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Research Article

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Optimization of fermentation conditions by *Trametes versicolor* FG-97 using response surface methodology for laccase production

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ABSTRACT

In order to improve the activity of laccase by Trametes versicolor FG-97, response surface methodology was applied to optimize the fermentation conditions. We first optimized the conditions by using Plackett-Burman design to screen for the significant factors, found that pH, temperature and incubation time were the main ones. After the steepest ascent test and Box-Behnken design, the optimum conditions for laccase production were found to be a temperature of 30 °C, pH of 5.2 , time of fermentation for 9 days (160r/min) and 2% (w/v) of inoculation density, the peak of laccase activity achieved at 306.33 $U{\cdot}L^{-1}$, and the experimental values agreed with the predicted values. These results suggested that the predicted model was reliable and available for the optimization of laccase fermentation conditions.

Key words: *Trametes versicolor*, laccase activity, Plackett-Burman, Box-Behnken

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INTRODUCTION

Laccases (E.C.1.10.3.2) are copper-containing polyphenol oxidases. They belong to a small family of the blue copper oxidases that are widely distributed in plants, insects, bacteria and fungus [1], expecially white-rot fungus, they are one of the most important laccase producing fungus [2]. The application of laccase made a great contribution to the degradation of pesticides [3] food industry [4] and biological detection [5]. In this study, we aimed at improving the activity of laccase, and using it to degrade bamboo, finally getting the bamboo fibers.

The optimal fermentation conditions are conducive to the production of laccase. There were some studies about the optimization of fermentation conditions of white-rot fungus, but most of these studies were using the single-factor experiments and orthogonal design experiments [6, 7]. The former one is only exploring one different factor at a time, and the latter can not find the optimal area of the response. However, response surface methodology (RSM) is much more efficient technique. We use it for investigating the relationship between many independent variables. Hence, the objective of this paper was to seek for the optimal fermentation of *Trametes versicolor* FG-97 by using RSM, which was isolated from the soil of a bamboo forest in Huangfengqiao Forestry Farm (Youxian, China)

EXPERIMENTAL SECTION

Micro-organism: *Trametes versicolor* strain (FG-97) used in this study is a highest activity laccase producing fungi among other strains we got from the soil of a bamboo forest.

Culture conditions: The basal medium for laccase production consisted of: glucose 5.0g/L, $(NH_4)_2C_4H_4O_6$ 0.2g/L, KH_2PO_4 1.0g/L, $CaCl_2$ 0.1g/L, $MgSO_4 \cdot 7H_2O$ 0.5g/L, V_{B1}0.1g/L, $CuSO_4 \cdot 7H_2O$ 0.007g/L, $MnSO_4$ 0.025g/L, $ZnSO_4 \cdot 7H_2O$ 0.06g/L, $FeSO_4 \cdot 7H_2O$ 0.005g/L, $CoCl \cdot 6H_2O$ 0.001g/L, Tween-80 1g/L [8]. First, the strain FG-97 was grown on PDA plate. Then two mycelial mats of 5-mm-diameter from the plate were transferred to the basal liquid medium for laccase production. Erlenmeyer flasks (500mL) containing 100mL of the liquid medium were incubated at 28°C in a rotary shaker at 160 r/min for 8 days.

Laccase assay: The culture broth was centrifuged at 10,000g for 10 min at 4°C, and the supernatant were retained for laccase assay [9].The detailed protocol for assaying laccase activity followed that described in Niladevie et al. [10]. Briefly, laccase activity assay was conducted in 6 ml reaction mixtures consisting of 5.4ml of 0.2M sodium acetate buffer (pH 4.5), 0.4 ml of 0.5mM 2,2-azino-bis-3- ethylbenzothiazoline-6-sulphonic acid (ABTS) solution, and 0.2 ml culture supernatant. The reaction was monitored by measuring the change of OD at A_{420} for 2 min at 30°C. One unit of enzyme activity is defined as the amount of enzyme that oxidized 1μM ABTS per minute under the assay conditions. The extinction coefficient of 3.6×10^{-4} $mol^{-1} \cdot cm^{-1}$ was used for oxidized ABTS.

