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Application of Box-Behnken design in optimisation for polysaccharides extraction from cultured mycelium of *Cordyceps sinensis*

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ABSTRACT

A three-level Box-Behnken design, combined with the canonical and ridge analyses, was employed to optimise the process parameters for polysaccharide extraction from cultured mycelium of *Cordyceps sinensis*, one of the most valued traditional Chinese medicines and health foods. The critical factors selected for the investigation were extraction temperature, duration of time and number of times. The experimental results were fitted with a second-order polynomial equation by a multiple regression analysis and more than 96% of the variation could be predicted by the models. The canonical analysis of surface responses revealed that the three eigenvalues had different signs, indicating a saddle stationary surface. The optimal conditions for extraction of polysaccharides from the cultured mycelium of *C. sinensis* were determined, using the ridge analysis, as extracting 110 min at 88.9 °C for three times. Under the optimal conditions the corresponding response value predicted for polysaccharide production was 15.85%, which was confirmed by validation experiments.

Keywords: *Cordyceps sinensis*; Polysaccharides; Box-Behnken design; Canonical analysis; Ridge analysis

1. Introduction

Cordyceps sinensis (Berk.) Sacc. is an entomogenous fungus belonging to *Ascomycota*, *Sordariomycetes*, *Hypocreales*, *Clavicipitaceae* (Lindau) O.E. Erikss., *Cordyceps* (Fr.) Link (Kirk et al., 2001). It is popularly referred to as the Chinese Caterpillar Fungus or 'Dong Chong Xia Cao' summer-plant, winter-worm) in Chinese. Early English translations of 'Hia Tsao Tong Tchong' and 'Hea Tsaon Tsong Chung' are also found in the western literature (Pegler et al., 1994). As a traditional Chinese medicine and health foods, *C. sinensis* is considered to have the similar medicinal effects of ginseng and deer velvet. It has been used in China for thousands of years and has been regarded as a celebrated drug in the Chinese Pharmacopeia since 1963 (CPCMH, 1964). It has been used to treat a wide range of conditions, including respiratory, liver and cardiovascular diseases, hyposexuality and hyperlipidemia.(Wang, 1995; Zhu et al., 1998). However, the natural resources of *C. sinensis* are very limited due to its confined geographic distribution on the Tibetan Plateau and over exploitation in recent years. For alternatives, culture of the fungus in submerged fermentation to produce mycelium in large quantity has been proved as a promising way to meet the needs of human consumption and to reduce the pressure on natural resources of the species which is in danger (Yao, 2004).

Many biologically active polysaccharides have been isolated from various fungi and some of them are now used in clinics, e.g. lentinan from *Lentinula edodes* (Berk.) Pegler (Zheng et al., 2005), schizophyllan from *Schizophyllum commune* Fr. (Krosl and Korbelik, 1994; Kubala et al., 2003) and protein-bound polysaccharide K (PSK) from *Coriolus versicolo* (L.) Quél. (Matsunaga et al., 1998; Kanazawa et al., 2004). These

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polysaccharides have been shown to enhance and stimulate the immune system of human and of mice, and are thus called biological response modifiers (Wasser, 2002).

Polysaccharides from *C. sinensis* have been demonstrated to have many biological activities, e.g. antitumour (Yang et al., 2005), antioxidation (Li et al., 2003), hypoglycaemic (Kiho et al., 1999) and hypocholesterolemic effects (Kiho et al., 1996). As polysaccharides are mainly present in the cell wall and within cytoplasm, improvement of the extraction rate of polysaccharides from cultured mycelium is evidently important to the efficiency of extraction process. Sun et al. (2003) studied the effects of granularity of mycelial powder, ratio of water to mycelium, duration of time of extraction and pH on the yield of polysaccharides with the one-variable-at-a-time method, for a so-called *C. sinensis* strain named '*Paecilomyces hepiali* chen et dai sp nov' [*sic!*] (An invalid fungal name, see Jiang and Yao (Jiang and Yao, 2002, 2003). It is clearly not a strain of real *C. sinensis*). Yu et al. (2002) optimised the extraction parameters, including duration of time of ethanol precipitation, the ratio of extraction solvent to fermentation broth and pH for the polysaccharide extraction from fermentation broth of *C. sinensis*. Numerous papers have been published in regard to polysaccharides from this fungus (Gong et al., 1990; Li et al., 2003; Wu et al., 2005), but none was on the optimisation of process parameters for polysaccharide extraction from the cultured mycelium of *C. sinensis*.

