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Optimization of a Culture Medium for Polysaccharide Production of *Antrodia camphorata* using Response Surface Methodology

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Antrodia camphorata is a uniquely preserved fungus that is indigenous to and found only in Taiwan. It only grows on a species of rainforest tree that is also native to Taiwan. A. camphorata has been used in traditional Chinese medicine for more than 100 years, and its main effective component are polysaccharides. A culture medium for increased polysaccharides was developed in this study. Soluble starch, wheat bran, and MgSO4 are the key factors of the culture medium based on the results obtained from the Plackett-Burman experimental design. The optimal concentration range of the three factors was examined by the steepest ascent path. Response surface methodology (RSM) was used to optimize the culture medium. A Box-Behnken design was used for the experimental design and analysis of the results to obtain the optimal culture medium. The 3D RS and contour plots derived from the mathematical models were applied to determine the optimal culture medium. The optimum culture medium is soluble starch (3.17%), wheat bran (0.79%), KH2PO4 (0.1%), MgSO4·7H2O (0.05%), and VB1 (10 mg/100 mL). The production was increased by 253% to 183.46 mg/L from 51.98 mg before optimization.

Key words: Antrodia camphorata, Polysaccharides, Plackett-Burman experimental design, Steepest ascent path, Response surface methodology.

Antrodia camphorata (or "Niu-chang-zhi") is a uniquely preserved fungus that is indigenous and found only Taiwan. It only grows in the hollow area of trunks of a mature rainforest tree species, Cinnamomum kanehirai, which is also native to Taiwan. This fungus is a newly discovered basidiomycete of the family Polyporaceae (Aphyllophorales) and has been identi?ed as a distinct species of the genus Antrodia (Zang and Su, 1990). Many researchers have studied *Antrodia camphorata* and found that it has an extensive range of biological activities, including anti-hepatic cirrhosis, anti-inflammatory, immunoregulation, anti-hepatitis B, anti-tumor, neuroprotection, antioxidant and vasorelaxation, hepatoprotection, liver protection, and anti-hypertension [1-4].

Many publications have been published on the effective components of this fungus. The effective components found in *Antrodia camphorata* are mainly polysaccharides and triterpenoids. Thus, many researchers have focused on the polysaccharide effect. Partially purified polysaccharides from *Antrodia camphorata* were reported to inhibit the proliferation of U937 cells through the activation of human mononuclear cells

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in an anti-tumor immunity model (Liu *et al*., 2004). The polysaccharides of *Antrodia camphorata* not only inhibit the expression of the surface antigen of hepatitis B virus (Lee *et al*., 2002) but also suppresses angiogenesis (Chen *et al*., 2005; Cheng *et al*., 2005). Researchers have reported that the polysaccharides have anti-angiogentic, immunomodulatory modulate, and inflammatory mediator expression effects, which have played important roles in the pharmacology action of *Antrodia camphorata*[5-9].

Antrodia camphorata is currently widely used in Taiwan as food additives or functional ingredients (Ao *et al*., 2009; Chen *et al*., 2007). However, the fruiting bodies of *Antrodia camphorata* are in large demand in Taiwan because of its host speci?city and rarity in nature. It is necessary that the arti?cial cultivation was developed as a substitute. Elevating the polysaccharide content in artificial cultivation for a more effective fungus is important. This study develops a method that can clearly elevate the polysaccharide content.

MATERIALS AND METHODS

Microorganism

Antrodia camphorate ATCC 200183 was obtained from SIMPON BIOTHCH CO, Ltd.

Determination of the polysaccharides

The polysaccharide content was detected using the phenol-sulfuric method. The dried mycelium was fully ground and poured into a round bottom flask. The *Antrodia camphorata* powder was extracted with 30 volumes of distilled water at 80 °C after 1 h twice. The filtered residues were then ultrasonically extracted after 30 min twice. The mixtures were then centrifuged, filtered, and the residue removed after the extraction with ultrasonic treatment. The pretreated extraction was mixed with four volumes of cold 95% ethanol (4 °C) to isolate the polysaccharides. The polysaccharide content is detected suing the anthrone sulfuric acid method.