Experimental design: Firstly, we use Minitab 16 to develop a Plackett-Burman design (PBD) to screen for the best key factors for the laccase production. The factors included inoculation density (%), liquid volume (mL), rotating speed (r/min), pH, temperature (°C) and incubation time (d). As shown in Table 1, the signs -1 and +1 represented the lower and higher levels. Generally, the higher levels of the factors were equal to 1.0-1.5-fold of the lower levels [11]. Secondly, steepest ascent experiments (Table 3) were carried out to investigate the change direction and step size of the key factors. Finally, based on the PBD and steepest ascent experiments, Box-Behnken design (BBD) was employed to establish the optimum levels of the three variables. The BBD with thtee variables at three levels was shown in Table 4.

RESULTS AND DISCUSSION

Plackett-Burman design: Statistical analysis was carried out by using ANOVA according to the results of PBD (Table 2). As shown in Figure 1 pH (D) and temperature (E) were found to be the key variables significantly influencing laccase activity (α=0.05), and incubation time (F) also had significant influence on laccase production when α=0.1. Especially, pH with P value of 0.014 was found to be the most important variable followed by temperature (P=0.036) and incubation time ((P=0.059). The first-order polynomial equation for the predicted response Y_1 of laccase yield was given as follow:

$Y_1 = 142.8 - 21.1\ A + 19.5\ B + 19.8\ C + 39.2\ D + 30.0\ E + 25.7\ F$

D, E, F had regression coefficient values of 39.2, 30.0, 25.7, respectively, which indicated pH, temperature and incubation time at high levels had positive effects on laccase production by *Trametes versicolor* FG-97.

Tab.1 six parameters used in the PBD

Factor	Variables with designate	Level	
		Low (-)	High (+)
A	Inoculation volume (%)	2	4
B	Liquid volume (mL)	50	100
C	Rotating speed (rad/min)	140	160
D	pH	3	4.5
E	Culture temperature (°C)	24	30
F	Incubation time (d)	7	10

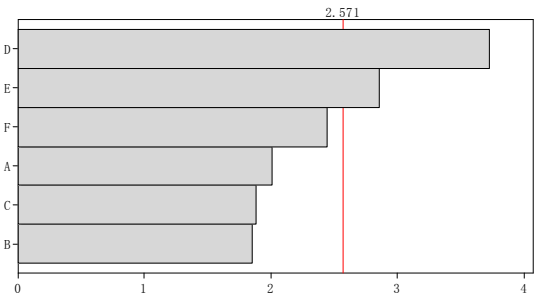


Fig.1: Pareto chart of six factors

Tab.2 Experiment design and results of PBD (with coded values)

Run	Variable code level						Laccase activity ($U \cdot L^{-1}$)
	A	B	C	D	E	F	
1	1	-1	1	-1	-1	-1	50.48
2	1	1	-1	1	-1	-1	126.95
3	-1	1	1	-1	1	-1	132.17
4	1	-1	1	1	-1	1	105.43
5	1	1	-1	1	1	-1	142.59
6	1	1	1	-1	1	1	198.44
7	-1	1	1	1	-1	1	251.04
8	-1	-1	1	1	1	-1	233.91
9	-1	-1	-1	1	1	1	227.68
10	1	-1	-1	-1	1	1	101.87
11	-1	1	-1	-1	-1	1	122.21
12	-1	-1	-1	-1	-1	-1	16.25

The steepest ascent experiments: After the key variables were identified, the steepest ascent experiments were carried out to explore the central point of these variables' values for subsequent response surface design. According to results from this experiment, the optimal fermentation conditions of the levels were trial 3. Hence, the levels of these three factors in trial 3 were chosen as a central point of the BBD in further optimization.

Tab.3 The design and results of the steepest ascent test

Trail	pH	Temperature (°C)	Incubation time (d)	Laccase activity ($U \cdot L^{-1}$)
1	4	26	7	153.03
2	4.5	28	8	192.67
3	5	30	9	226.85
4	5.5	32	10	189.23
5	6	34	11	79.23

Box-Behnken design: The BBD and the coded levels of each factor were shown in Table 4. Experimental results for the three-factor-three-level response surface analysis were shown in Table 5. The total number of trial runs for this design was 15, included three repeats at the centre point of this BBD.

