.0001
$X4$	3.066667	0.441228	6.950307	0.0001
$X1 * X1$	−3.025	0.661841	−4.57058	0.000643
$X1 * X2$	−1.75	0.764228	−2.28989	0.04094
$X1 * X3$	−1.75	0.764228	−1.50479	0.158238
$X1 * X4$	0.8755	0.764228	1.144946	0.274554
$X2 * X2$	−6.5125	0.661841	−9.83997	0.0001
$X2 * X3$	−3.025	0.764228	−3.95824	0.001899
$X2 * X4$	−0.775	0.764228	−1.01409	0.330549
$X3 * X3$	−2.65	0.661841	−4.00398	0.001749
$X3 * X4$	−1.15	0.764228	−1.50479	0.158238
$X4 * X4$	−3.0875	0.661841	−4.66502	0.000546

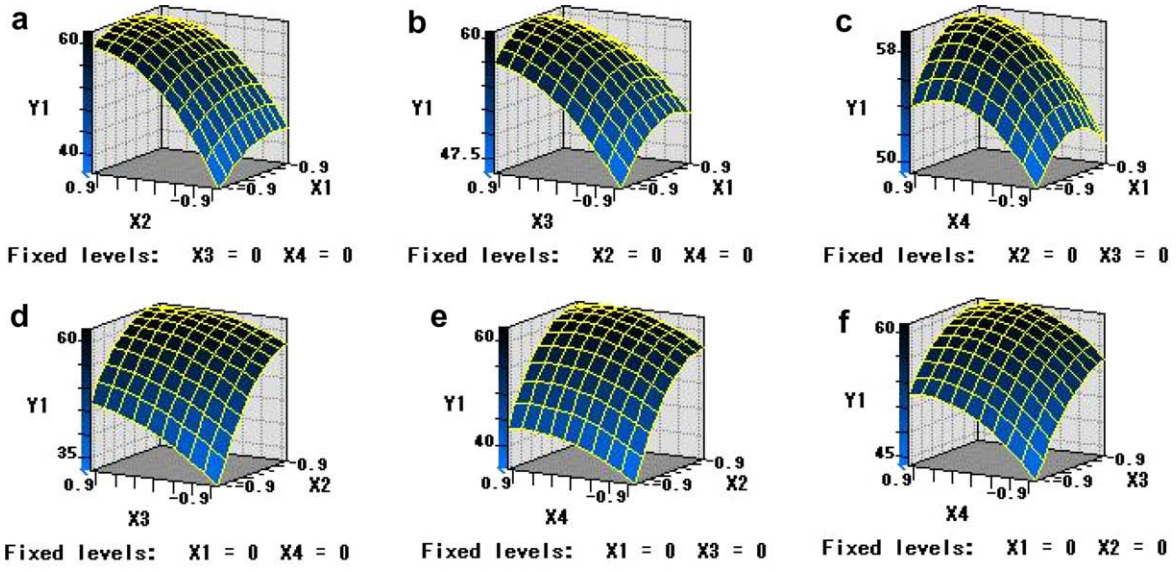

Fig. 2. Response surface polts (3-D) showing the effects of variables ($X1$: extraction temperature, °C; $X2$: ratio of water to raw material; $X3$: number of extraction; and $X4$: extraction time, h) on the response *Y*.

