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Optimization of polysaccharides (ABP) extraction from the fruiting bodies of *Agaricus blazei* Murill using response surface methodology (RSM)

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ABSTRACT

Response surface methodoloty (RSM) was used to optimize the extraction conditions of polysaccharides (ABP) from the fruiting body of *Agaricus blazei*. A central composite design (CCD) was used for experimental design and analysis of the results to obtain the optimal processing parameters. 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 3-D response surface plot and the contour plot derived from the mathematical models were applied to determine the optimal conditions. The optimum extraction conditions were as follows: extraction temperature 91 °C, ratio of water to raw material 14, number of extraction 6, and extraction time 2.1 h. Under these conditions, the experimental value was 65.8 ± 1.42, which is well in close agreement with value predicted by the model.

1. Introduction

In recent years, mushroom polysaccharides have drawn the attention of chemists and immunobiologists on account of their immunomodulatory and antitumor properties. *Agaricus blazei* is an edible mushroom distributed originally in Brazil and presently cultivated in other areas, including Japan, China, and Indonesia, which is an Agaricaceae fungus belonging to the Basidiomycotina. In China its fruiting body is generally called "Baxi Mogu" and is used as a health food and a home remedy. Its chemical components have been widely studied, including steroids (Kawagishi, Katsumi, et al., 1988; Kawagishi, Nomura, et al., 1988), lipids (Takaku, Kimura, & Okuda, 2001), lectins (Kawagishi, Katsumi, et al., 1988; Kawagishi, Nomura, et al., 1988), and various polysaccharides (Dong, Yao, Yang, & Fang, 2002; Kawagish et al., 1989; Mizuno et al., 1990). Some polysaccharides and protein-bound polysaccharides isolated from *A. blazei* have been shown to have anticancer activity directly or through immunomodulation (Fujimiya, Suzuki, Katakura, & Ebina, 1999; Fujimiya et al., 1998; Mizuno et al., 1999; Ohno et al., 2001; Sorimachi et al., 2001; Takaku et al., 2001). More biological activities of *A. blazei*, such as anti-mutagenic (Delmanto et al., 2001), anti-bacterial (Osaki, Kato, Yamamoto, Okubo, & Miyazaki, 1994), anti-oxidant (Tetsuo, Yuki, & Tetsuya, 2008), anti-diabetic, and anti-angiogenic activities (Kim, Kim, Choi, & Lee, 2005; Kimura, Kido, Takaku, Sumiyoshi, & Baba, 2004), have been reported. Additionally, polysaccharides form *A. blazei* significantly induces apoptosis *via* reduction of Bcl-2 levels, caspase-3 activation, and Poly ADP Ribose Polymerase degradation (Jin, Moon, Choi, Lee, & Kim, 2007). However, so far there is not any information published about the optimization of extraction conditions for polysaccharides from the fruiting bodies of *A. blazei*.

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 central composite design (CCD) or Box–Behnken design (BBD) 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 determine the interrelationship among the variables.

The purpose of the present study was to optimize the production process of ABP from the fruiting body of *A. blazei* using RSM.

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

Independent variables	Factor level				
	−2	−1	0	1	2
*X*1, Extraction temperature (°C)	80	85	90	95	100
*X*2, Ratio of water to raw material	8	10	12	14	16
*X*3, Number of extraction	2	3	4	5	6
*X*4, Extraction time (h)	1	1.5	2	2.5	3

Employing a CCD (four factors and five levels) to study the effects of extraction temperature, ratio of water to raw material, number of extraction, and extraction time on the purity of ABP from the fruiting body of *A. blazei*.

2. Materials and methods

2.1. Materials

The fruiting body of *A. blazei* was purchased from Fuzhou Eaststar Biotechnology Co., Ltd. Phenol was from Beijing Dingguo Biotechnology Co., Ltd. D-glucose was form Amresco Inc. All other reagents were of analytical grade.

