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Optimization of medium composition for exopolysaccharide production by *Phellinus nigricans*

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ABSTRACT

Using Plackett–Burman design, steepest ascent method, and Box–Behnken design, we carried out sequential statistical optimization of the *Phellinus nigricans* culture medium for maximizing the production of exopolysaccharides. The concentrations of the constituents of culture medium as optimized by this method were: 51.67 g/L glucose, 6.96 g/L peptone, 1.0 g/L yeast extract, 5 mg/L thiamine, 1.0 g/L KH_2PO_4, and 0.5 g/L $MgSO_4$. The yield of exopolysaccharides obtained using the optimized culture medium was 1.89 ± 0.13 g/L—2.3-fold higher than that obtained when unoptimized culture medium was used. The exopolysaccharide of *P. nigricans* (PNEP) is comprised by glucose and mannose with the molar ratio 1:2.63. PNEP thus produced scavenged superoxide anion radical, 1,1-diphenyl-2-picrylhydrazyl radical, and hydroxyl radical; showed antioxidant properties; and had reducing power.

1. Introduction

Mushrooms have been used in traditional medicine of Asian countries such as China, Korea, and Japan. Advancements in cell biology, immunology, and molecular biology provided tools that were valuable in confirming that polysaccharides and their composites were the active ingredients present in these medicinal mushrooms. Polysaccharides from medicinal mushroom have attracted much attention in the recent years due to their diverse and potentially significant pharmacological properties, including antioxidant, immunomodulatory, anti-tumor, and anti-inflammatory activities (Ajith & Janardhanan, 2003; Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Chen et al., 2011; Ooi & Liu, 2000).

Phellinus igniarius is one of the most well known of all mushrooms used in traditional medicine (Guo, Zou, & Sun, 2010). Polysaccharides from *P. igniarius* have been found to inhibit tumor growth and metastasis, and showed low toxicity (Chen et al., 2011; Song, Lin, Yang, & Hu, 2008; Wang et al., 2012). However, little is known about *Phellinus nigricans* (Fr.) P. Karst, which belongs to the same family as that of *P. igniarius*, a perennial wood-decaying fungus. Fruit bodies of *P. nigricans* are precious because of their rarity and difficulty of cultivation. The submerged fermentation technology is a promising alternative for producing exopolysaccharides of *P. nigricans* (PNEP). This technology compensates for the limited availability of fungal resources. Further, artificial cultivation is difficult and requires extensive training, whereas submerged fermentation is a fast and efficient way to obtain PNEP.

Optimal medium composition is vital for enhancing the yield of PNEP production using submerged cultures. Statistical experimental design provides a systematic and efficient means of realizing desired goals. Response surface techniques are important statistical optimization methods, where many parameters can be optimized simultaneously and much quantitative information can be derived from a limited number of experimental trials (Houng, Hsu, Liu, & Wu, 2003). These methods have been successfully applied for improving the culture medium and cultivation process of many edible and medicinal mushrooms (Cui et al., 2006; Mao, Eksriwong, Chauvatcharin, & Zhong, 2006; Tang et al., 2008).

Using Plackett–Burman design (PBD), path of steepest ascent, and Box–Behnken design (BBD), we attempted to establish an optimal medium composition for PNEP production in submerged cultures. Here we report the development of an improved culture medium and antioxidant properties of PNEP produced.

2. Materials and methods

2.1. Chemicals

Ascorbic acid, pyrogallic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals (St. Louis, MO, USA). Unless otherwise stated, all chemicals used were of analytical grade.

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2.2. Microorganism

The *P. nigricans* strain was isolated in the laboratory from the fruiting body obtained from the Institute of Biology located at Jilin province. The strain was maintained on potato dextrose agar (PDA) slant at 4 °C and activated once every 2 months.

2.3. Preparation of inoculum and liquid culture

P. nigricans was initially grown on PDA medium in a Petri dish for 6–7 days. A 10-mm^2 area of the agar-plate culture was excised with a sterilized cutter and inoculated into 100 mL basal liquid medium contained in a 500 mL flask. The basal liquid medium (pH 6.8) consisted of glucose (20 g/L), peptone (2.0 g/L), yeast extract (1.0 g/L), thiamine (VB_1) (10 mg/L), KH_2PO_4 (1.0 g/L), and $MgSO_4$ (0.5 g/L). Experiments involving flask-culture of *P. nigricans* were performed using 200 mL medium in 500 mL flasks at 28 °C for 6 days on a rotary shaker at 150 rpm (Luo et al., 2009).