Plackett–Burman design

Different carbon and nitrogen sources, as well as the main factors that affect polysaccharide production, were evaluated for screening. Each factor was examined in two levels based on the PB factorial design: ?1 means a low level and +1 means a high level. Table 1 shows the factors under investigation and levels of each factor used in the experimental design, whereas Table 2 shows the design matrix. The PB experimental design is based on the first order polynomial model:

$$Y= \beta o +\sum \beta i \chi i \quad ...(1)$$

where Y is the response (polysaccharide yield), ?o is the model intercept, ?i is the linear coefficient, and ?i is the independent variable level. This model can not reflect the interaction among factors. It is only used to screen and evaluate the important factors that influence the response. Approximately 14 assigned variables were screened in 20 experimental designs in the present work (Table 2). The variables that were significant at 90% level ($P < 0.1$) were considered to have a larger impact on polysaccharides production based on the regression analysis[11-12].

Path of the steepest ascent experiment

The fitting of RSM is useful near the neighborhood of the optimum. We used the steepest ascent method to rapidly move toward the neighborhood of the optimum response. According to the increasing or decreasing the variable concentrations in PB test, a suitable direction could be determined.(Gheshlaghi *et al*., 2005)[13-15].

Box–Behnken

When the critical factors were found, a BB design for three independent variables (i.e., each at three levels with three replicates at the center points) was employed to fit a normal model:

$$Y =\beta 0+\sum \beta i \chi i +\sum \beta_{i\,j} \chi_i \chi_j+\sum \beta_i i \chi_i^2$$

Design Expert(version 6.0.5 State-Ease Inc Minneapls, MN USA) was used for the experimental design and regression analysis of the data obtained[15-18].

RESULTS AND DISCUSSION

Carbon and nitrogen source screening

The data in Table 2 indicate a wide variation in polysaccharide production from 0.054 mg/L to 114.791 mg/L in the 20 trials. This difference shows the importance of the carbon and nitrogen sources to attain higher productions. Variables with confidence levels larger than 90% were treated as significant. Soluble starch (? = 20.363) and wheat bran (? =11.1) showed a positive effect for polysaccharide production based on the regression coefficient (R2) and t-value analyses of 14 ingredients. Besides, carbamide (? = ?13.790), peptone (? = ?10.063), and ammonium citrate (? = ?10.39) showed a negative effect. The results are presented in Table 3.

Neglecting the variables that were insignificant, the model equation for polysaccharide production can be written as follows:

$$Y=28.384+20.363\,X5+11.152\,X7-17.571\,X8-10.063\,X10-10.39\,X12$$

where X5 is sucrose, X7 is wheat bran, X8 is urea, X12 is ammonium citrate, and X10 is peptone.

The R2 of the model was 0.981, which indicates that nearly 98.1% of the variability in the response can be explained by the model.

Optimization of culture medium by PB design

Media optimization was performed, and the results are shown in Table 4.

A first-order model was analyzed by Design-Expert and fitted to the results obtained from the 12 experiments as follows:

$$Y=72.414+27.299\,X1+25.136\,X2+12.196\,X3$$

where X1 = sucrose, X2=K_2HPO_4 and X3 = $MgSO_4$

The R2 of the first-order model was 0.925, which indicates that nearly 92.5% of the variability in the response can be explained by the model.

The steepest ascent experiment and analysis

A set of the steepest ascent experiments was arranged based on the results of the PB design to determine the orientation of predictive higher responses (Table 5). The variables [i.e., soluble starch (x1), wheat bran (x3), and MgSO4] were adjusted because they significantly affect the polysaccharide production. Table 5 shows that the highest production was achieved, which was higher than that of other combinations. The corresponding variable levels should also be used in further studies.

The BB experimental design and RSM were used to further examine the optimal level point

Table 1. Assigned concentrations of variables at different levels in Plackett–Burman design for polysaccharides production

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	Response Polysaccharides Production mg/L
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	27.322
2	-1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	1	25.687
3	-1	-1	1	-1	-1	1	1	1	1	-1	1	-1	-1	-1	3.482
4	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	1	-1	5.362
5	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	1	4.903
6	-1	1	1	-1	-1	1	-1	-1	1	1	1	1	-1	1	8.996
7	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	-1	15.093
8	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	-1	-1	2.962
9	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	-1	-1	76.165
10	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	-1	27.134
11	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	0.877
12	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	-1	0.054
13	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	-1	-1	1	8.676
14	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	28.422
15	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	1	1	4.661
16	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	1	92.388
17	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	5.872
18	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	114.791
19	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	31.608
20	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	1	-1	83.216

of soluble starch, wheat bran, and MgSO4. The low and high levels for each factor were coded and shown in Table 6. The results are shown in Table 7.