2.2. Extraction and purity determination of ABP

The fruiting body of *A. blazei* (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 (ABP) 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, Gilles, Hamilton, Rebers, & Smith, 1956). The purity (%) of ABP 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 CCD 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 five levels was performed (Box & Behnken, 1960). For statistical calculation, the variables were coded according to

$$x_i = (X_i - X_0)/\Delta X_i \tag{1}$$

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 centre 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 ABP was the dependent variables. As seen from Table 2, the complete design consisted of 31 experimental points (16 factorial points, eight axial points and seven center points), and the experiment was carried out in a random order.

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

$$Y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} x_i + \sum_{i=1}^{4} \beta_{kii} x_i^2 + \sum_{i<j=2}^{4} \beta_{kij} x_i x_j \tag{2}$$

Table 2
Response surface central composite design and results for the purity of ABP.

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(85)	−1(10)	−1(3)	−1(1.5)	54.24
2	−1(85)	−1(10)	−1(3)	1(2.5)	57.33
3	−1(85)	−1(10)	1(5)	−1(1.5)	57.47
4	−1(85)	−1(10)	1(5)	1(2.5)	60.23
5	−1(85)	1(14)	−1(3)	−1(1.5)	60.36
6	−1(85)	1(14)	−1(3)	1(2.5)	61.34
7	−1(85)	1(14)	1(5)	−1(1.5)	61.12
8	−1(85)	1(14)	1(5)	1(2.5)	62.12
9	1(95)	−1(10)	−1(3)	−1(1.5)	58.65
10	1(95)	−1(10)	−1(3)	1(2.5)	57.02
11	1(95)	−1(10)	1(5)	−1(1.5)	60.21
12	1(95)	−1(10)	1(5)	1(2.5)	60.43
13	1(95)	1(14)	−1(3)	−1(1.5)	61.34
14	1(95)	1(14)	−1(3)	1(2.5)	62.28
15	1(95)	1(14)	1(5)	−1(1.5)	62.23
16	1(95)	1(14)	1(5)	1(2.5)	63.67
17	−2(80)	0(12)	0(4)	0(2)	57.53
18	2(100)	0(12)	0(4)	0(2)	59.45
19	0(90)	−2(8)	0(4)	0(2)	57.90
20	0(90)	2(16)	0(4)	0(2)	64.22
21	0(90)	0(12)	−2(2)	0(2)	60.04
22	0(90)	0(12)	2(6)	0(2)	65.38
23	0(90)	0(12)	0(4)	−2(1)	57.54
24	0(90)	0(12)	0(4)	2(3)	58.02
25	0(90)	0(12)	0(4)	0(2)	63.72
26	0(90)	0(12)	0(4)	0(2)	63.76
27	0(90)	0(12)	0(4)	0(2)	63.74
28	0(90)	0(12)	0(4)	0(2)	63.71
29	0(90)	0(12)	0(4)	0(2)	63.75
30	0(90)	0(12)	0(4)	0(2)	63.75
31	0(90)	0(12)	0(4)	0(2)	63.76

Y_k represent 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 x_i, x_{ii}, and x_{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 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 purity of ABP

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 ABP when the other reaction conditions were set as follows: ratio of water to raw material 12, number of extraction 4, and extraction time 2 h. Fig. 1a indicated that the purity of ABP increases with the increasing extraction temperature and reached the peak value from 90 to 95 °C. And then there is no increase when extraction temperature continued to rise. Therefore, 80–100 °C was considered to be optimal extraction temperature in this experiment.

3.2. The effect of different ratio of water to raw material on the purity of ABP

The purity of ABP affected by different ratio of water to raw material (6, 8, 10, 12, 14, 16, and 18) was seen in Fig. 1b, when the other three factors (extraction temperature, number of extraction, and extraction time) were fixed at 90 °C, 4 times, and 2 h. The result implied the purity of ABP was enhanced to the critical value (64.2 ± 0.29%) at the ratio of 14, and then it maintain a mild slope when the ratio of water to raw material increasing.

3.3. The effect of different number of extraction on the purity of ABP

The purity of ABP 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 90 °C, 12, and 2 h. The purity of ABP unexpectedly gets the value (64.5 ± 0.45%) when the samples were extracted for 4 times. However, the purity of ABP no longer obviously changed, when the number of extraction continued to increase.

