

Optimization of culture medium for production of melanin by *Auricularia auricula*

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Abstract

Melanin is a natural high molecular weight pigment with the huge application value and development potential in food industry. In the present study, medium composition for melanin production by fungus *Auricularia auricula* was investigated. Wheat bran extract, L-tyrosine, and CuSO₄ were determined to optimize medium composition by response surface methodology with Box–Behnken design. Results indicated that the optimal medium composition was 26.80% (v/v) wheat bran extract, 1.59 g/L L-tyrosine, and 0.11 g/L CuSO₄, and the maximum melanin yield was 519.54 mg/L. Melanin production through *A. auricula* fermentation avoided expensive enzymatic or complicated chemical methods for melanin extraction from tissues of plant or animal, which had the huge application value and development potential for efficient production of melanin.

Keywords: optimization; culture medium; melanin; *Auricularia auricula*.

Practical Application: Melanin production through *A. auricula* fermentation is a new preparation method of melanin.

1 Introduction

Auricularia auricula, a non-toxic macro-fungus, is widely distributed in China and has been used as cuisine materials for a long time (Zou et al., 2015a). The black-brown fruit body of *A. auricula* is rich in natural melanin and is increasingly popular in China due to its biological activities, including immunomodulatory activity (Sava et al., 2001), anti-HIV activity (Manning et al., 2003; Montefiori & Zhou, 1991), and antioxidant activity (Liu et al., 2011; Tu et al., 2009; Wu et al., 2008). Thus, melanin is regarded as the main functional components in *A. auricula* fruit body. As a result, there has been a strong consumer demand to use melanin in food industry as a natural colorant, due to its healthful functions and safety, especially compared with synthetic colorant. However, preparation process of melanin from fruit body of *A. auricula* is complex and costly (Zou et al., 2015b). In addition, the time to complete fruit body of *A. auricula* is long and quality of fungus product is instability (Wu et al., 2006).

The production of melanin through microbial fermentation has been considered to be a efficient preparation method of natural melanin. *Escherichia coli* is firstly used for producing melanin (Lagunas-Muñoz et al., 2006). However, some hidden troubles and potential dangers still exist during fermentation process of *E. coli*. Some shiga toxin-producing *E. coli* are the main food-borne pathogens and their propagation may lead to potentially severe disease (Vu-Khac & Cornick, 2008). Therefore, melanin produced by these bacteria can not be used in food industry.

Melanin can also be produced by *A. auricula* through submerged culture. Furthermore, *A. auricula* does not produce fruiting bodies and melanin is secreted into fermentation medium, which make extraction of melanin easier. However, fermentation medium for production of melanin by *A. auricula* is little explored. Response surface methodology (RSM) is a practical statistical method for dealing with complex relationship and optimizing

various factors. Medium composition has been optimized by RSM with Box-Behnken experimental design for production of valuable compounds (Wang et al., 2009). In this study, effect of wheat bran extract, L-tyrosine, and CuSO₄ on melanin yield was investigated. RSM was used to optimize culture medium for attaining the maximum melanin yield.

2 Materials and methods

2.1 Materials and reagents

Wheat bran was obtained from Jiangnan Co. (Nanjing, China), crushed into powder and sieved (opening 0.42 mm). Nutrient content from wheat bran was extracted using deionized water at a liquid-solid ratio of 4 mL/g for 5 h at 60 °C, then incubated for 0.5 h at 100 °C. Wheat bran extract (centrifuged at 4000 rpm for 5 min) was obtained and used as the main nutrients in culture medium. Synthetic melanin and L-tyrosine used in the study were obtained from Sigma-Aldrich Chemicals Co. (St. Louis, USA). The other reagents were obtained from Sinopharm Chemicals Co. (Shanghai, China).

2.2 Strain

Fungus *A. auricula* (RF201) was obtained from Jiangsu Academy of Agricultural Sciences (Nanjing, China). It was maintained on PDA slants and cultured at 25 °C for 7 d, then stored at 4 °C.

2.3 Preparation of inoculum and media

Potato dextrose broth medium was used as seed medium for preparation of inoculum. Four discs (diameter 6 mm) from PDA plates containing fungal mycelia were inoculated to 50 mL seed medium in a 250 mL Erlenmeyer flask and then cultured at 25 °C for 5 d on a 100 rpm reciprocating shaker. Inoculum

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(10%, v/v) was subcultured in a 250 mL Erlenmeyer flask containing 50 mL basal medium for *A. auricula* fermentation. The basal fermentation medium contained 40% (v/v) wheat bran extract, 0.1 g/L CuSO₄, 2 g/L l-tyrosine, 0.1 g/L vitamin B₁, 1 g/L KH₂PO₄, and 1 g/L MgSO₄. Medium composition was varied according to experimental design. All media were incubated for 5 d at pH 8.0, 25 °C, and rotation speed of 100 rpm.

