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Optimization of Extraction Procedure for Chinese Truffle *Tuber sinense* Polysaccharides and Effect of the Polysaccharides on Phagocytosis of Macrophage *in vivo*

[1,2]Ling Zhao, [1,2]Kaiyu Wang, [3]Xun Wang, [1]Dan Huang, [1,2]Juchun Lin,
[3]Defang Chen, [1,2]Gang Ye and [1,2]Cheng Lv
[1]College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, China
[2]Key Laboratory of Animal Disease and Human Health of Sichuan Province,
Sichuan Agricultural University, Ya'an, China
[3]College of Animal Science and Technology, Sichuan Agricultural University, Ya'an, China

Abstract: In this study, Response Surface Methodology (RSM) was employed to optimize the extraction conditions (extraction temperature, ratio of water to raw material and extraction time) of polysaccharides from the fruiting bodies of Chinese truffle *Tuber sinense*. A Box-Behnken design was used for experimental design and analysis of the results to obtain the optimal processing parameters. The optimal conditions were extraction temperature 94.75°C, ratio of water to raw material 16.41:1, extraction time 2.67 h. According to analysis, extraction temperature was the most significant factor to affect the yield of polysaccharides from Chinese truffle *Tuber sinense*. Pharmacological experiments indicated that CTP had potent immunomodulatory effects in immunosuppression mice model. At the dose of 50, 100 and 200 mg kg^{-1} body weight, a significant increase ($p<0.01$) in phagocytosis of macrophage was observed.

Key words: Chinese truffle *Tuber sinense*, polysaccharides, extraction, optimization, macrophage phagocytosis

INTRODUCTION

Truffle, known as underground gold are hypogeous and ectomycorrhizal fungi which lived in symbiosis with trees and some shrubs. Truffle as an edible mushroom was considered one of expensive delicacies in the world because of its organoleptic properties such as unique and wonderful aroma. In Europe, truffle has been used for >1000 years. The commercial value of truffle exceeds some rare edible fungi such as *Tricholoma matsutake* and *Cordyceps sinensis*. Truffle is mainly distributed in some European countries especially France, Spain and Italy. Recently, a great deal of Chinese truffle *Tuber sinense* is found in Sichuan, Yunnan in China and Panzhihua city have been the distribution center of Chinese truffle *Tuber sinense*.

Previous reports have showed that black truffles are rich in protein, 17 amino acids, unsaturated fatty acids, vitamins, zinc, manganese, iron as well as sphingolipids, cerebroside, truffles polysaccharides and other large metabolites. Many ingredients from the fruiting-bodies of truffles have been isolated and they showed important biological activities (Al-Laith, 2010; Gao *et al*., 2004; Splivallo, 2007). Polysaccharide is one of important ingredients which has payed great attention to its bioactivities. In the last few years, modern pharmacological research about truffle polysaccharides clearly demonstrated its immunomodulating, antitumor (Hu *et al*., 1994) and antioxidant activities (Guo *et al*., 2010; Qiang *et al*., 2010).

Response Surface Methodology (RSM) is an affective statistical technique for optimizing complex processes. It is less laborious and time-consuming than other approaches required to optimize a process (Giovanni, 1983).

The main aim of this study was to optimize extraction condition of the polysaccharides from the fruiting body of Chinese truffle *Tuber sinense* using RSM. Besides, enhancing effect of truffle polysaccharide on mice macrophage *in vivo* was investigated.

MATERIALS AND METHODS

Dried fruiting bodies of Chinese truffle *Tuber sinense* were obtained from Panzhihua, Sichuan province, China. Cyclophosphamide was from Depu pharmaceutical Co., Ltd. (Shanxi province, China). Phenol was from Beijing Solarbio S and T Co., Ltd. (Beijing, China), sulfuric

Corresponding Author: Kaiyu Wang, Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an, China

acid, ethanol and glucose were from the Cheng Du Kelong Chemical Factory (Chengdu, China). All other chemicals were of analytical grade.