3.4. The effect of different extraction time on the purity of ABP

The purity (%) of ABP affected by different extraction time (0.5, 1, 1.5, 2, 2.5, 3, and 3.5 h) was seen in Fig. 1d, when the other three factors (extraction temperature, ratio of water to raw material and number of extraction) were fixed at 90 °C, 12 and 4 times. The purity of ABP reached a maximum percentage of 63.1 ± 0.23 when the extraction time was 2.5 h. After this point, the purity of ABP started to maintain a descending dynamic equilibrium with increasing the extraction time. This situation maybe due to the polysaccharide hydrolyses under some temperature and long extraction time. Therefore, extraction time range of 1–3 h was adopted 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 raw material. There were a total of 31 runs for optimizing the four individual parameters in the CCD. Table 2 shows the experimental

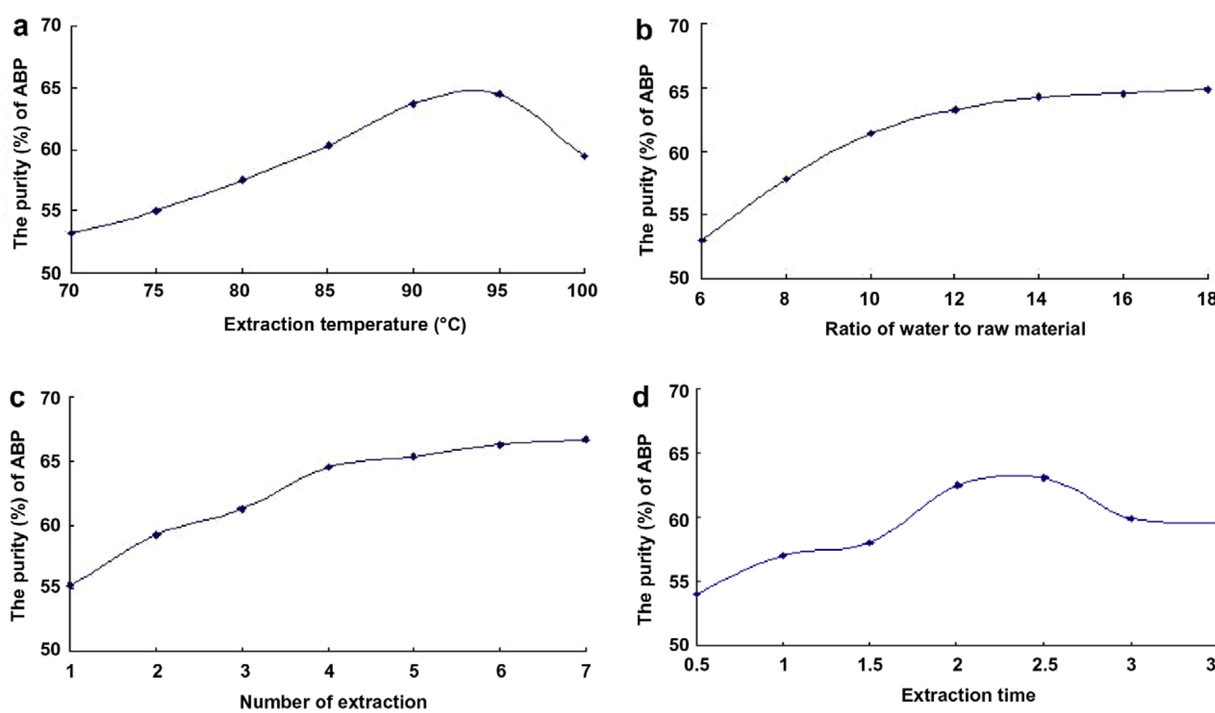

Fig. 1. Effect of different extraction parameters on the purity of ABP (extraction temperature, °C; ratio of water to raw material; number of extraction; and extraction time, h).

conditions and the results of purity of ABP according to the factorial design. Maximum purity of ABP (65.38%) was recorded under the experimental conditions of extraction temperature 90 °C, ratio of water to raw material 12, number of extraction 6, and extraction time 2 h. By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following second-order polynomial equation:

$$Y = 63.74143 + 0.644167 * X1 + 1.73 * X2 + 1.066667 * X3 + 0.406667 * X4 - 1.314107 * X1 * X1 - 0.15375 * X1 * X2 - 0.02625 * X1 * X3 - 0.42875 * X1 * X4 - 0.671607 * X2 * X2 - 0.455 * X2 * X3 - 0.005 * X2 * X4 - 0.259107 * X3 * X3 + 0.1275 * X3 * X4 - 1.491607 * X4 * X4 \quad (3)$$