2.4. Optimization of culture medium for PNEP production

2.4.1. Plackett–Burman experimental design

The Plackett–Burman experimental design is an efficient method for the optimization of medium composition. Such a design was employed to evaluate the effect of various factors, assuming that there are no interactions between the constituents X_i in the range of variables under consideration (Reddy, Wee, Yun, & Ryu, 2008). The results of PBD were fitted by the first-order model as follows:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the estimated target function, β_0 is the model intercept, β_i is the regression coefficient, and X_i is the coded independent factor.

Table 1 illustrates the selected levels of each variable used in our experimental design. Each independent variable was defined at two levels, high level (+1) and low level (−1), respectively. High levels were 1.5 times the low levels. The six assigned variables were screened in twelve experimental designs. All experiments were carried out in duplicate and the averages of PNEP were taken as response (Table 2).

2.4.2. Path of steepest ascent method

The path of steepest ascent is a procedure for moving sequentially along the path of steepest ascent to the region of the optimum, namely, in the direction of the maximum increase in response. Based on the results of the PBD, the optimum level scope of each selected factor was examined by path of steepest ascent method. A path of steepest ascent method was designed using the direction of PBD experimental value as the uphill direction. The changing step size was confirmed according to the effects value of PBD. High value was selected when the effect value of variable was positive, and low value was selected when the effect value of variable was negative.

Table 2
The designs and results of Plackett–Burman design.

No.	X_1	X_2	X_3	X_4	X_5	X_6	PNEP (g/L)
1	1	−1	1	−1	−1	−1	0.96
2	1	1	−1	1	−1	−1	0.94
3	−1	1	1	−1	1	−1	1.03
4	1	−1	1	1	−1	1	0.83
5	1	1	−1	1	1	−1	0.98
6	1	1	1	−1	1	1	1.21
7	−1	1	1	1	−1	1	0.84
8	−1	−1	1	1	1	−1	0.78
9	−1	−1	−1	1	1	1	0.75
10	1	−1	−1	−1	1	1	1.03
11	−1	1	−1	−1	−1	1	1.14
12	−1	−1	−1	−1	−1	−1	0.77

The variables with that were predicted to produce highest PNEP yield were used as center-point for Box–Behnken design.

2.4.3. Box–Behnken design and response surface methodology

The Box–Behnken design was used to further derive the optimal level of the key factors required for the maximal production of PNEP. The results obtained were analyzed by the response surface methodology. The experimental variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (2)$$

where x_i is a coded value of the independent variable, X_i is the actual value of the independent variable, X_0 is the actual value of X_i at the center-point, and ΔX_i is the value of the step change.

The BBD data were analyzed by multiple regression to fit the following quadratic polynomial model:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta k_{ij} x_i x_j \quad (3)$$

where Y is the predicted response (herein it was yield of PNEP), β_0 is an intercept, β_i, β_{ii}, and β_{ij} are the coefficients of the linear, quadratic, and interactive terms, respectively, while x_i and x_j represent the coded independent variables.

The regression coefficients of the linear, quadratic, and interaction terms were determined according to the variable analysis and were then used in statistical calculations to generate three-dimensional (3D) surface and contour plots from the fitted polynomial equation. The second-order polynomial coefficients were calculated and analyzed using the Design Expert software (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA). $P < 0.05$ was considered statistically significant.

2.5. Production and measurement of PNEP

The culture mediums after cultivating *P. nigricans* according to the experimental design were centrifuged at 3000 × *g* for 20 min. The supernatant (5 mL) was treated with ethanol to a final concentration of 75% (v/v) to induce precipitation. The precipitates

Table 1
Plackett–Burman design and effect of each variable on the PNEP production.