Table 2. Plackett-Burman design for 14 variables with coded values along with the observed results for polysaccharides production

Factors	+1(%)	-1(%)
X1 glucose	1	0
X2 sucrose	1	0
X3 lactose	1	0
X4 maltose	1	0
X5 soluble starch	1	0
X6 yeast powder	0.1	0
X7 wheat bran	1	0
X8 urea	0.1	0
X9 soybean flour	0.1	0
X10 peptone	0.1	0
X11 ammonium citrate	0.1	0
X12 ammonium sulfate	0.1	0
X13 corn meal	1	0
X14 tryptone	0.1	0

Fitting of the experimental data by regression analysis yielded a second-order polynomial equation model as follows:

$$Y = 157.77 + 17.18A + 7.37B - 3.53C + 3.89AB + 3.64AC - 12.27BC - 50.13A^2 - 10.59B^2 - 6.73C^2$$

where Y is the predicted polysaccharide yield, and A, B, and C are the coded values of soluble starch, wheat bran, and MgSO4.

Table 8 shows the significance of the equation fitting, which was tested by Analysis of Variance.

The P-value obtained from an F-test was 0.0001, which implies that the model was adequate.

$$\text{Yield of polysaccharides} = 157.77+17.18A+7.37B-3.53C+3.89AB+3.64AC-12.27BC-50.13A^2-10.59B^2-6.73C^2$$

The R2 was 0.972 and the adjusted determination coefficient (Adj R2) was 0.9359, which indicate that the model has a high

Table 3. Statistical analysis of Plackett–Burman design showing coefficient values,t and P-value for significant variables

Model term	Estimate	Stand Error	*T* value	*P* value
intercept	28.384	2.090	13.58	<0.0001
soluble starch	20.363	2.090	9.74	0.0002
urea	-17.571	2.090	-8.41	0.0004
wheat bran	11.152	2.090	5.34	0.0031
ammonium citrateX12	-10.39	2.090	-4.97	0.0042
peptone	-10.063	2.090	-4.82	0.0048

Table 4. Plackett–Burman design for 5 variables with coded values along with the observed results for polysaccharides production

Number	Soluble starch X1	wheat bran X2	$MgSO_4$ X3	KH_2PO_4 X4	VB_1 X5	Polysaccharides Production mg/L
1	1	1	1	1	1	121.362
2	-1	1	-1	1	1	55.945
3	-1	-1	1	-1	1	24.741
4	1	-1	-1	1	-1	50.913
5	-1	1	-1	-1	1	55.738
6	-1	-1	1	-1	-1	49.893
7	-1	-1	-1	1	-1	19.355
8	1	-1	-1	-1	1	44.878
9	1	1	-1	-1	-1	134.481
10	1	1	1	-1	-1	152.755
11	-1	1	1	1	-1	65.019
12	1	-1	1	1	1	93.89

significance. The 3D RS curves and respective contour plots are presented in Figs. 1 to 3.

The interactions of the three components and their optimal level in the polysaccharide production were further analyzed using the RSM. The 3D RS curves and respective contour plots are presented in Figs. 1to 3. The RSs of Figs. 1 and 2 were convex, which suggests that the optimal conditions were well defined and a maximum exists for each variable. Moreover, Figs. 1 and 2 demonstrate better ellipses, whereas Fig 3 almost displayed circularity. These results suggest that the interactions among wheat bran and soluble starch, magnesium sulfate, and soluble starch were significant, whereas the interactions among magnesium sulfate and wheat bran were almost insignificant. This result is consistent with the P-test results.