Extraction of crude polysaccharides of truffle: To remove lipids and pigments, the dried fruiting bodies of truffle were first grinded to powder by a miller and the powder was submerged in 4 volumes of 95% ethanol twice for 12 h at room temperature. The pretreated samples were separated from the organic solvent and dried at 40°C. The dried powder (5.0 g) was extracted with distilled water for three times and then mixed the filter liquor of three extraction times together. The supernatant was concentrated in a rotary evaporator under reduced pressure and then precipitated by the addition of ethanol to final concentration of 80% (v/v). The precipitate was collected by sucking filtration and washed with 100% ethanol, acetone and finally dried at 40°C to obtain Crude Polysaccharide (CTP). The total sugar content of polysaccharide was quantified by the phenol-sulfuric acid method, glucose was used as standard and the results were then expressed as glucose equivalents (DuBois *et al*., 1956). The yield of CTP is calculated as follows:

$$\text{The yield of CTP}(\%) = \frac{\text{The polysaccharides content of extrction (g)}}{\text{Weight of truffle powders (g)}} \times 100 \quad (1)$$

Experimental design: RSM with Box-Behnken Design (BBD) was used to determine the influence of three major independent parameters and the optimal conditions of crude polysaccharides from Chinese truffle *Tuber sinense*. Three independent parameters (extraction temperature (X_1), ratio of water to raw material (X_2) and extraction time (X_3) at three different levels each were employed. The range of independent variables and their levels were shown in Table 1. Extraction yield (y) was taken as the response for the combination of the independent variables shown in Table 2. Experimental runs were randomized to minimize the effects of unexpected variability in the observed response. The behavior of the system was explained by the following quadratic equation:

Table 1: Indepedent variables and their levels used in the response surface design

	Levels		
Factors	-1	0	1
X_1 extraction temperature/°C	80	90	100
X_2 ratio of water to raw material	15:1	20:1	25:1
X_3 extraction time/h	2.5	3.0	3.5

$$y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_{ij} \quad (2)$$

Where:

y = The dependent variable
A_0 = Constant
A_i, A_{ii} and A_{ij} = Coefficients estimated by the model
X_i, X_j = Levels of the independent variables

They represent the linear, quadratic and cross-product effects of the X_1-X_3 factors on the response, respectively. The model evaluated the effect of each independent variable to a response. The fitness of the second-order model was expressed by the regression coefficient R^2 and its statistical significance was determined by F-test and p-test.

Animals treatment: Young adult Kunming mice (18-22 g, 7-9 weeks old) were provided by the Chengdu Dossy Experimental Animals Co., Ltd. China. The mice were housed under normal laboratory conditions (room temperature, 12/12-h light-dark cycle with free access to standard rodent chow and water). After fed for 1 week, 50 mice were randomly divided into 5 groups (each group contained 10 mice).

One group was normal control group received orally 0.9% normal saline once daily for 7 days. The second group was model control group injected intraperitoneally Cyclophosphamide (CY) at a dosage of 80 mg kg^{-1} body weight on days 1st, 3rd, 5th and 7th and received orally 0.9% normal saline once daily for 7 days. In the other three groups, CTP was received orally at doses of 50, 100 or 200 mg kg^{-1} body weight once daily for 7 days together and CY was administered by intraperitoneal injection at dosage of 80 mg kg^{-1} body weight on days 1st, 3rd, 5th and 7th, respectively.

Phagocytosis of macrophage assay: About 24 h after the last drug administration, all of the mice were killed by

Table 2: Box-Behnken experimental design and results for extraction yield

Run	X_1/temperature (°C)	X_2/ratio	X_3/extraction time (h)	Yield (%)
1	1/100	1/25	0/3.0	9.26
2	1/100	0/20	-1/2.5	9.93
3	1/100	0/20	1/3.5	9.00
4	1/100	-1/15	0/3.0	10.18
5	0/90	1/25	-1/2.5	9.80
6	0/90	1/25	1/3.5	9.30
7	0/90	-1/15	-1/2.5	10.10
8	0/90	-1/15	1/3.5	9.20
9	-1/80	1/25	0/3.0	8.71
10	-1/80	0/20	1/3.5	8.65
11	-1/80	0/20	-1/2.5	8.87
12	-1/80	-1/15	0/3.0	8.84
13	0/90	0/20	0/3.0	10.34
14	0/90	0/20	0/3.0	10.27
15	0/90	0/20	0/3.0	9.92