The results of the analysis of variance, goodness-of-fit and the adequacy of the models are summarized in Table 3. The determination coefficient ($R^2 = 0.97$) was showed by ANOVA of the quadratic regression model, indicating that only 3.0% of the total variations was not explained by the model. The value of the adjusted determination coefficient (Adj $R^2 = 0.9437$) also confirmed that the model was highly significant. At the same time, a very low value 1.09 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 is 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 are 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 is the value of P, the more significant is the corresponding coefficient. It can be seen from this table that the linear coefficients ($X1$, $X2$, $X3$, $X4$), a quadratic term coefficient ($X1^2$, $X2^2$, $X4^2$) and cross product coefficients ($X1 * X4$, $X2 * X3$) were significant, with very small P values ($P < 0.05$). The other term coefficients are not significant ($P > 0.05$). The full model filled Eq. (3) was made three-dimentional and contour plots to predict the relationships between the independent and dependent variables.

3.5.2. Optimization of extraction conditions of ABP

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 ABP affected by extraction temperature, ratio of water to raw material, number of extraction, and extraction time are 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 ABP 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 correspond with the optimum values of the dependent variables obtained by the equations.

Table 3
Fit statistics for *Y*.

	Master model	Predictive model
Mean	60.85	60.85
R-square	97.00%	97.00%
Adjusted *R*-square	94.37%	94.37%
Coefficient of variation	1.08891	1.08891

Table 4
Regression coefficients of the predicted quadratic polynomial model.

Parameter	Estimate	Standard error	*t* Ratio	*P* Value
$X1$	0.644167	0.13525	4.762779	0.000212
$X2$	1.73	0.13525	12.79111	<0.0001
$X3$	1.066667	0.13525	7.88662	<0.0001
$X4$	0.40667	0.13525	3.006774	0.008361
$X1 * X1$	−1.314107	0.123906	−10.6057	<0.0001
$X1 * X2$	−0.15375	0.165647	−0.92818	0.367106
$X1 * X3$	−0.02625	0.165647	−0.15847	0.87607
$X1 * X4$	−0.42875	0.165647	−2.58834	0.019804
$X2 * X2$	−0.671607	0.123906	−5.42029	<0.0001
$X2 * X3$	−0.455	0.165647	−2.74681	0.014328
$X2 * X4$	−0.005	0.165647	−0.03018	0.976293
$X3 * X3$	−0.259107	0.123906	−2.09116	0.052825
$X3 * X4$	0.1275	0.165647	0.769709	0.452687
$X4 * X4$	−1.491607	0.123906	−12.0382	<0.0001

In Figs. 2a and 3a, when the 3-D response surface plot and the contour plot were developed for the purity of ABP 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 ABP increased with the increasing ratio of water to raw material, and reached the peak value rapidly at extraction temperature 91 °C, then dropped from 91 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 ratio of water to raw material (0 level) and extraction time (0 level). The purity of ABP increased with the increasing number of extraction and reached the maximum value when extraction temperature at the threshold level of 91 °C. Beyond this level, purity of ABP slightly decreased. The purity of ABP 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 ABP can be achieved when extraction temperature and extraction time are 91 °C and 2.1 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 ABP extraction, ratio of water to raw material and number of extraction used both had a positive impact on the purity of ABP. There was a linear increase in the purity of ABP 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 ABP. 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 ABP could be significantly achieved with the increases of ratio of water to raw material. It is obvious that the purity of ABP was increased with the increasing extraction time from 1 to 2.1 h, meaning that further increases of extraction time would not increase the purity of ABP 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 ABP increase with increase in number of extraction, and purity of ABP is found to increase rapidly with increase of extraction time from 1 to 2.1, but beyond 2.1 h, the purity of ABP decreased with increasing extraction time.