Verification experiments were conducted under the following conditions to validate the model's adequacy: soluble starch (3.17%); wheat bran (0.79%); KH2PO4 (0.1%); MgSO4·7H2O (0.05%); VB1 (10 mg/100 mL). Polysaccharide production

Table 5. Experimental design and results of steepest ascent experiment

Number	soluble starch(%)	wheat bran(%)	$MgSO_4$ (%)	Polysaccharides Production(mg/L)
1	1	0.2	0.03	57.445
2	2	0.4	0.05	90.826
3	3	0.6	0.07	158.435
4	4	0.8	0.09	135.665
5	5	1.0	0.11	104.035

Table 6. Level and code of variables chosen for Box–Behnken design

Variables	Symbol	Coded levels		
		1	0	1
Soluble starch(%)	A	2	3	4
Wheat bran(%)	B	0.4	0.6	0.8
$MgSO_4$ (%)	C	0.05	0.07	0.09

Table 7. The levels of each variable and corresponding Polysaccharides Production obtained from the Box–Behnken design

Groups	soluble starch X1	wheat bran X2	$MgSO_4$ X3	Polysaccharides Production(mg/L) Observed	Predicted
1	-1	-1	0	77.324	76.39
2	1	-1	0	99.872	102.96
3	-1	1	0	86.443	83.35
4	1	1	0	124.561	125.50
5	-1	0	-1	82.775	90.91
6	1	0	-1	113.873	117.98
7	-1	0	1	80.659	76.55
8	1	0	1	126.334	118.20
9	0	-1	-1	131.533	124.34
10	0	1	-1	168.669	163.63
11	0	-1	1	136.781	141.82
12	0	1	1	124.823	132.02
13	0	0	0	160.075	157.77
14	0	0	0	158.473	157.77
15	0	0	0	163.983	157.77
16	0	0	0	150.431	157.77
17	0	0	0	155.87	157.77

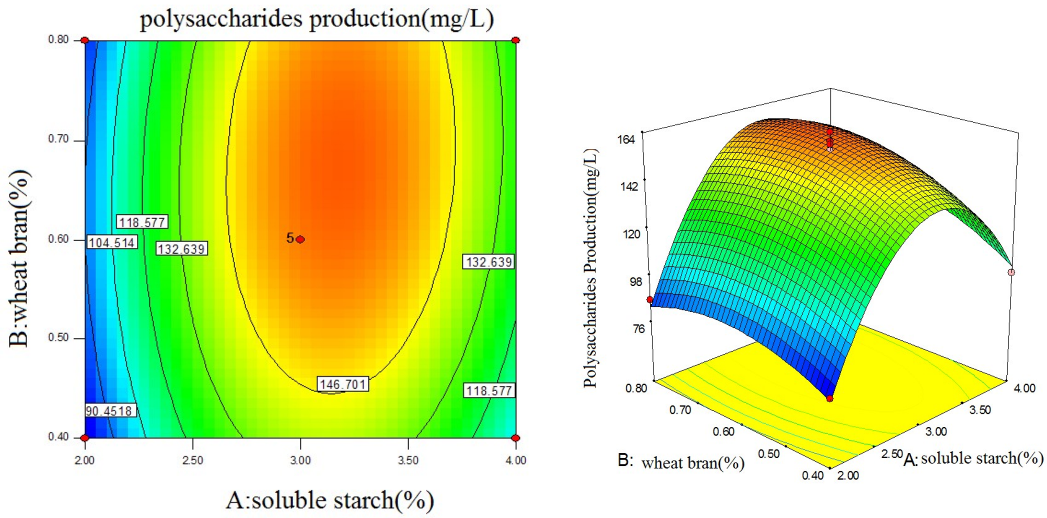

Fig. 1. Contour plot and respective response surface plot of Antrodia camphorata polysaccharides versus soluble

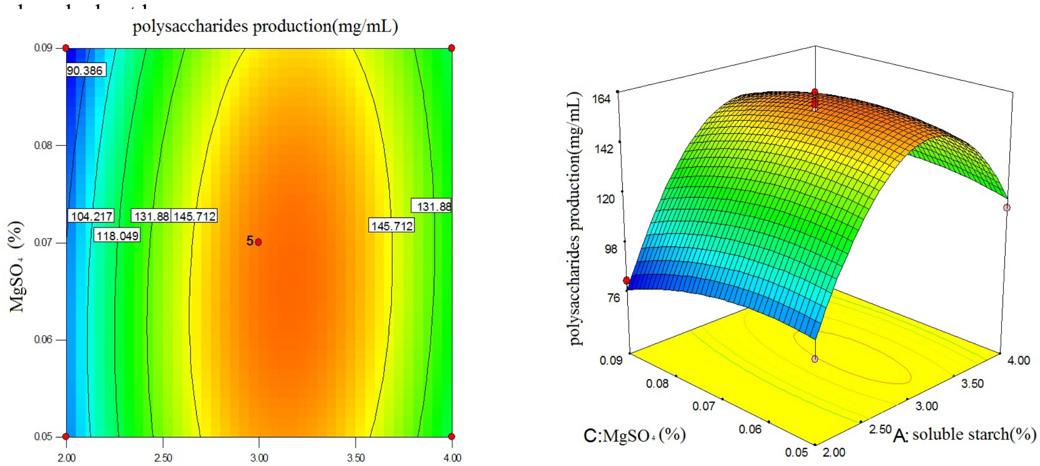

Fig. 2. Contour plot and respective response surface plot of *Antrodia camphorata* polysaccharides versus soluble starch and $MgSO_4$