decapitation. The mice were injected intraperitoneally with 1.0 mL 6% starch broth 2 days prior to sacrifice. On day 7, the mice were injected intraperitoneally with 1.0 mL 2% Chicken Red Blood Cells (CRBC). After 60 min, the mice were killed by decapitation and then injected 2.0 mL Hank's. The activated macrophages were collected, fixed with methanol and stained with Giemsa. The percentages of macrophages ingesting CRBC were determined and expressed as Phagocytosis Rate (PR) (Kui *et al*., 2010). The number of CRBC ingested by 200 macrophages were counted and expressed as Phagocytosis Index (PI):

$$PR(\%) = \frac{\text{Number of macrophages ingesting CRBCs}}{\text{Total number of macrophages}} \times 100 \quad (3)$$

$$PI = \frac{\text{Number of CRBCs ingested by macrophages}}{\text{Total number of macrophages}} \quad (4)$$

Statistical analysis: The Design Expert Software (Version 8.0.6) and SPSS Statistical Software (Version 17.0) were performed to analyze the experimental data. For multiple comparisons, one-way Analysis of Variance (ANOVA) was used.

RESULTS AND DISCUSSION

Fitting the model: The experimental data for extraction yield of the polysaccharides under different treatment conditions were shown in Table 2. The results of ANOVA were shown in Table 3. The statistical analysis indicated that the proposed regression model for yield was adequate, possessing to significant lack of fit and with satisfactory values of the R^2 for all the responses. The R^2 value was 0.956 for yield. The model can fit well with the actual data when approaches one.

Optimization of the process: The graphical representations of the regression Eq. 2, called the response surfaces and the contour plots were obtained using Design-Expert 8.0. 6. As shown in Fig. 1-3, 3D response surface plots and 2D contour plots were useful to see interaction effects of the factors on the responses. They provide a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables.

Table 3: Fit statistics for y

Statistics	Master model	Predictive model
Mean	9.490	9.490
R^2	0.956	0.956
Adj. R^2	0.877	0.877
RMSE	0.210	0.210
CV (%)	2.250	2.250

Predicted response y for the yield of polysaccharides could be expressed by the following second-order polynomial equation in terms of coded values:

$$y = 10.18 + 0.41X_1 - 0.16X_2 - 0.32X_3 - 0.20X_1X_2 - 0.18X_1X_3 + 0.10X_2X_3 - 0.71X_1^2 - 0.22X_2^2 - 0.36X_3^2 \quad (5)$$

Where:

y = The yield of CTP

X_1-X_3 = The coded values for extraction temperature, ratio of water to raw material and extraction time, respectively

According to Table 4, the linear coefficients (X_1, X_3), the quadratic term coefficient (X_1^2, X_3^2) and were found significant ($p<0.05$). The other coefficients (X_2, X_1X_2, X_1X_3, X_2X_3, X_2^2) were not significant ($p>0.05$). The full model filled Eq. 5 was made 3D and contour plots to predict the relationships between the independent variables and the dependent variables.

Figure 1 shows the effects of extraction temperature and ratio of water to raw material on the extraction yield of CTP at extraction time of zero level. It can be seen that the temperature had significant effects on the yield of CTP. Polysaccharides yield was increased significantly when the temperature was increased in the range from 80-94.75°C and then decreased. The yield of CTP increased slightly with increasing of ratio of water to raw material from 15:1 to 16.41:1 but beyond 16.41:1, the extraction yield of polysaccharides decreased slightly.

Figure 2 shows the effects of extraction time and extraction temperature on the extraction yield of CTP at ratio of water to raw material of zero level. The extraction yield of polysaccharides increased slightly with increasing of extraction temperature from 80-94.75°C and then decreased. The yield of CTP increased with increasing of extraction time from 3 min to a threshold level, beyond this level, polysaccharides yield slightly decreased.