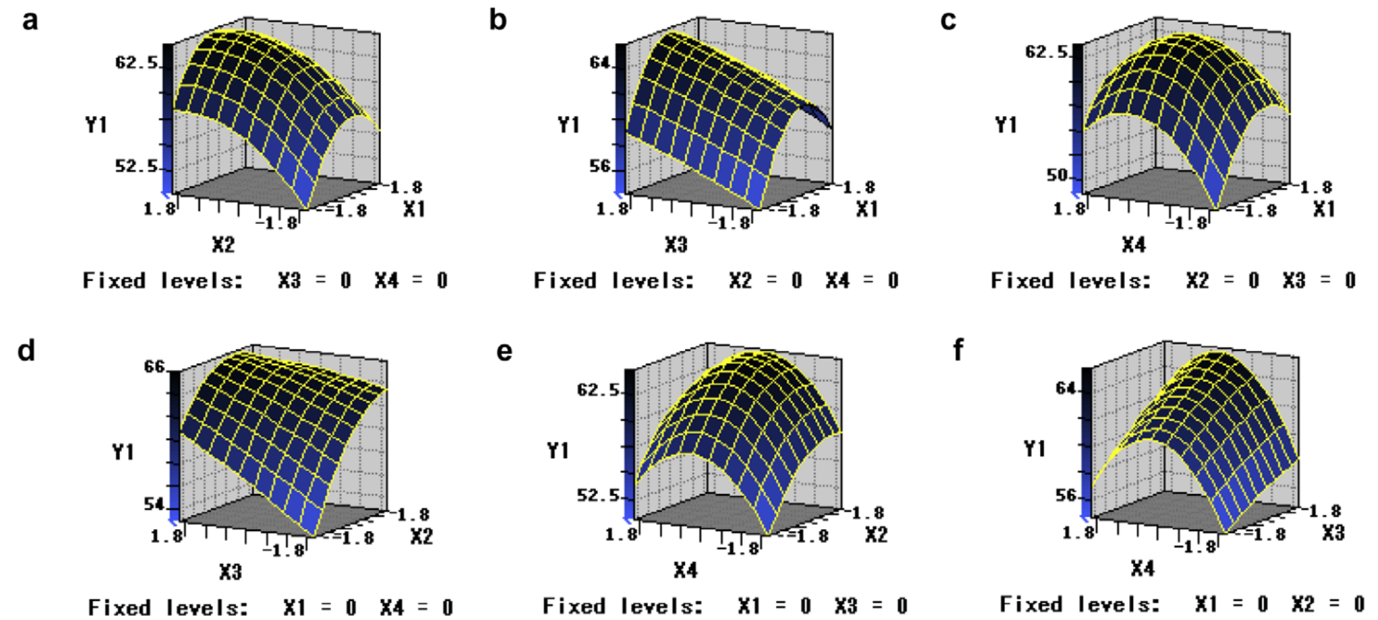

Fig. 2. Response surface plots (3-D) showing the effects of variables (*X*1: extraction temperature, °C; *X*2: ratio of water to raw material; *X*3: number of extraction; and *X*4: extraction time, h) on the response *Y*.

