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Optimization of extraction and characterization of polysaccharides from medicinal mushroom *Ganoderma lucidum* using response surface methodology

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Submerged fermentation for the production of polysaccharides has received the greatest interest in recent years due to its increased demand as drug. Response surface methodology was employed to optimize the conditions for extraction of polysaccharides from *Ganoderma lucidum*. Central composite rotatable design was employed to optimize temperature, time, sodium hydroxide (NaOH) concentration and volume of NaOH for polysaccharide extraction. The various effects of the factors were studied by β-coefficient and Fischer's F-test for analysis of variance (ANOVA) and a second order model was developed. The results indicated that the optimum conditions were an extraction temperature of 100°C, an extraction time of 3 h, NaOH concentration of 6% and ratio of liquid to solid of 20 ml. The experimental polysaccharide production at predicted optimum conditions was 4.96% that validates the high degree of accuracy. Fourier transform infrared spectroscopy (FTIR) was used to obtain vibrational spectra of GL-2 mycelium and fruit body. The bands in the range of 1200 to 800 cm^{-1} indicated the presence of polysaccharides.

Key words: Central composite rotatable design (CCRD), Fourier transform infrared spectroscopy (FTIR), *Ganoderma lucidum*, polysaccharides, response surface methodology.

INTRODUCTION

Ganoderma lucidum (Red Reishi) is currently the most popular among all mushroom species, because of its high medicinal values (Wang et al*.,* 2005). It has been used in both Chinese and Japanese traditional medicine for the prevention and treatment of various types of diseases, such as cancer, hepatopathy, arthritis, hypertension, neurasthenia and chronic hepatitis (Liu and Zhang, 2005; Lin, 2007). The main functional components of *G. lucidum* include polysaccharides, proteins, peptides, amino acids, triterpenes, steroids, alkaloids, nucleotides, lactones, and fatty acids (Tim et al*.,* 2004). Fungal fruiting bodies, fungal mycelium or the culture fluid in which the mycelium has been cultivated have all been explored for the biological activity. Submerged culture of mushrooms is a promising alternative for efficient production of mycelium and metabolites and has received increasing attention around the world (Tang et al*.,* 2007). Mycelial biomass powder has been used to formulate various types of health tablets and capsules (Chen and Jie, 2001). Extraction of polysaccharides is an important process for their application or further research and development, and this has prompted the publication in recent years of numerous research papers on the technology for extraction of polysaccharides from plants or fungi. Polysaccharides yield extracted from fruiting bodies and mycelia is relatively low and its cost is high.

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By comparison, exopolysaccharides (EPS) have several advantages: the use of harsh extraction steps can be avoided in EPS production, thus lessening product degradation during recovery. Various types of polysaccharides, with molecular weights ranging from 4×10^5 to 1×10^6 have been identified in *G. lucidum* mostly in the fruiting body and mycelia and a few have been found in the spores (Sanodiya et al*.,* 2009). The main bioactive polysaccharides isolated from *Ganoderma* species are D-glucans with β-1,3 and β-1,6 glycosidic bonds. Structural analysis shows that polysaccharides of *G. lucidum* are all hetropolymers (Chan et al*.,* 2007). Glucose forms the major part of polysaccharides with xylose, mannose and galactose in different conformations. The solubility characteristics and different branching conformations affect the antitumor properties of these polysaccharides (Kim et al*.,* 1993). Glucans are known to have strong anti-tumor activity and better absorption than other medicinal components of *G. lucidum* (Sone et al*.,* 1985).

A number of methods have been developed to extract the anticancer polysaccharides from mushroom mycelium, fruit bodies and liquid media (Mizuno, 1999). Dong et al*.* (2009) optimized the hot water extraction (HWE) process of polysaccharides from cultured mycelium of *Cordyceps sinensis* using Box-Behnken design. Ultrasonic technology was employed by Yang et al. (2008) to extract polysaccharides from longan fruit pericarp and obtained the optimal extracted condition by response surface methodology. They found that the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of polysaccharides could be improved by application of ultrasonic treatment. Huang et al*.* (2007) studied the microwave-assisted extraction (MAE) of polysaccharides from spores of *Ganoderma atrum* with response surface analysis. Response surface methodology (RSM) is a useful technique for optimization studies and it is better acknowledged than traditional one. RSM is a collection of mathematical and statistical techniques useful for the modelling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 2005). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. The most common applications of RSM are in industrial, biological and clinical, social, food, and physical and engineering sciences. Since RSM has an extensive application in the real-world. The objective of this study was to improve the yield of polysaccharides from *G. lucidum*, using a RSM design, which explores the relationships between several variables and one or more response variable and uses designed experiments to obtain an optimal response and thus optimize the alkaline extraction conditions. This work also investigated the chemical constituents present in the mycelium of *G. lucidum* by Fourier Transform Infrared (FTIR) spectroscopy.