Table 4: ANOVA for response surface quadratic model

Parameters	Sum of squares	df	Mean square	F-value	p-value
Model	4.96	9	0.550	12.07	0.0068
X_1	1.36	1	1.360	29.83	0.0028
X_2	0.20	1	0.200	4.28	0.0934
X_3	0.81	1	0.810	17.81	0.0083
X_1X_2	0.16	1	0.160	3.42	0.1237
X_1X_3	0.13	1	0.130	2.76	0.1575
X_2X_3	0.04	1	0.040	0.88	0.3922
X_1X_1	1.85	1	1.850	40.59	0.0014
X_2X_2	0.18	1	0.180	3.95	0.1037
X_3X_3	0.47	1	0.470	10.24	0.0240
Residual	0.23	5	0.046	-	-
Lack of fit	0.13	3	0.042	0.84	0.5852
Pure error	0.10	2	0.051	-	-
Cor total	5.19	14	-	-	-

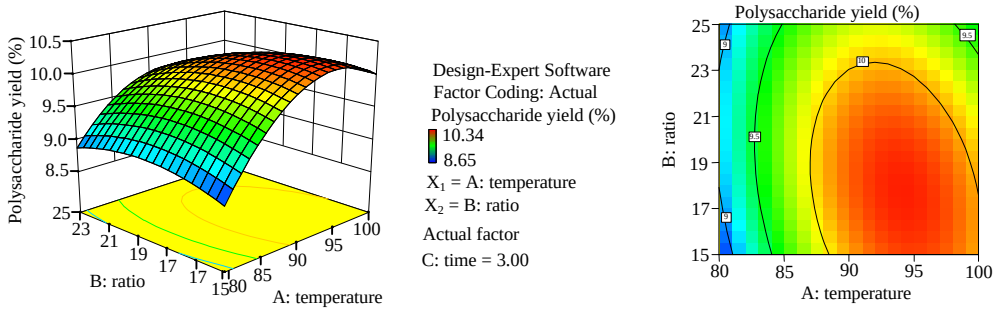

Fig. 1: Response surface plot and contour of ratio of water to raw material and temperature and their mutual interactions on the yield of polysaccharides

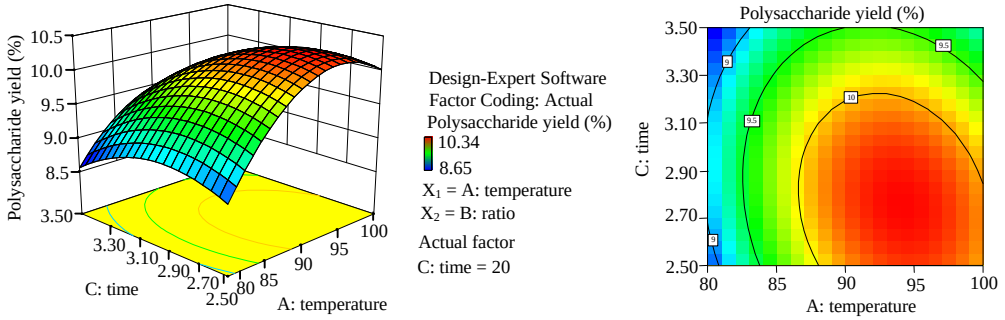

Fig. 2: Response surface plot and contour of extraction time and temperature and their mutual interactions on the yield of polysaccharides

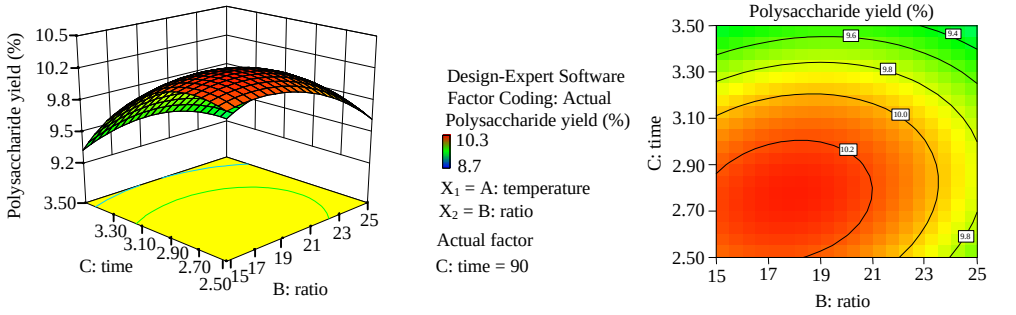

Fig. 3: Response surface plot and contour of extraction time and ratio of water to raw material and their mutual interactions on the yield of polysaccharides