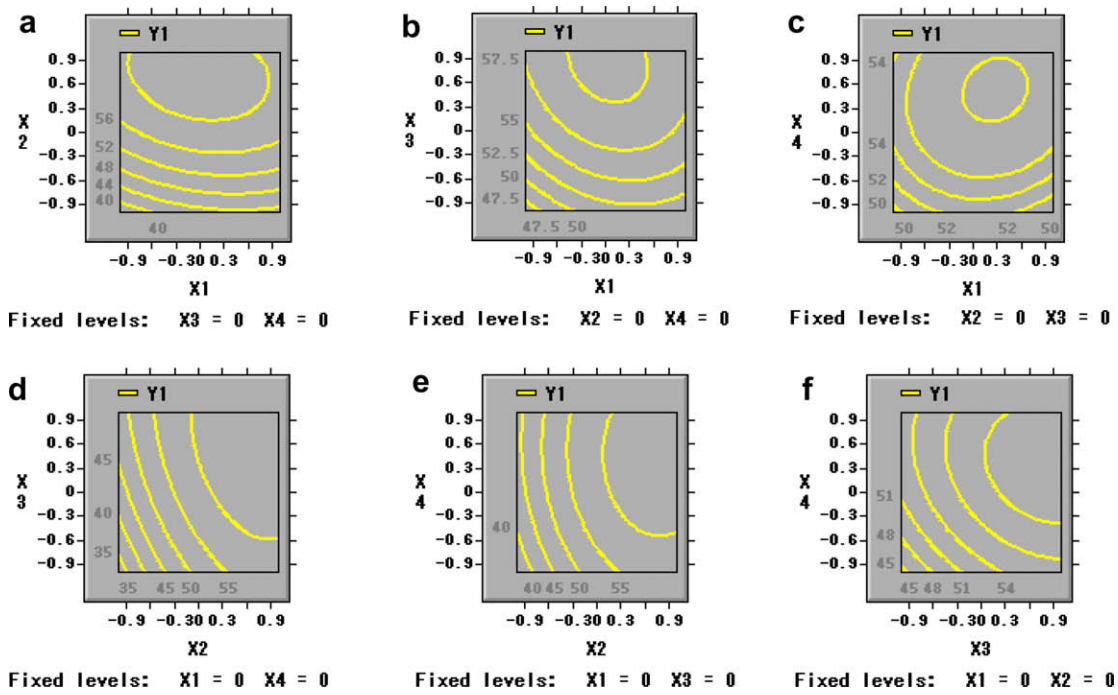

Fig. 3. Contour polts (2D) showing the effects of variables (*X*1: extraction temperature, °C; *X*2: ratio of water to raw material; *X*3: number of extraction; and *X*4: extraction time, h) on the response *Y*.

ratio of water to raw material (0 level) and extraction time (0 level). The purity of POP increased with the increasing number of extraction and reached the maximum value when extraction temperature at the threshold level of 94.9 °C. Beyond this level, purity of POP slightly decreased. The purity of POP affected by different extraction temperature and extraction time was seen in Figs. 2c and 3c, when the other two variables (ratio of water to raw material and number of extraction) were fixed at 0 level. It can be seen that maximum purity of POP can be achieved when extraction temperature and extraction time are 94.5 °C and 2.7 h, respectively. The Figs. 2d and 3d showed the 3-D response surface plot and the contour plot at varying ratio of water to raw material and number of extraction at fixed extraction temperature (0 level) and extraction time (0 level). As in the case of POP extraction, ratio of water to raw material and number of extraction used both had a positive impact on the purity of POP. There was a linear increase in the purity of POP with increase in the ratio of water to raw material and number of extraction. The contours were slightly inclined to the horizontal showing that there was a significant interaction between the two parameters. Thus the ratio of water to raw material and number of extraction were significantly positive correlated to the purity of POP. The 3-D response surface plot and the contour plot based on independent variables ratio of water to raw material and extraction time were shown in Figs. 2e and 3e, while the other two independent variables, extraction temperature and number of extraction were kept at a zero level. An increase in the purity of POP could be significantly achieved with the increases of ratio of water to raw material. It was obvious that the purity of POP was increased with the increasing extraction time from 2 to 2.7 h, meaning that further increases of extraction time would not increase the purity of POP any longer. The Figs. 2f and 3f showed the 3-D response surface plot and the contour plot at varying number of extraction and extraction time at fixed extraction temperature (0 level) and ratio of water to raw material (0 level). From two figures, we can conclude that the purity of POP increased with increase in number of extraction, and purity of POP was found to increase rapidly with increase of extraction time from 2 to 2.7, but beyond 2.7 h, the purity of POP decreased with increasing extraction time.

According to Figs. 2 and 3, and the above single parameter study, it can be concluded that optimal extraction condition of POP from the fruiting body of *P. ostreatus* were extraction temperature 94.9 °C, ratio of water to raw material 22, number of extraction 4 and extraction time 2.7 h. Among the four extraction parameters studied, ratio of water to raw material was the most significant factor to affect the purity of POP, followed by number of extraction, extraction time and extraction temperature according to the regression coefficients significance of the quadratic polynomial model (Table 4) and gradient of slope in the 3-D response surface plot (Fig. 2).

3.5.3. Verification of predictive model

The suitability of the model equations for predicting optimum response values was tested under the conditions: extraction temperature 94.9 °C, ratio of water to raw material 22, number of extraction 4 and extraction time 2.7 h. This set of conditions was

Table 5
Predicted and experimental values of the responses at optimum conditions.

Optimum condition				Purity of POP (%)	
Extraction temperature	Ratio of water to raw material	Number of extraction	Extraction time	Experimental[a]	Predicted
94.9 °C	22	4	2.7 h	64.1 ± 1.17	63.2

[a] Mean ± standard deviation ($n = 3$).

determined to be optimum by the RSM optimization approach and was also used to validate experimentally and predict the values of the responses using the model equation. A mean value of 64.1 ± 1.17 (N = 3), obtained from real experiments, demonstrated the validation of the RSM model, indicating that the model was adequate for the extraction process (Table 5).

4. Conclusion

The extraction conditions have significant effects on the purity of POP. Using the contour and surface plots in RSM was effective for estimating the effect of four independent variables (extraction temperature, °C; ratio of water to raw material; number of extraction; and extraction time, h). The optimum set of the independent variables was obtained graphically in order to obtain the desired levels of crude polysaccharides extraction. The optimal experimental purity of 64.1 ± 1.17% was obtained when the optimum conditions of POP extraction was extraction temperature 94.9 °C, ratio of water to raw material 22, number of extraction 4 and extraction time 2.7 h. Under these optimized conditions the experimental purity of POP agreed closely with the predicted yield.

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