MATERIALS AND METHODS

Microorganism

The higher fungi used in the study *G. lucidum* (GL-2 strain) was procured from Directorate of Mushroom Research, Solan, India. For characterization of polysaccharides, DXN capsule as standard was used for the characterization of polysaccharides.

Standard inoculum preparation

The *G. lucidum* GL-2 strain was cultivated in mushroom complete medium (MCM) having the following composition (g/L): glucose 20, peptone 2.0, yeast extract 2.0, KH_2PO_4 0.5, K_2HPO_4 1.0, and $MgSO_4.7H_2O$ 0.5 having pH of 6.5. After inoculation, the flasks were incubated at 30±2°C till the growth of the mycelium was completed. These flasks were then shaken to cut the fully grown mycelium into small pieces. This mycelium is used as standard inoculum. Two milliliters of this standard inoculum is taken to inoculate fresh media.

Experimental design for process optimization

The mycelium of *G. lucidum* was subjected to analytical assay for the extraction of biomedicinal components. RSM was carried out in order to find the optimum condition for the extraction of polysaccharide with modification in Mizuno (1999) methods. Mushroom mycelium was heated in 80% ethanol for 1 h in water bath at 50°C. The low molecular compounds were eliminated as filtrate, while the residue was boiled at 100°C with sterilized water for 3 h. The filtrate was discarded and the residue obtained was treated for 6 h with 1% ammonium oxalate at 100°C on water bath. The ammonium oxalate extract was further heated at 80°C with 5% sodium hydroxide for 6 h. Purification of the polysaccharides was done with acetic acid.

The percentage yield obtained was then calculated by the following formula:

$$\text{Yield (\%)} = \frac{\text{Amount of polysaccharide obtained after extraction (g)}}{\text{Amount of sample used for extraction (g)}} \times 100$$

Central composite rotatable design (CCRD) was used in order to optimize the dependent variable (polysaccharide yield) as a response while the independent variables chosen were extraction temperature (80 to 100°C), extraction time (3 to 7h), ratio of liquid to solid (10 to 20 ml) and NaOH concentration (4 to 6%).

According to this design, the total number of treatment combinations was calculated as $2^k + 2k + n_0$ where k is the number of independent variables and n_0 is the number of experiment repetitions at the centre point. Therefore, for four variables:

Total number of experiments: $2^4 + 2 \times 4 + 6 = 30$ experimental points

The experiment was carried out in duplicate which are necessary to estimate the variability of experimental measurements, that is, the repeatability of the phenomenon. The coded levels of the independent variables are given in Table 1. A 2^4 factorial CCD was developed by Design Expert (Stat Ease, Minneapolis, MN) with 8 axial points and 6 replicates at the centre points leading to 30 runs. Thirty experimental points were obtained for the optimization of

Table 1. Levels of different variables tested in CCRD.

Independent variables and symbol code		Coded levels		
		-1	0	+1
Temperature (°C)	X_1	80	90	100
Time (h)	X_2	3	5	7
Volume (ml)	X_3	10	15	20
NaOH concentration (%)	X_4	4	5	6

Table 2. Experimental design in coded and un-coded form process variables and experimental values for extraction of polysaccharides in GL-2 strain.

S/N	Un-coded process variable				Response	
	Temperature (x_1)	Time (x_2)	Volume (x_3)	NaOH concentration (x_4)	Yield (g)	Yield (%)
1	100	7	20	4	0.74	4.97
2	80	3	20	6	0.64	4.29
3	80	3	10	6	0.57	3.84
4	80	3	10	4	0.68	4.56
5	90	1	15	5	0.83	5.56
6	80	7	10	4	0.74	4.98
7	100	3	10	4	0.88	5.89
8	100	3	20	4	0.64	4.28
9	90	5	15	5	0.78	5.67
10	80	7	10	6	0.59	3.98
11	90	5	15	7	0.82	5.47
12	110	5	15	5	0.58	3.89
13	100	3	10	6	0.81	5.43
14	80	3	20	4	0.68	4.56
15	80	7	20	6	0.59	3.98
16	90	5	15	3	0.86	5.78
17	100	3	20	6	0.76	5.12
18	80	7	20	4	0.88	5.89
19	90	9	15	5	0.86	5.78
20	100	7	20	6	0.83	5.55
21	100	7	10	6	0.93	6.24
22	90	5	15	5	0.88	5.89
23	90	5	5	5	0.53	3.56
24	70	5	15	5	0.38	2.54
25	100	7	10	4	0.70	4.64
26	90	5	15	5	0.88	5.21
27	90	5	15	5	0.85	5.89
28	90	5	25	5	0.87	5.82
29	90	5	15	5	0.85	4.56
30	90	5	15	5	0.68	5.67

extraction procedure for the enhancement of polysaccharides yield (Table 2).