Factor	Variable	Levels		Effect	Standard error	*t*-Value	*P*-value
		−1	1				
Glucose (g/L)	X_1	20	30	0.1067	0.0178	3.00	0.030
Peptone (g/L)	X_2	2	3	0.1700	0.0178	4.78	0.005
Yeast extract (g/L)	X_3	1	1.5	0.0067	0.0178	0.19	0.859
Thiamine (mg/L)	X_4	10	15	−0.1700	0.0178	−4.78	0.005
KH_2PO_4 (g/L)	X_5	1	1.5	0.0500	0.0178	1.41	0.219
$MgSO_4$ (g/L)	X_6	0.5	0.75	0.0567	0.0178	1.59	0.17

collected by centrifugation at 3000 × *g* for 20 min were solubilized in deionized water and lyophilized to obtain PNEP. The yield of PNEP was measured by phenol–sulfuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.6. Preparation of PNEP

PNEP was dissolved in distilled water and deproteinized using Sevag reagent. Following the removal of Sevag reagent, the aqueous fraction was lyophilized and was analyzed for analysis and antioxidant activities in vitro.

2.7. Analysis of PNEP

Monosaccharide compositions of PNEP were determined by gas chromatography (GC). The sample was hydrolyzed with 2 M trifluoroacetic acid (120 °C, 3 h). After hydrolysis, the neutral monosaccharides were successively reduced with $NaBH_4$ and acetylated with 1:1 pyridine acetic anhydride at 90 °C for 1 h. The alditole acetates were analyzed by GC on a Shimudzu GC-14C instrument equipped with an Rtx 2330 column (30 m × 0.32 mm × 0.2 μm). The column temperature was maintained at 170 °C for 2 min, increased to 240 °C at a rate of 8 °C/min held for 1 min, and then increased to 265 °C at a rate of 8 °C/min held for 20 min. Uronic acid content was determined using the m-hydroxybiphenyl colourimetric procedure with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

Infrared (IR) spectral analysis of PNEP was obtained using a PerkinElmer Spectrum GX (USA). The purified polysaccharides were mixed with KBr powder and pressed into pellets for FTIR measurement within the frequency range of 4000–400 cm^{-1}.

2.8. Antioxidant activity test in vitro

Antioxidant activity was determined based the efficacy of scavenging superoxide anion radical, hydroxyl radical, and DPPH radical, as well as from the reducing power of the respective compounds. The data of antioxidant activity assay were presented as mean ± SEM. Statistical analyses were performed using Student's *t*-test and one-way analysis of variance. All computations were done by employing the statistical software (SPSS, version 13.0).

2.8.1. Superoxide radical scavenging assay

Approximately 4.5 mL of 50 mM Tris–HCl (pH 8.2) was mixed with 4.2 mL deionized water and incubated at 25 °C for 20 min. Following this, 1 mL PNEP solution and 0.4 mL pyrogallicacid were added and the mixture was shaken thoroughly. After incubating the mixture at 25 °C for 5 min, reaction was terminated by the addition of 8 mM HCl. The absorbance of the solution at 320 nm was measured and ascorbic acid was used as positive control (Wang, Wang, & Quan, 2014). The superoxide radical scavenging activity was calculated using the following formula:

$$\text{Scavenging rate}\,(\%) = \left[\frac{A_c - A_s}{A_c}\right] \times 100\% \quad (4)$$

where A_c is the absorbance of blank and A_s is the absorbance corresponding to PNEP or ascorbic acid.

2.8.2. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was estimated according to the method of Winterbourn and Sutton (1984). The reaction mixture containing 1 mL of 0.15 M phosphate buffer (pH 7.4), 1 mL of 40 μg/mL safranin, 1 mL of 0.945 mM EDTA–Fe(II), 1 mL of 3% (v/v) H_2O_2, and 0.5 mL of PNEP solution was incubated at 37 °C for 30 min. Following this, the absorbance of the solution at 560 nm was measured. Ascorbic acid was used as positive control. The EC_{50} value (mg/L) of PNEP or ascorbic acid was defined as the effective concentration required for scavenging 50% of the hydroxyl radicals. The hydroxyl radical scavenging activity was expressed as follows:

$$\text{Scavenging rate}\,(\%) = \left[\frac{A_c - A_s}{A_c}\right] \times 100\% \quad (5)$$

where A_c is the absorbance of blank and A_s is the absorbance of PNEP or ascorbic acid.