The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum condition of factors for desirable responses (Box et al., 1978). The optimisation process of this methodology involves studying the response of the statistically designed combinations, estimating the coefficients by fitting it in a mathematical model that fits best the experimental conditions, predicting the response of the fitted model and checking the adequacy of the mode. The most common designs, i.e. central composite design (CCD) and Box-Behnken design (BBD), of the principal response surface methodology have been widely used in various experiments (Box et al., 1978; Dean and Voss, 1999). Box-Behnken, a spherical and revolving design, has been applied in optimisation of chemical and physical processes (Oscar et al., 1999; Qiu and Chen, 1999; Muthukumar et al., 2003) because of its reasoning design and excellent outcomes.

In the present work, Box-Behnken design, followed by canonical and ridge analyses, was employed to optimise the process parameters of polysaccharide extraction from the cultured mycelium of *C. sinensis* so as to facilitate the further and reasonable exploration of this treasured fungus.

2. Materials and methods

2.1. Fungal strain

The strain, No. 762, used in this study was originally isolated from *C. sinensis* collected from Sichuan, China by this laboratory. It was maintained on potato dextrose agar (PDA) supplemented with 5% wheat bran and 0.5% peptone at 4 °C. The Internal Transcribed Spacer (ITS1-5.8S-ITS2) of nuclear ribosomal DNA (nrDNA) was amplified from the culture and 496 bp of the fragment were obtained from DNA sequencing. The sequence was compared with a data set generated in this laboratory containing ITS sequences from dried specimens and living strains of *C. sinensis* obtained from various regions of the Tibetan Plateau to confirm the identity of the strain.

2.2. Inoculum preparation and submerged culture

The strain was first incubated on the same medium as for the stock at 18 °C for 45 d in Petri dish and then transferred to 500 ml Erlenmeyer flasks with the same medium without agar (Dong and Yao, 2005) by punching a 5-mm agar disc from the 45-d culture with a sterilized cutter. The flasks, containing 100 ml of liquid medium, were rotated at 100 rpm, at 18 ± 1 °C for 45 d.

The mycelium was harvested by centrifugation for 15 min at $8000 \times g$ to separate it from the liquid medium. After repeated washing with distilled water, the mycelial pellets were lyophilized using a VirTis freeze dryer (VirTis Co., Gardiner, New York) for later experiments.

2.3. Extraction of mycelial polysaccharides

The powder of lyophilized mycelium (1.000 g) was extracted several times with 20 volumes of distilled water at 70–90 °C for 1–3 h each time. After vacuum filtration, the aqueous extracts were combined and concentrated to one-third of its total volume in vacuum. The resulting concentrated liquor was mixed with three times of its volume of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated polysaccharides were centrifuged at $8000 \times g$ for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 65 °C to a constant weight and the polymer was weighted by a scale (Sartorious ALC-110.4, Germany). The product yield was measured at the (w/w) % of polysaccharides per unit mass of lyophilized mycelium.