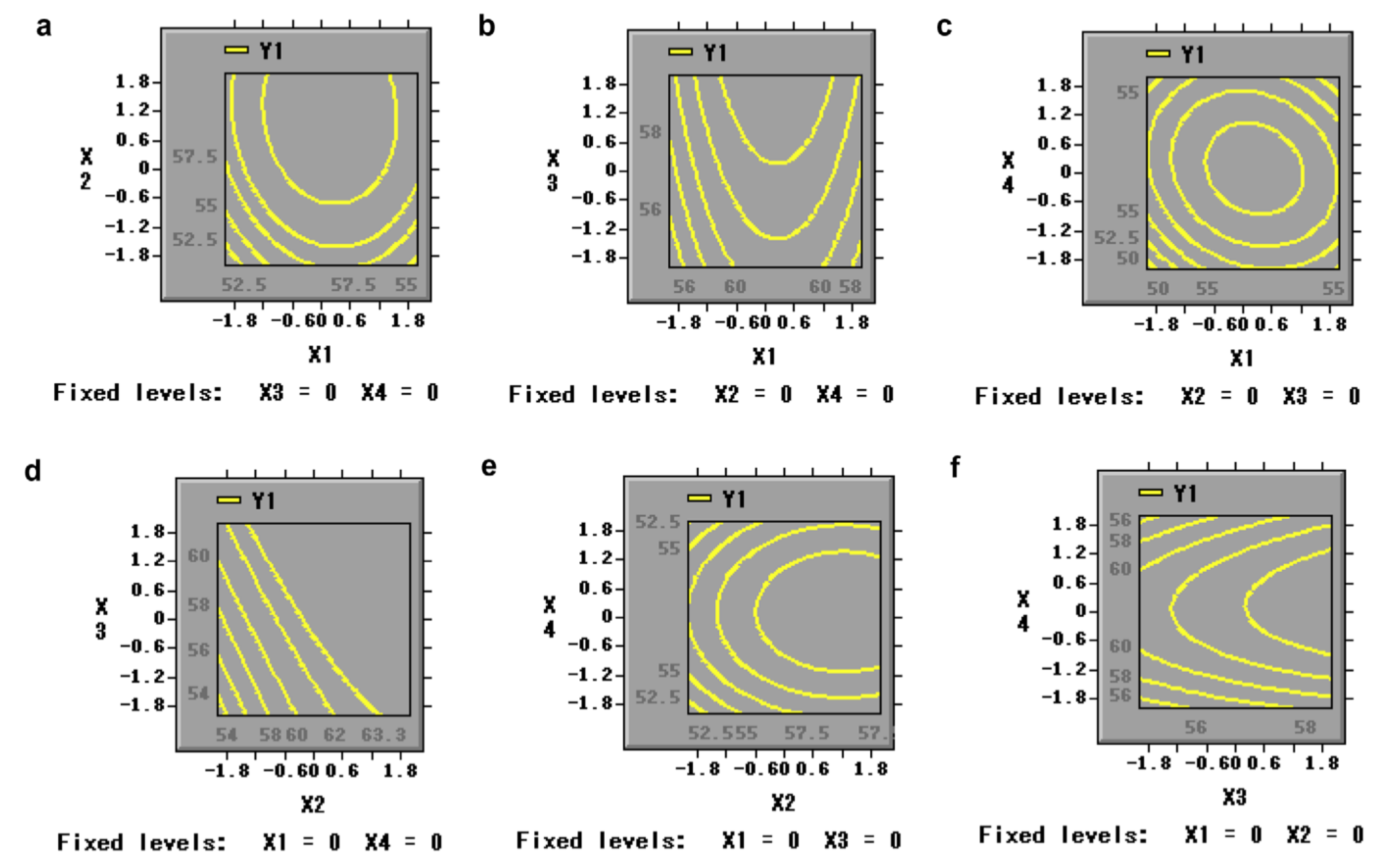

Fig. 3. Contour plots (2-D) showing the effects of variables (*X*1: extraction temperature, °C; *X*2: ratio of water to raw material; *X*3: number of extraction; and *X*4: extraction time, h) on the response *Y*.

According to Figs. 2 and 3, and the above single parameter study, it can be concluded that optimal extraction condition of ABP from the fruiting body of *A. blazei* are extraction temperature 91 °C, ratio of water to raw material 14, number of extraction 6 and extraction time 2.1 h. Among the four extraction parameters studied, ratio of water to raw material is the most significant factor to affect the purity of ABP, followed by number of extraction, extraction temperature and extraction time 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).

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

Optimum condition				Purity of ABP (%)	
Extraction temperature	Ratio of water to raw material	Number of extraction	Extraction time	Experimental[a]	Predicted
91 °C	14	6	2.1 h	65.8 ± 1.42	65.47

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

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 91 °C, ratio of water to raw material 14, number of extraction 6 and extraction time 2.1 h. This set of conditions was 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 65.8 ± 1.42 ($N = 3$), 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 ABP. 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 65.8 ± 1.42% was obtained when the optimum conditions of ABP extraction was extraction temperature 91 °C, ratio of water to raw material 14, number of extraction 6 and extraction time 2.1 h. Under these optimized conditions the experimental purity of ABP agreed closely with the predicted yield.

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