2.8.3. DPPH scavenging assay

DPPH radical scavenging activity was measured according to the method of Shimada, Fujikawa, and Yahara (1992) with minor modifications. The reaction mixture containing 2 mL of DPPH (0.1 μM in 95% ethanol) solution and 2 mL of PNEP solution was incubated at 25 °C for 15 min and the absorbance of the mixture at 517 nm was measured. Ascorbic acid was used as a positive control. The EC_{50} value (mg/L) of PNEP was defined as the effective concentration required for scavenging 50% of the DPPH radicals.

The antioxidant activity of PNEP was evaluated according to the following formula:

$$\text{Scavenging rate}\,(\%) = \left[1 - \frac{A_s}{A_c}\right] \times 100\% \quad (6)$$

where A_s is the absorbance of PNEP or ascorbic acid solution and A_c is the absorbance of DPPH solution.

2.8.4. Determination of the PNEP reducing power

The reducing power of PNEP was evaluated according to the method of Deng et al. (2011). The reaction mixture containing 2.5 mL of phosphate buffer (pH 6.6, 0.2 M), 2.5 mL of potassium ferricyanide (1%, w/v), and PNEP solution was incubated at 50 °C for 20 min. Following this, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. The mixture was centrifuged at 1200 × *g* for 10 min. Approximately 2.5 mL of the supernatant was collected and mixed with 2.5 mL deionized water and 0.5 mL of $FeCl_3$ solution (0.1%, w/v). After incubating at room temperature for 15 min, the absorbance of the solution at 700 nm was measured. Ascorbic acid was used as positive control.

3. Results and discussion

3.1. Plackett–Burman experimental design

To optimize the culture medium for the production of PNEP, we first analyzed the constituents of the medium. The upper and lower limits of the variables for each factor was then chosen based on the medium used for the culture of *Phellinus baumii* (Luo et al., 2009) (Table 1). The Plackett–Burman experimental design for 12 trials with two levels for each variable, and the corresponding PNEP production are presented in Table 2. To approach the neighborhood of the optimum response, a fitted first-order model for PNEP production was obtained from the Plackett–Burman experimental design as follows:

$$Y = 0.9383 + 0.0533X_1 + 0.0850X_2 + 0.0033X_3 - 0.0850X_4 + 0.025X_5 + 0.0283X_6 \quad (7)$$

The coefficient of each variable in Eq. (7) represents the extent of the effect of this variable on the yield of PNEP. The linear regression coefficient R^2 was 0.9222 and the adjusted determination coefficient (Adj R^2) was 0.8288 for the model. These results indicated that the model was suitable for Plackett–Burman experimental design. The effect of each variable and the results of statistical analyses are shown in Table 1. The *P*-values for glucose (X_1), peptone (X_2),

Table 3
The designs and results of path of steepest ascent.

No.	Glucose (g/L)	Peptone (g/L)	Thiamine (mg/L)	PNEP (g/L)
1	30	3	10	1.14
2	35	4	9	1.28
3	40	5	8	1.47
4	45	6	7	1.58
5	50	7	6	1.72
6	55	8	5	1.43
7	60	9	4	1.24

and thiamine (X_4) were less than 0.05 with a 95% confidence. These results clearly suggested that, of all the examined factors, glucose, peptone, and thiamine were the most significant for PNEP production.

3.2. The path of steepest ascent

Our results indicated that compared with other factors, glucose, peptone, and thiamine could significantly influence the PNEP production. Further, the coefficients of X_1 and X_2 in Eq. (7) were positive while the coefficient of X_4 was negative, suggesting that an increase in concentrations of glucose and peptone and decrease in that of thiamine could have a positive effect on the production of PNEP. Subsequently, path of the steepest ascent was employed to search the proper direction for altering the levels of these three factors, keeping the rest of the factors constant at zero level. The experimental design and corresponding results are shown in Table 3. The results showed that the yield of PNEP was the highest when the concentrations of glucose, peptone, and thiamine were 50 g/L, 7 g/L, and 6 mg/L, respectively. Thus, our results suggested that PNEP production response could be near maximum at this concentrations of glucose, peptone, and thiamine.