The variables were coded according to the following equation:

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

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of Xi at the centre point and ΔX is the step change.

A second-order polynomial model was used to fit the quadratic resulting in the equation:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2$$

where Y_i = response, Y_1 = yield of polysaccharides (%), X_i = independent variables, X_1 = temperature (°C), X_2 = time (h), X_3 = ratio of liquid to solid (ml), X_4 = NaOH concentration (%), β_0 = value of fitted response at central point of design, that is (0, 0, 0), β_i, β_{ii}, β_{ij} = linear, quadratic and cross product regression coefficients, respectively.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability P(F), correlation coefficient R, and determination coefficient R^2 which measures the goodness of fit of regression model. It also includes the Student's t-value for the estimated coefficients and associated probabilities, P (t). The relative effect of each process parameter on individual response was compared from the β values corresponding to that parameter. The quadratic models were represented as response surface graphs, which gives infinite number of combinations of the two factors selected keeping the other constant. The optimization of the process was aimed at finding the optimum values of independent variables (temperature, time, NaOH concentration and volume), which would give maximum polysaccharide production. The optimum values of the selected variables were obtained by solving the regression equation.

Characterization of polysaccharides from *G. lucidum* by FTIR spectroscopy

FTIR spectroscopy of *G. lucidum* strain (GL-2) mycelial biomass and fruit body along with the standard DXN capsule were tested using Nicolet FTIR Spectrometer (Nicolet 6700, Thermo Scientific). This helped to analyze different sulphate, carboxyl and hydroxyl groups of these sample molecules. One part of sample was mixed with 99% of dried potassium bromide (KBr) powder and compressed to prepare a salt disc of 3 mm diameter. These discs were subjected to FTIR spectrum measurement in the frequency range of 400 to 4000 cm^{-1}. A signal integration time of 30 to 120 s per sample was used. Spectra were corrected for wave number dependent signal-detection efficiency of the set up using the white light spectrum of a temperature calibrated tungsten band lamp (Romer et al., 1998; Grosev et al., 2001).

RESULTS AND DISCUSSION

The experimental data of the process variables for yield of polysaccharides under different extraction conditions are shown in Table 2. After the response surface regression (RSREG) procedure, the results of the analysis of variance, regression coefficient, along with the corresponding P-value, and the adequacy for the models of polysaccharides yield from the *G. lucidum* showed that the model data could adequately predict the experimental polysaccharide yield. The analysis of variance showed that this regression model was highly significant ($P<0.01$) with an F-value of 3.55, implying a good fit between the predicted model and the experimental data. The value of 1.51 for lack of fit ($P>0.05$) implied that it is not significant, compared to the pure error. The yield of polysaccharides changed significantly with all the quadratic term and linear coefficients X_1, X_1X_4, $(X_1)^2$. The importance of the independent variables on the yield could be ranked in the following order: extraction temperature (X1) > ratio of liquid to solid (X3) > extraction time (X2) > NaOH (sodium hydroxide) concentration (X4) according to the F-value of analysis of variance (Table 3).

The results were analysed by polynomial quadratic regression method which describes the effect of variables in the model derivatized. The regression coefficients were obtained after fitting the experimental data in the selected model given in Table 2. The individual effect of each variable and also the effect of the interaction terms in coded level of variables were determined by polynomial quadratic equation.

Yield of polysaccharides (%) = $+ 5.48 + (0.37\ x_1) + (0.11\ x_2) + (0.15\ x_3) - (0.083\ x_4) - (0.054\ x_1x_2) - (0.23\ x_1x_3) + (0.40\ x_1x_4) - (0.12\ x_2x_3) - (9.375E\text{-}003\ x_2x_4) - (9.375E\text{-}003x_3x_4) - 0.55(x_1)^2 + 0.062\ (x_2)^2 - 0.18\ (x_3)^2 + 0.051\ (x_4)^2$

The magnitude of β coefficients as given in Table 3 revealed that out of all the linear terms, temperature shows maximum effect on yield of polysaccharides (β = 0.37) followed by ratio of liquid to solid (β=0.15), time (β=0.11) and NaOH concentration (β=0.083). All the linear terms had positive β-values, which show that with increasing time, temperature, ratio of liquid to solid and NaOH concentration, there is increase in yield of polysaccharides. Therefore, all linear terms were found critical factors affecting yield of polysaccharides.