The second-order polynomial equation to explain the model for laccase activity by multiple regression analysis was given below (Y_2 is laccase activity):

$$Y_2 = 303.62 - 13.80A - 3.822B - 19.237C - 37.823A^2 - 67.34B^2 - 56.56C^2 - 4.058AB - 5.94AC + 25.02BC$$

Tab.4 The range and the levels of the parameters

Factor	Variables with designate	Level		
		-1	0	1
A	pH	4	5	6
B	Culture temperature (°C)	28	30	32
C	Incubation time (d)	8	9	10

Tab.5 Design and experimental results of Box-Behnken design

Trail	Factors			Laccase activity ($U \cdot L^{-1}$)
	A	B	C	
1	1	1	0	167.63
2	-1	1	0	202.33
3	1	-1	0	202.70
4	0	1	-1	177.63
5	1	0	1	167.63
6	-1	0	1	208.10
7	0	-1	-1	216.01
8	0	-1	1	131.77
9	0	0	0	306.67
10	1	0	-1	222.26
11	0	1	1	193.48
12	0	0	0	292.32
13	-1	-1	0	221.17
14	0	0	0	301.88
15	-1	0	-1	238.98

The data were processed by using software Minitab 16, and Table 6 presented the model coefficient, F values and determination coefficient (R^2) for the second-order polynomial equation. The results of ANOVA indicated that the model of the equation for laccase activity was significant (P=0.002, Lack-of-fit=0.294), the coefficient of determination (R^2) was calculated to be 97.39%, this indicated there was 97.39% of the total variation could be explained by the model for laccase activity. The value of the adjusted determination coefficient (Adjusted R^2=92.70%) also indicated that the model was highly reliable. From Table 6, it can be seen that the factors with greater significance were A, C and squared of A^2, B^2, C^2, and the interaction terms BC.

Figure 2 showed the response surfaces and contour plots provided by the regression equation. All of those plots indicated that the effects of variables were consistent with the predictions.

Tab.6 ANOVA results for the equation

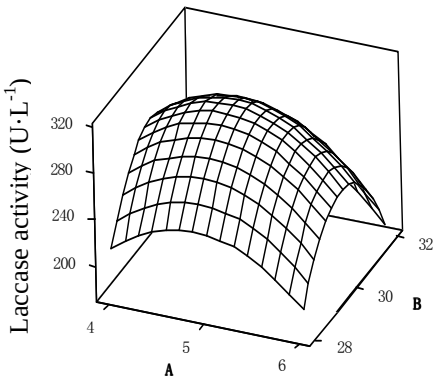
Source	DF	Seq SS	Adj MS	F	P
Model	9	36994.5	4110.5	20.77	0.002
Linear	3	4600	1533.3	7.75	0.025
A	1	1522.4	1522.4	7.69	0.039
B	1	116.9	116.9	0.59	0.477
C	1	2960.7	2960.7	14.96	0.012
Squure	3	29683.2	9894.4	49.99	<0.001
A^2	1	3133.9	5282.1	26.69	0.004
B^2	1	14738.4	16744.9	84.6	<0.001
C^2	1	11810.9	11810.9	59.67	0.001
Interaction	3	2711.4	903.8	4.57	0.068
AB	1	65.9	65.9	0.33	0.589
AC	1	141	141	0.71	0.437
BC	1	2504.5	2504.5	12.65	0.016
Residual	5	989.6	197.9		
Lack-of-fit	3	784.4	261.5	2.55	0.294
Pure Error	2	205.2	102.6		
Total	14	37984.2			
R^2=97.39%			R^2-Adj=92.70%		

According to the results of BBD, optimized analysis was carried out. And then, applying the equation, we could work out the independent variable real value [12]:

$$x_i = \frac{X_i - X_o}{\Delta X_o} \quad (i = A, B, C)$$

Where X_i is the actual value of independent variable, X_o is the actual value of independent variable at the central point, ΔX_o is the steep change value, and x_i is the coded value of independent variable. The optimal levels were as follows: X_A= -0.1717, X_B= -0.0505 and X_C= -0.1717, which means that pH 5.2, at 30°C cultured for 9 days, and other conditions stayed the same as before. Under this fermentation conditions, the maximal laccase activity was 306.5406 $U \cdot L^{-1}$ and the composite desirability was 98.353% which means the laccase activity was likely to achieved at 310.00 $U \cdot L^{-1}$.