3.3. Box–Behnken design and response surface methodology (RSM)

The Box–Behnken design and response surface methodology were used employed to further examine and refine the optimal levels of glucose, peptone, and thiamine, respectively required for the production of PNEP. The concentrations of the three factors derived from the steepest ascent path (Table 3) were taken as the center points. The low and high levels for each factor were coded as shown in Table 4. Fitting of the experimental data by regression analysis gave rise to a second-order polynomial equation model as follows:

Table 5
ANOVA for the PNEP production according to response surface quadratic model.

Source	Statistical analysis				
	Sum of squares	df	Mean square	*F*-value	*P*-value
Model	0.22	9	0.025	33.34	<0.0001
X_1	0.044	1	0.044	58.86	0.0001
X_2	0.0098	1	0.0098	13.26	0.0083
X_4	0.021	1	0.021	28.42	0.0011
$X_1\,X_2$	0.0043	1	0.0043	5.71	0.0481
$X_1\,X_4$	0.0081	1	0.0081	10.96	0.0129
$X_2\,X_4$	0.0056	1	0.0056	7.61	0.0282
X_1^2	0.00853	1	0.00853	11.53	0.0115
X_2^2	0.11	1	0.11	150.39	<0.0001
X_4^2	0.011	1	0.011	14.24	0.0070
Residual	0.0052	7	0.0007		
Lack of fit	0.0032	3	0.0016	2.12	0.2409
Pure error	0.002	4	0.0005		
Cor total	0.23	16			

$$Y = 1.72 + 0.074X_1 + 0.035X_2 - 0.051X_4 - 0.033X_1X_2 + 0.045X_1X_4 + 0.038X_2X_4 - 0.045X_1^2 - 0.16X_2^2 - 0.05X_4^2 \quad (8)$$

where Y was the predicted PNEP production, X_1, X_2, and X_4 were the coded values of glucose, peptone, and thiamine, respectively.

Table 5 shows the significance of the equation fitting that was tested by ANOVA. The *F*-value obtained from an *F*-test was 33.34, suggesting that the model was adequate. *P* values were used to determine the significance of each coefficient. These values indicated the pattern of the interactions between variables. *P* values less than 0.05 indicated that the coefficient was significant (Guo et al., 2010). As shown in Table 5, the linear coefficients (X_1, X_2, and X_4), the interaction term coefficients ($X_1\,X_2$, $X_2\,X_4$, and $X_1\,X_4$), and the coefficients of the quadratic term (X_1^2, X_2^2, and X_4^2) were significant, as their *P* values were below 0.05.

The value of the regression coefficient R^2 was 0.9772 and that of adjusted determination coefficient (Adj R^2) was 0.9479, suggesting that the model was highly effective. The interactions of the three components and their optimal levels for PNEP production were further analyzed by the response surface methodology. The three-dimensional (3D) response surface curves and the respective contour plots are presented in Fig. 1. The 3D surface and contour plots constructed based on the independent variables (glucose and peptone) are shown in Fig. 1a and d. The independent variable, thiamine, was kept at zero level. The highest PNEP yield was achieved

Table 4
The levels of each variable and corresponding production of PNEP obtained from BBD.

No.	Coded variable level			Real variable level			PNEP (g/L)	
	X_1	X_2	X_4	Glucose (g/L)	Peptone (g/L)	Thiamine (mg/L)	Predicted	Experimental
1	−1	−1	0	45	6	6	1.37	1.35
2	1	−1	0	55	6	6	1.59	1.57
3	−1	1	0	45	8	6	1.51	1.52
4	1	1	0	55	8	6	1.59	1.61
5	−1	0	−1	45	7	5	1.78	1.76
6	1	0	−1	55	7	5	1.81	1.81
7	−1	0	1	45	7	7	1.56	1.55
8	1	0	1	55	7	7	1.79	1.78
9	0	−1	−1	50	6	5	1.66	1.67
10	0	1	−1	50	8	5	1.658	1.63
11	0	−1	1	50	6	7	1.486	1.51
12	0	1	1	50	8	7	1.632	1.62
13	0	0	0	50	7	6	1.72	1.72
14	0	0	0	50	7	6	1.72	1.71
15	0	0	0	50	7	6	1.72	1.73
16	0	0	0	50	7	6	1.72	1.75
17	0	0	0	50	7	6	1.72	1.69