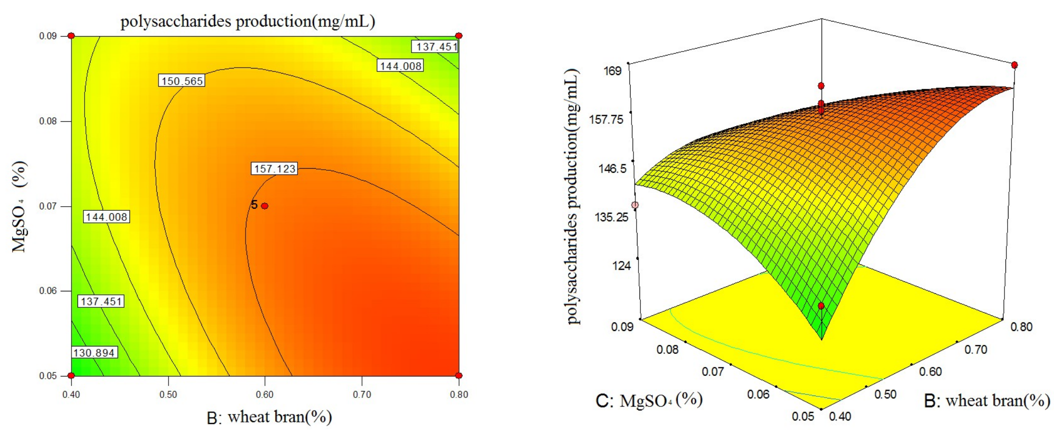

Fig. 3. Contour plot and respective response surface plot of *Antrodia camphorata* polysaccharides versus wheat bran and $MgSO_4$

Table 8. ANOVA for the polysaccharides production according to the response surface quadratic model.

Model term	Coefficient Estimate	Standard Error	Sum of squares	Mean square	F Value	Prob > F
Intercept	157.766	3.558	-	-	-	-
A	17.180	2.813	2361.185	2361.185	37.306	0.0005
B	7.373	2.813	434.919	434.919	6.872	0.0343
C	-3.532	2.813	99.779	99.779	1.576	0.2496
A^2	-50.129	3.877	10580.628	10580.628	167.171	< 0.0001
B^2	-10.588	3.877	471.986	471.986	7.457	0.0293
C^2	-6.727	3.877	190.555	190.555	3.011	0.1263
AB	3.893	3.978	60.606	60.606	0.958	0.3604
AC	3.644	3.978	53.122	53.122	0.839	0.3901
BC	-12.274	3.978	602.555	602.555	9.520	0.0177

reached 183.46 mg/L, which is approximately thrice higher than 51.98 mg/mL before optimization.

CONCLUSION

This study determined the fermentation medium of the high polysaccharide production of *Antrodia camphorata*. Soluble starch, MgSO4, and wheat bran are pivotal factors based on the results obtained from the PB experimental design. The optimal scope of their levels was determined by the steepest ascent path. The optimal concentration for the three factors was further optimized and determined using the BB design and RSM. Unlike other fermentation media, the polysaccharide production clearly increased. The optimal medium contained the following: soluble starch (3.17%); wheat bran (0.79%); KH2PO4 (0.1%); MgSO4 and 7H2O (0.05%); VB1 (10 mg/100 mL). The optimal culture conditions are as follows: temperature (28.8 °C), liquid volume (130.24 mL/250 mL), fermentation time (8.4 d), pH (2), speed (150 rpm), and inoculation amount (12%). The optimal culture medium and optimal fermentation conditions were verified thrice. The experimental results show that the optimal fermentation conditions and liquid fermentation of *Antrodia camphorata* polysaccharide yield increased by 253% to 183.46 mg/L, which is higher than that before optimization.

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