2.4. Box-Behnken design

According to the principle of Box-Behnken design, extraction temperature, duration of time and number of times, which were identified to have strong effects on the response in preliminary one-factor-at-a-time experiments, were taken as the variables tested in a 15-run experiment to determine their optimum levels. As shown in Table 1, the three factors chosen for this study were designated as X_1, X_2, X_3 and prescribed into three levels, coded +1, 0, −1 for high, intermediate and low value, successively. Three test variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X} \quad i = 1, 2, 3$$

where x_i is the coded value of an independent variable; X_i is the actual value of an independent variable; X_0 is the actual value of an independent variable at centre point; and ΔX is the step change value of an independent variable. Table 2 shows the Box-Behnken design matrix of the experiment of 15 trials. All experiments were performed in triplicate and the averages of polysaccharide yield were taken as response.

For predicting the optimal point, a second-order polynomial model was fitted to correlate relationship between independent variables and response (polysaccharide yield). For the three factors, the equation is

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \\ & + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 \end{aligned}$$

Table 1 – Levels and code of variable chosen for Box-Behnken design

Variables	Symbol		Coded levels		
	Uncoded	Coded	−1	0	+1
Temperature (°C)	X_1	x_1	70	80	90
Extraction duration of time (h)	X_2	x_2	1	2	3
Extraction number of times	X_3	x_3	1	2	3

where Y is the predicted response; β_0 is model constant; x_1, x_2 and x_3 are independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross-product coefficients; and β_{11}, β_{22} and β_{33} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

2.5. *Statistical analyses of data*

The analyses of regression and variance were carried out using the RSREG procedure of the Statistical Analysis System (SAS) (Version 8.01, SAS Institute Inc., Cary, NC, USA). Both canonical and ridge analyses were also conducted.

The following SAS program was performed:

```
Data polysaccharides;
input x1-x3 y1@;
cards;
;
Proc RsReg data=polysaccharides;
Model Y1=x1 x2 x3/lackfit;
ridge max;
Run;
```

3. Results

3.1. *Model building and statistical significance test*

A 15-run Box-Behnken design with three factors and three levels, including three replicates at the centre point, was used for fitting a second-order response surface. The three centre point runs were added to provide as a measure of process stability and inherent variability. The considerable variation in the polysaccharide yield from the cultured mycelium of *C. sinensis* under different conditions was shown in Table 2.

Table 2 – Box-Behnken design with experimental and predicted values of polysaccharide yield

Run	x_1	x_2	x_3	Polysaccharide yield (%)		
				Predicted	Experimental	Difference
1	−1	−1	0	9.93	9.36	−0.57
2	−1	1	0	10.83	10.32	−0.51
3	1	−1	0	15.15	15.66	0.51
4	1	1	0	12.05	12.62	0.57
5	0	−1	−1	9.74	9.88	0.14
6	0	−1	1	11.42	12.37	0.95
7	0	1	−1	8.64	9.74	1.10
8	0	1	1	10.32	10.18	−0.14
9	−1	0	−1	12.67	13.60	0.93
10	1	0	−1	12.75	12.61	−0.14
11	−1	0	1	11.21	12.36	1.15
12	1	0	1	17.57	17.66	0.09
13	0	0	0	10.66	11.82	1.16
14	0	0	0	10.66	11.73	1.07
15	0	0	0	10.66	11.21	0.55

The model adequacy was checked by an F-test and the determination coefficient R^2. The analysis of variance (Table 3) showed that this regression model was highly significant ($P < 0.01$) with F-value of 14.90. The value of 7.39 for lack of fit implies that it is not significant comparing to the pure error. The fitness of the model was further confirmed by a satisfactory value of determination coefficient, which was calculated to be 0.9638, indicating that 96.38% of the variability in the response could be predicted by the model. Furthermore, the predicted polysaccharide yields by the final quadratic model, along with the corresponding values observed, were given in Table 2. The agreement between the yield predicted by the model and the experimental data is very strong, with a difference less than 1.30% of extraction rate.