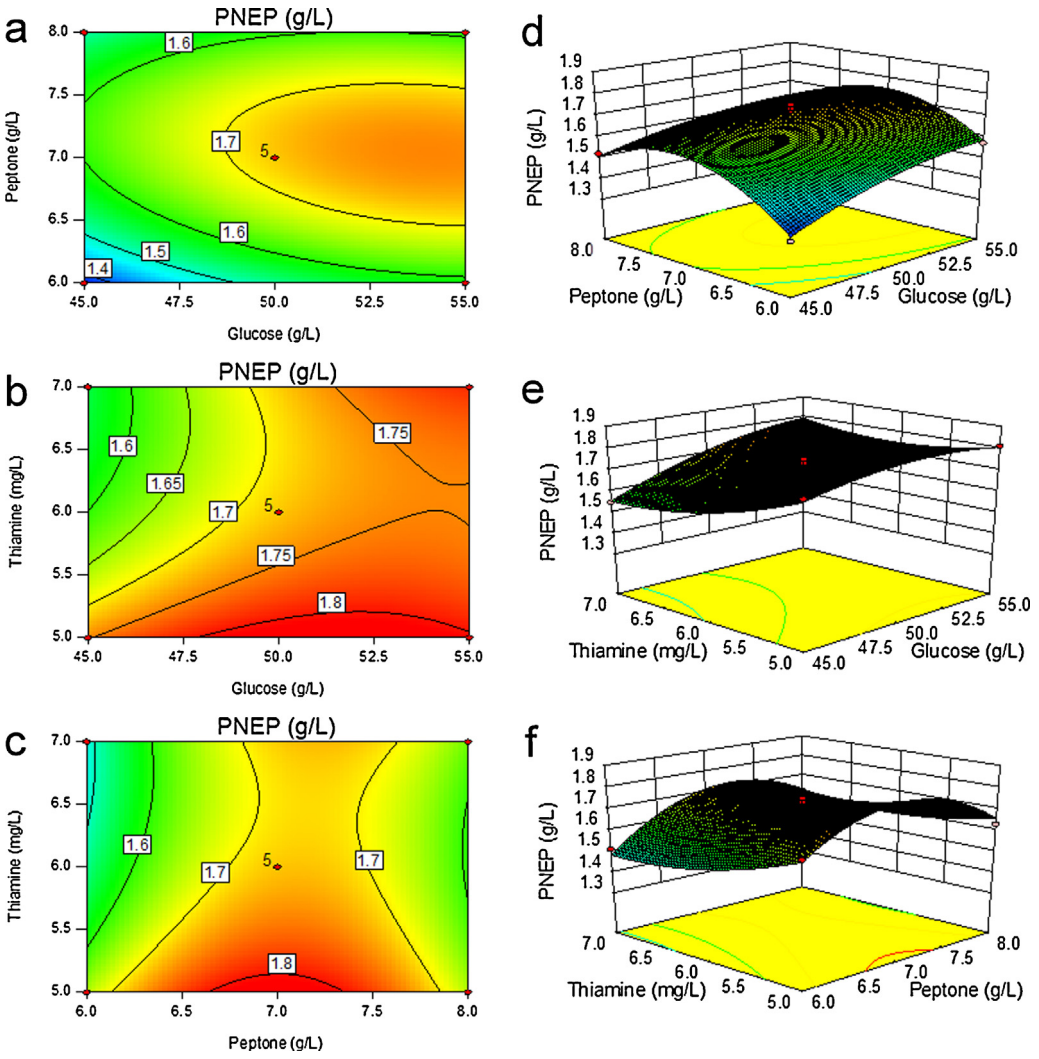

Fig. 1. Three-dimensional and plots response surface showing the effects of variables (X_1, glucose; X_2, peptone; X_4 thiamine) on the response (yield of PNEP).

when the concentrations of glucose and peptone were 51.67 g/L and 6.96 g/L, respectively. Fig. 1b and e shows that the PNEP yield varied with peptone and thiamine concentrations when glucose concentration was kept constant at zero level. The highest PNEP yield was achieved when the concentrations of peptone and thiamine were 6.96 g/L and 5 mg/L, respectively. Fig. 1c and f shows the effects of different glucose and thiamine concentrations on the PNEP yield when peptone concentration was kept constant at zero level. The maximum PNEP yield was achieved when glucose and thiamine was set at 51.67 g/L and 5 mg/L, respectively.