In interactive terms "temperature and NaOH concentration" had maximum, positive and significant effect on yield of polysaccharides (β=0.40) followed by "time and ratio of NaOH used for solid" (β = 0.12). Other interactions had negative effect on yield of polysaccharides. In quadratic terms, NaOH concentration (β=0.051) and time (β=0.062) had maximum positive effect on yield of polysaccharides followed by negetive effect of temperature(β = -0.55) and ratio of liquid to solid (β = -0.18).

The goodness of fit for the model was expressed by the coefficient of determination R^2 and was found to be 0.7682, indicating that 76.82% of the variability in the response could be explained by the model. This suggests that the predicted values exhibits a good correlation with the experimental data and that the model is suitable and practicable.

Verification of models

In order to optimize the process conditions for yield of polysaccharide extraction, equal importance was given to all the process variables and response (% yield). The optimum operating conditions for yield of polysaccharide extraction were of temperature 100°C, time 7 h, and the use of 20 ml of 6% NaOH. Corresponding to these optimized values of process variables, the predicted value for yield of polysaccharide were 5.70% and 0.85 g which was in agreement with the observed value of polysaccharide yield 4.96% and 0.74 g. The excellent correlation between predicted and measured values of

Table 3. Regression summary and ANOVA table for the yield of polysaccharides uncoded value of process variable of GL-2 strain

Source	Sum of squares	β-coefficient	Df	Mean squares	F-value	P value (Prob>F)
Model	17.72	5.48	14	1.27	3.55	0.0102
Temperature	3.20	0.37	1	3.20	8.99	0.0090
Time	0.31	0.11	1	0.31	0.87	0.3654
Volume	0.53	0.15	1	0.53	1.49	0.2411
NaOH concentration	0.17	-0.083	1	0.17	0.46	0.5066
Temperature × Time	0.047	-0.054	1	0.047	0.13	0.7207
Temperatue × Volume	0.84	-0.23	1	0.84	2.36	0.1452
Temperature × NaOH concentration	2.58	0.40	1	2.58	7.25	0.0167
Time × Volume	0.25	0.12	1	0.25	0.69	0.4178
Time × NaOH concentration	1.406E-003	-9.375E-003	1	1.406E-003	3.945E-003	0.9507
Volume × Extraction concentration	1.406E-004	-9.375E-003	1	1.406E-003	3.945E-003	0.9507
Temperature^2	8.35	-0.55	1	8.35	23.43	0.0002
Time^2	0.11	0.062	1	0.11	0.30	0.5947
Volume^2	0.92	-0.18	1	0.92	2.58	0.1292
NaOH concentration ^{2}	0.071	0.051	1	0.071	0.20	0.6627
Residual	5.35	-	15	0.36	-	-
Lack of fit	4.02	-	10	0.40	1.51	0.3386
Pure error	1.33	-	5	0.27	-	-
Cor total	23.06	-	29	-	-	-
R-Squared	0.7682	-	-	-	-	-
Adj R-Squared	0.5518	-	-	-	-	-

these experiments justifies the validity of the response model and the existence of an optimum point.

Interaction between factors influencing yield of polysaccharide production

Three dimensional response surface (3D) and contour (2D) plots for responses were generated to study the effect of independent variables and their interactions on polysaccharide yield according to the results of regression equations. Figure 1 shows the effect of NaOH concentration and temperature on yield of polysaccharide. Gradual increase in polysaccharide yield was observed as temperature increased from 80 to 95°C and thereafter, decline in polysaccharide yield was witnessed as temperature further increases from 95 to 100°C. As far NaOH concentration increased, no observed change was noticed in polysaccharide yield.

FTIR spectroscopy

FTIR spectroscopy is currently being used to investigate the vibrations of molecules and polar bonds between different atoms on the basis of wave number of bands. The absorption intensity can be used for calculating the relative concentration. The FTIR spectra exhibit characteristic features in three regions. The first region between 4000 and 1800 cm^{-1}, presents a prominent broad band, corresponding to the absorption due to stretching mode of hydroxyl bond (Grosev et al., 2001). The second region between 1800 and 1500 cm^{-1}, is composed of the vibrational mode of carbonyl and C=C double bond. The third region between 1500 and 750 cm^{-1}, is associated with the vibration of proteins, lipids and carbohydrates. The absorption bands in the mid-infrared region 1200 to 800 cm^{-1} are useful for the identification of polysaccharides with different structures and composition (Kacurakova et al., 2000).