Figure 3 shows the effects of extraction time and ratio of water to raw material on the extraction yield of CTP at extraction temperature of zero level. The extraction yield of polysaccharides increased gradually with increasing of ratio of water to raw material from 15:1 to 16.41:1 then decreased. The yield of CTP increased with increasing of extraction time from 2.5-2.67 h. Beyond 2.67 h, the yield of CTP decreased rapidly with increasing of extraction time. According to Fig. 1-3, optimal extraction condition of CTP were extraction temperature 94.75°C, ratio of water to raw material 16.41:1, extraction time 2.67 h, respectively.

Among the three extraction parameters studied, the temperature was the major factor affecting the extraction yield of CTP followed by extraction time and ratio of water to raw material. The study about *Plantago asiatica* L. also indicated the important of extraction temperature in polysaccharide extraction procedure (Ye and Jiang, 2011).

Validation of the models: In order to validate the adequacy of the model equations (Eq. 5), a verification experiment was carried out using the recommended optimum conditions. Under the conditions, the

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

Conditions	Temperature (°C)	Ratio of water to raw material	Extraction time (h)	Yield (%)
Optimum	94.75	16.41:1	2.67	10.44
Modified	95.00	16.40:1	2.70	10.23±0.28

Table 6: Effect of polysaccharide from *Crassula argentea* on phagocytosis ($\bar{X}$±SD)

Groups	Dosage (mg kg^{-1})	Phagocytic rate (%)	Phagocytic index
CTP	200	52.69±7.72^{AB}	1.31±0.16^{AB}
CTP	100	32.81±9.48^{AB}	0.74±0.22^{AB}
CTP	50	20.88±2.89^{B}	0.47±0.06^{B}
Model control	-	7.38±3.56^{A}	0.14±0.83^{A}
Normal control	-	21.13±5.52	0.44±0.13

$^{A}p<0.01$, significantly different from the normal control group.$^{B}p<0.01$, significantly different from the model control group

experimental yield of polysaccharide was 10.23±0.28% (n = 3) which was close to the predicted value (10.44%) (Table 5).

Effects of CTP on macrophage phagocytosis in mice: As shown in Table 6, the phagocytic activity of macrophages in model control group injected cyclophosphamide was lower significantly than that in normal control group ($p<0.01$). The macrophages phagocytosis in three CTP groups all significantly enhanced as compared with that in model control group ($p<0.01$). Phagocytic rate and phagocytic index of macrophage both increased within the range from 50-200 mg kg^{-1} and they reach the level of normal control group at the concentration of the 50 mg kg^{-1}. The maximum phagocytic rate and the maximum phagocytic index were 52.69±7.72 and 1.31±0.16%, respectively at the concentration of 200 mg kg^{-1}. Activated macrophages participate in both specific and nonspecific immune reactions but also are the bridge cell of these two kinds immune reactions (Gan *et al*., 2004). A great deal of reports have been reported that polysaccharides from mushroom can enhance macrophage host defense responses (Igor and Schepetkin, 2006; Zheng *et al*., 2005).

In the present study, CTP enhanced significantly the macrophages phagocytosis in immunosuppression mice. The result was similar with previous investigation (Hu *et al*., 1994).

CONCLUSION

In this study, RSM was successfully used to determine the extraction conditions for CTP. The optimal extraction conditions for the polysaccharides were as follows: extraction temperature 94.75°C, extraction time 2.67 h, ratio of water to raw material 16.41:1. CTP had potent immunomodulatory properties in mice models, enhanced significantly on macrophage phagocytosis.

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