According to the results of the response surface analysis, the predicted maximum production of PNEP was 1.83 g/L when the concentration of glucose, peptone, and thiamine were 51.67 g/L, 6.96 g/L, and 5 mg/L, respectively, and the rest of the variables were kept at the levels found in basal liquid medium.

The effectiveness of the model equations in successfully predicting the optimum response values was examined by culturing *P. nigricans* in the optimized medium. The set of conditions that was predicted by response surface methodology as optimum was used for experimental validation. The yield obtained from experiments using *P. nigricans* culture was 1.89 ± 0.13 ($n = 3$), a value that was similar to that predicted by RSM model (1.83), indicating that the model could be indeed used for optimizing culture conditions for PNEP production.

3.4. Comparison of optimized culture medium and basal culture medium

To compare the performance of the media, we cultured *P. nigricans* using optimized culture medium and basal culture medium. The yield of PNEP in basal culture medium was 0.82 ± 0.11 g/L. Thus, the yield of PNEP (1.89 ± 0.13 g/L) in optimized culture medium

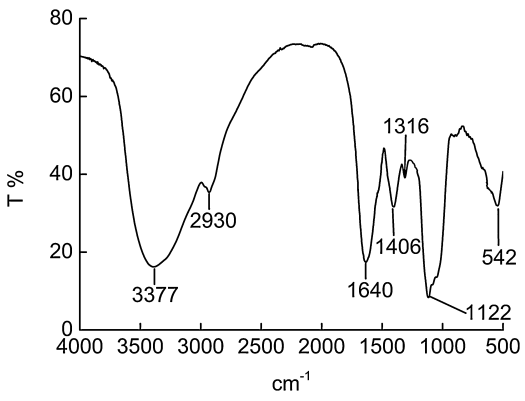

Fig. 2. IR spectrum of PNEP.

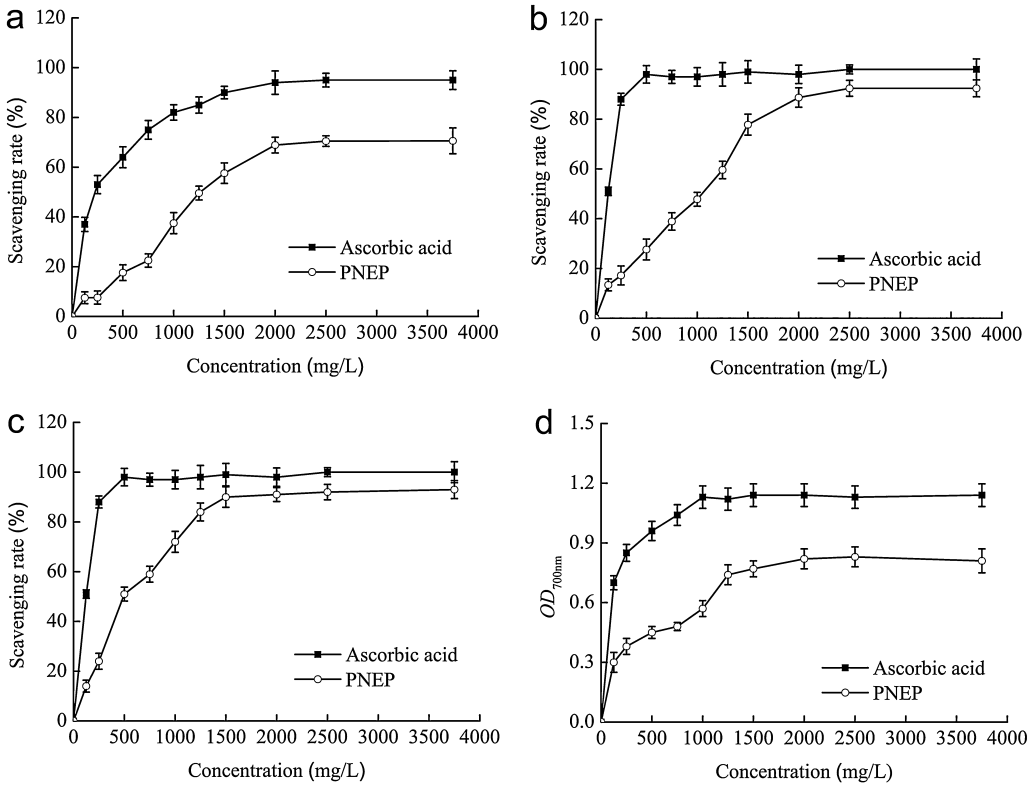

Fig. 3. Antioxidant activity assay of PNEP and ascorbic acid. (a) Scavenging effects on superoxide anion radicals. (b) Scavenging effects on hydroxyl radicals. (c) Scavenging effects on DPPH. (d) Reducing power of PNEP and ascorbic acid.

was approximately 2.3-fold of that obtained when basal culture medium was used.