The spectral comparison of standard with GL-2 mycelial biomass was given as: standard mycelium (DXN capsule): 3629.6, 3394.3, 2927.6, 1654.9, 1637.7, 1458.5, 1419.7,1155.2, 1078.8, 1023.0,708.4, 669.3, 572.4, 525.7; standard fruit body (DXN capsule): 3386.1, 1654.7, 1637.7, 1156.0, 1076.2, 1043.2, 559.7; GL-2 mycelium: 3422.4, 1654.9, 1048.6; GL-2 fruit body: 921.7, 751.1, 697.3, 672.0, 662.9, 645.3, 605.6, 588.8, 568.8, 547.5, 528.4, 520.2.

The IR spectra of standard mycelium and fruit body were given as shown in Figures 2 and 3, respectively. The spectra of fruit body and mycelium extract of GL-2 and the standard showed several main absorption bands. *G. lucidum* strain and the standard showed almost similar bands. A prominent band was seen at 1654 cm^{-1} in GL-2 mycelium and standard of mycelium and fruit body. This

may be due to the presence of some complex chromophores present in these compounds. Moreover, a prominent band in the range of 1200 to 800 cm^{-1} indicated the presence of polysaccharides in GL-2 mycelium and fruit body (Figures 4 and 5). IR spectra of six water soluble polysaccharides GTM1 to GTM6 in *Ganoderma tsugae* were observed by Peng et al. (2005). GTM1 exhibited the typical absorption peaks at 870 and 810 cm^{-1} for mannan (Mathlouthi and Koenig, 1986). The appearance of obvious characteristic peaks both at 850 and 920 cm^{-1} for α-D-glucan and at 890 cm^{-1} for β-D-glucan in GTM3 and GTM4 implied the co-existing of α- and β-D-glucans. They observed that GTM5 and GTM6 exhibited the main absorption peak at 890 cm^{-1} for the β configuration of D-glucan. Carbonero et al. (2008) observed a heteropolysaccharide, a fucomannogalactan, with a main chain of (1-6)-linked α-D-galactopyranosyl units, partially substituted at O-2 by single-unit β-D-mannose or α-L-fucose side chains. He et al. (2010) studied IR characteristic peaks of *G. lucidum* from different places. They observed that there was obviously a wide and strong absorption peak in 3377.8 to 3396.5 cm^{-1}, a small acromion in 2924.2 to 2925.1 cm^{-1}, a medium intensity absorption peak in 1635.8 to 1650.3 and 1372.5 to 1375.2 cm^{-1}, a strong absorption bifurcate peak in 1074.8 to 1075.3 and 1043.2 to 1045.2 cm^{-1}, an obvious weak peak in fingerprint regions 891.0 to 894.8 cm^{-1}, and a medium intensity absorption peak in 563.10 to 574.7 cm^{-1}.

Conclusion

The extraction of polysaccharides from *G. lucidum* and its characterization by FTIR have studied in this study. The importance of the independent variables on the yield could be ranked in the following order: extraction temperature (X1) > ratio of NaOH to polysaccharide (X3) > extraction time (X2) > NaOH concentration (X4). The dependent variable and independent variable are related by the following second-order polynomial equation: $Y (\%) = +5.48 + (0.37\ x_1) + (0.11\ x_2) + (0.15\ x_3) - (0.083\ x_4) - (0.054\ x_1x_2) - (0.23\ x_1x_3) + (0.40\ x_1x_4) - (0.12\ x_2x_3) - (9.375E\text{-}003\ x_2x_4) - (9.375E\text{-}003x_3x_4) - 0.55(x_1)^2 + 0.062\ (x_2)^2 - 0.18\ (x_3)^2 + 0.051\ (x_4)^2$. The optimal experimental conditions for the alkaline extraction of polysaccharides from *G. lucidum* were: a temperature of 100°C, a time of 3 h, NaOH concentration of 6% and ratio of liquid to solid of 20 ml, respectively. The yield of polysaccharides from *G. lucidum* was expected to be 4.96% by FTIR spectroscopy. Moreover, a prominent band in the range of 1200 to 800 cm^{-1} indicated the presence of polysaccharides.

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