The regression coefficients, along with the corresponding P-values, for the model of polysaccharide yield from the cultured mycelium of *C. sinensis* were shown in Table 4. The P-values were used as a tool to check the significance of each coefficient, which also indicates the interaction effects between each independent variable. The regression of all the linear term and quadratic coefficients of x_1^2 and x_2^2 were significant and two cross-products (x_1 x_2 and x_1 x_3) were also significant.

Table 3 – Analysis of variances in the regression model for optimisation of polysaccharide extraction from cultured mycelium of *Cordyceps sinensis*

Source of variations	Degree of freedom	Sum of square	Mean square	F-value	Determination coefficient (R^2)
Regression	9	69.74	7.75	14.90**	0.9638
Residual	5	2.62	0.52		
Pure error	14	72.36			
Linear	3	28.94	9.65	87.73*	
Quadratic	3	25.86	8.62	78.36*	
Cross-product	3	14.94	4.98	45.27*	
Lack of fit	3	2.41	0.80	7.39	
Total error	2	0.22	0.11		

$F_{0.05}(9, 5) = 4.78$; $F_{0.01}(9, 5) = 10.15$, $F_{0.05}(3, 2) = 19.16$; $F_{0.01}(3, 2) = 99.17$.

* Significant at 5 % level.

** Significant at 1 % level.

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Table 4 – Regression analysis of a full second-order polynomial model for optimisation of polysaccharide extraction from the cultured mycelium of *C. sinensis*

Variables	Coefficient based on actual value	Standard error	P-value	Coefficient based on coded value
Intercept	126.414167	25.677685	0.0044^{**}	11.586667
x_1	−3.087792	0.612149	0.0040^{**}	1.613750
x_2	14.697083	3.354507	0.0071^{**}	−0.551250
x_3	−12.759167	3.354507	0.0126^{*}	0.842500
$x_1 \times x_1$	0.019592	0.003769	0.0035^{**}	1.959167
$x_2 \times x_1$	−0.100000	0.036208	0.0397^{*}	−1.000000
$x_2 \times x_2$	−1.555833	0.376861	0.0091^{**}	−1.555833
$x_3 \times x_1$	0.157250	0.036208	0.0074^{**}	1.572500
$x_3 \times x_2$	−0.512500	0.362077	0.2161	−0.512500
$x_3 \times x_3$	0.511667	0.376861	0.2326	0.511667

** Significant at 1% level.
* Significant at 5% level.

Table 5 – Canonical analysis based on the coded and actual values

Variables	Coded value	Actual value	Predicted response
X_1	−0.215977	77.840228	11.21%
X_2	−0.024768	1.975232	
X_3	−0.503814	1.491860	

Table 6 – Eigenvalues and eigenvectors

Eigenvalues	Eigenvectors		
	x_1	x_2	x_3
2.38	0.91	−0.14	0.40
0.17	−0.41	−0.02	0.91
−1.64	0.12	0.99	0.07

The polynomial model for polysaccharide yield (Y) was regressed by considering only the significant terms and shown as below:

$$Y = 11.59 + 1.61x_1 - 0.55x_2 + 0.84x_3 + 1.96x_1^2 - x_1x_2 - 1.56{x_2}^2 + 1.57x_1x_3$$

3.2. *Canonical and ridge analyses*

To determine the shape of the fitted response and the estimated stationary point, the canonical analysis of response surface was performed with SAS. According to the model, the predicted response at the stationary point ($X_1 = 77.84$, $X_2 = 1.98$, $X_3 = 1.50$, shown in Table 5) was 11.21%.

As shown in Table 6, the three eigenvalues had different signs, indicating that the stationary point for this model was a saddle point. Therefore, the estimated surface did not have a unique optimum and a ridge analysis was performed to determine the optimum.

The results of ridge analysis (Table 7) indicated that all the three variables tested, i.e. extraction temperature, duration of time and number of times, were positively related to the response, and the optimal level of them was determined as 88.9 °C, 110 min and three times, successively, with a predicted extraction rate of 15.85%.