To confirm the optimal conditions, three replicate experiments with the optimal of concentrations were carried out, and the maximal laccase activities were: 305.89$U \cdot L^{-1}$, 306.38 $U \cdot L^{-1}$, 306.73 $U \cdot L^{-1}$, similar to that predicted based on the model. The comparison of laccase activities by *Trametes versicolor* FG-97 in Erlenmeyer flasks with the control and the optimized conditions was shown in Figure 3.



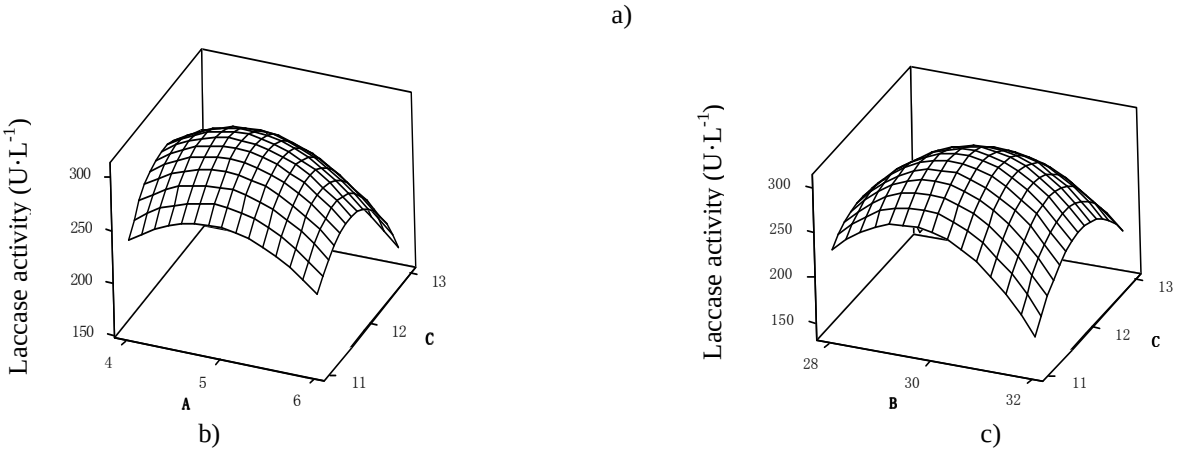


Fig.2: Surfaces and contours plots for the effect of a) pH and temperature for the laccase activity, b) pH and incubation time for the laccase activity, c) temperature and incubation time for the laccase activity by FG-97

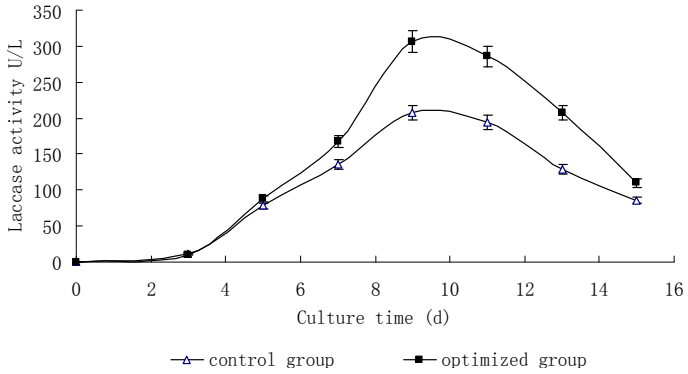


Fig.3: Comparative of laccase activity with control and optimized conditions

CONCLUSION

In this study, optimization of fermentation conditions of *Trametes versicolor* FG-97 was explored by using RSM, the results of PBD showed that, among these six factors, pH, temperature and incubation time all had significantly positive effect on laccase activity. Further study based on the steepest ascent test and BBD, indicated that when the initial pH 5.2, inoculation density of 2%, 100mL liquid volume, then incubated at 30°C in a rotary shaker (160r/min) for 9 days, the peak of laccase activity achieved at 306.33 $U \cdot L^{-1}$, and significantly higher than that from the control conditions, an approximate 1.48-fold improvement over the previous activity (206.98 $U \cdot L^{-1}$). Thus by using the RSM, it is possible to determine the accurate values of the fermentation conditions where maximum production of laccase production.

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