3.5. PNEP analysis

The monosaccharide composition analysis by GC showed PNEP is only comprised by two monosaccharides (glucose and mannose) and the molar ratio of them was 1:2.63.

As shown in Fig. 2, the band (3377 cm^{-1}) between 3600 and 3200 cm^{-1} represented hydroxyl group stretching. The small band (2930 cm^{-1}) between 3000 and 2800 cm^{-1} can be attributed to C—H stretching and bending vibrations. The band at 1640 cm^{-1} was due to the bound of water. The absorbance at 1316 cm^{-1} was due to the existence of sulfate radical. The band at 1122 cm^{-1} was dominated by ring vibrations overlapped with stretching vibrations of C—OH side groups and C—O—C glycosidic band vibration.

3.6. Antioxidant activity

Anti-oxidative activities of PNEP were evaluated in vitro by assessing the reducing power and the radical scavenging activities.

Superoxide anion is one of the precursors of singlet oxygen and hydroxyl radicals. By contributing to the production of highly reactive radical species, superoxide anions cause extensive cellular damage (Athukorala, Kim, & Jeon, 2006). Fig. 3a shows the results of superoxide radical scavenging assay. Within the range of 0–1500 mg/L, the superoxide anion radical scavenging activity of PNEP was directly proportional to its concentration and reached the peak at 1500 mg/L. However, the scavenging activity of PNEP was substantially lower than that of ascorbic acid. Within this concentration range, the EC_{50} values of PNEP and ascorbic acid were 1123.6 ± 12.7 mg/L and 205.2 ± 4.6 mg/L, respectively.

Hydroxyl radicals easily traverse the cell membranes, readily react with most biomolecules, and cause cell death and tissue damage. Thus, removal of hydroxyl radicals protects tissues from oxidative damage (Yuan, Zhang, Fan, & Yang, 2008). As shown in Fig. 3b, the hydroxyl radical scavenging activity of PNEP was concentration-dependent. The EC_{50} value of PNEP was 1044.6 ± 12.3 mg/L, which was substantially higher than that of ascorbic acid (124.2 ± 5.7 mg/L).

The DPPH free radical is relatively stable and are readily scavenged by antioxidants. Thus, the DPPH free radical has been widely used as a tool for evaluating the free radical scavenging activities of natural products (Leong & Shui, 2002). The DPPH radical scavenging activities of PNEP and ascorbic acid were measured and compared (Fig. 3c). The DPPH radical scavenging activity of PNEP was concentration-dependent. The EC_{50} value of PNEP was 493.6 ± 7.6 mg/L, significantly higher than that of ascorbic acid (125.9 ± 4.6 mg/L).

The reducing power of a compound is an indicator of its potential antioxidant potential (Kallithraka, Bakker, & Clifford, 2001). Fig. 3d shows the reducing power of PNEP and ascorbic acid. The reducing power of PNEP gradually increased with concentration, but was lower than that of ascorbic acid.

4. Conclusions

In this work, culture medium for production of polysaccharide by *P. nigricans* was optimized utilizing a series of experimental designs. Compared with the basal culture medium, the use of optimized culture medium resulted in a 160% increase in PNEP production. The sequential application of PBD, steepest ascent method, and BBD was found was highly effective in predicting the composition of the culture medium required for maximizing the yield of

PNEP. This method may be extended for the production of other bioactive molecules. PNEP was able to scavenge superoxide anion, hydroxyl, and DPPH radical, and showed great potential as a novel antioxidant in vitro. Our study provides important information for the large-scale production of bioactive natural products such as exopolysaccharides from *P. nigricans*, which can be used as functional food or antioxidant.

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