In order to confirm the predicted results, three further experiments using the optimum extraction parameters determined above were performed and the value of 15.9–16.2% (mean value 16.10%) of polysaccharides was obtained, with relative deviation of 1.90% between the mean and the predicted values.

4. Discussion

It is well known that the conventional optimisation technique, e.g. one-factor-at-a-time method, is not only tedious and time-consuming, but also misleading of result interpretation, especially for the interactions among different factors which they are unable to detect. The orthogonal array method, coupled with variance analysis, has proved to be a cost-effective optimisation strategy that can be used to assign experimental factors in a series of experimental trials (Wang and Yang, 2003), but it cannot fit the results into a regression equation to locate the optimum level through the entire space of the tested independent variables. The response surface methodology is an efficient statistical technique for the

Table 7 – Estimated ridge of maximum response for polysaccharide yield

Radii	Yield (%)	Standard error	Actual value		
			x_1	x_2	x_3
0.0	11.586667	0.418090	80.000000	2.000000	2.000000
0.1	11.799986	0.416796	80.864628	1.975345	2.043776
0.2	12.060170	0.413058	81.747009	1.955476	2.086586
0.3	12.367659	0.407336	82.637462	1.937750	2.128697
0.4	12.722621	0.400444	83.532344	1.921162	2.170323
0.5	13.125137	0.393609	84.429945	1.905249	2.211609
0.6	13.575251	0.388517	85.329346	1.889770	2.252646
0.7	14.072986	0.387306	86.230003	1.874586	2.293500
0.8	14.618359	0.392432	87.131575	1.859612	2.334212
0.9	15.211379	0.406350	88.033834	1.844792	2.374814
1.0	15.852055	0.431090	88.936622	1.830090	2.415329

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optimisation of multiple variables in order to predict the best conditions with a minimum number of experiments. In comparison with orthogonal design and variance analysis, Box-Behnken design, one of the designs of the response surface methodology, allows calculations to be made of the response at intermediate levels which were not experimentally studied. A three-level Box-Behnken design was employed in the present study and the optimal conditions were determined through a minimal experiment number compared with other designs.

The strong agreement between the yield predicted by the final quadratic model and the experimental results (Table 2), and the variance analysis of the second-order polynomial model and the value for lack of fit (Table 3) indicate that the accuracy and general ability of the polynomial model are very good. The analysis of response trends using the model is considered to be reasonable.

Although many experiments are completed at the canonical analysis (Liu et al., 1999; Kincl et al., 2005) the ridge analysis is very useful to find out the maximum response which may occur in the experiment when the results of the canonical analysis shows a saddle, stationary point and no unique optimum in the estimated surface (Lin, 1992; Zhuang et al., 2000). Through the ridge analysis, the optimal extraction temperature, duration of time and number of times for polysaccharide extraction were determined in this study with a good predicted extraction rate without further experimental work. A mean value of 16.10% of polysaccharides was obtained in three further validation experiments using the optimum extraction parameters, with a relative deviation of 1.90% comparing with the predicted value (15.85%). The good correlation between these two results verifies the validity of the response model and the existence of optimal point.

The biological activities of polysaccharides are closely related to their dimensional structure (Christophe et al., 1998). In addition to the improvement of extraction rate, the influence of extraction parameters on the structure and thus on the bioactivity of polysaccharides should also be taken into consideration in the process optimisation. The relationship between the structure and the activities of mycelial polysaccharides from *C. sinensis* is an on-going project in this laboratory.

5. Conclusion

In order to optimise the process parameters of polysaccharide extraction from the cultured mycelium of *C. sinensis*, Box-Behnken design was applied to investigate three critical variables, *viz.* temperature, duration of time and number of times of extraction, in the present work. Through the canonical and ridge analyses of the second-order polynomial model, a maximum extraction rate 16.10% was obtained under the following conditions: extracting three times at 88.9 °C, with 110 min per time.

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