2.4 Box-Behnken design

RSM with Box-Behnken experimental design was used to optimize medium composition for obtaining the maximum melanin yield. Three factors (wheat bran extract, l-tyrosine, and CuSO₄) were determined in experimental design (Table 1). Based on the single-factor experiments, wheat bran extract (20%, 30%, and 40%), l-tyrosine (1, 1.5, and 2 g/L), and CuSO₄ (0.05, 0.1, and 0.15 g/L) were chosen as three critical levels with great impact on melanin yield.

Experimental data was employed by multiple regression to fit quadratic polynomial Equation 1:

$$Y = \beta_0 + \sum_{i=1}^3 B_i X_i + \sum_{i=1}^3 B_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=1}^3 B_{ij} X_i X_j \tag{1}$$

where Y stands for predicted response, X_i and X_j for independent variables, β₀, B_i, B_{ii}, and B_{ij} for intercept and regression coefficients of the model, respectively.

2.5 Measurement of melanin

Measurement of melanin was carried out using the method previously (Zou et al., 2010). Culture medium was firstly centrifuged for 5 min at 4000 rpm and filtered to remove impurities. The obtained supernatant was regulated pH to 2.0 using 3 M HCl, then centrifuged for 20 min at 10000 rpm to separate melanin. Crude melanin was successively washed using ethyl acetate, chloroform, and ethanol. Finally, melanin was dissolved in 0.01 M NaOH and optical density of solution at 400 nm was assayed by a Unico UV-2802 spectrophotometer (Princeton, USA) and compared with synthetic melanin.

2.6 Statistical analysis

Box-Behnken design and analysis of data were done using software Design-Expert 7.0.0 (Minneapolis, USA). Statistical analysis was conducted using Student's t-test, and p < 0.05 and p < 0.01 were regarded as significant and very significant, respectively.

Table 1. Factor and level in experimental design.

Factor	Level		
	-1	0	+1
Wheat bran extract (A, %)	20	30	40
l-Tyrosine (B, g/L)	1	1.5	2
CuSO ₄ (C, g/L)	0.05	0.1	0.15

3 Results and discussion

3.1 Response surface analysis

Medium components (wheat bran extract, l-tyrosine, and CuSO₄) were optimized in order to attain the maximum melanin yield. A Box-Behnken design of RSM and experimental results are shown in Table 2.

A quadratic polynomial model was fitted to results obtained from Box-Behnken experiment and reduced to Equation 2:

$$Y = -787.09 + 34.22A + 707.37B + 5659.41C - 0.64A^2 - 176.08B^2 - 16520.51C^2 - 1481.21BC \tag{2}$$

Statistical significance of polynomial model was analyzed with t-test and results are shown in Table 3. Analysis of variance (ANOVA) showed that the polynomial model was very significant (p < 0.01) and lack of fit was not significant (p > 0.05). Meanwhile, coefficient of determination (R²) was 0.9775 suggesting that melanin yield variation of 97.75% was attributed to medium components. Results showed that quality of model was adequately good and might describe real relationship among medium components.

3.2 Effect of wheat bran extract, l-tyrosine, and CuSO₄ on melanin production

Response surface and contour plots were employed to explain interaction of medium components (wheat bran extract, l-tyrosine, and CuSO₄) and the optimal concentration of each component required for melanin production by *A. auricula*. Response surface and contour plots are plotted in Figures 1-3.

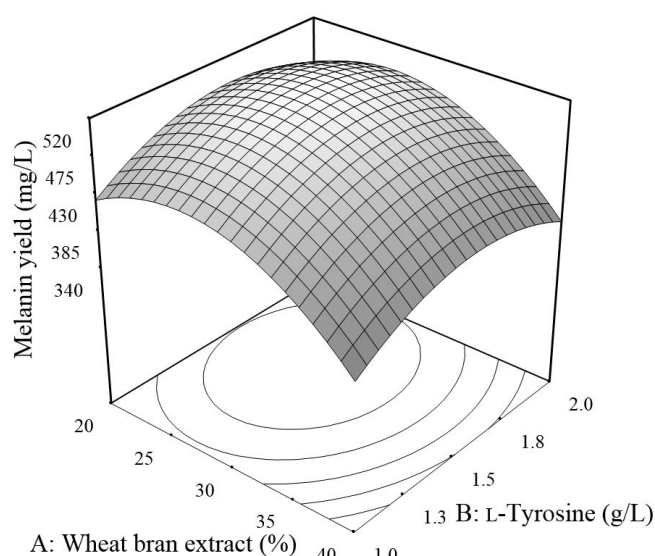
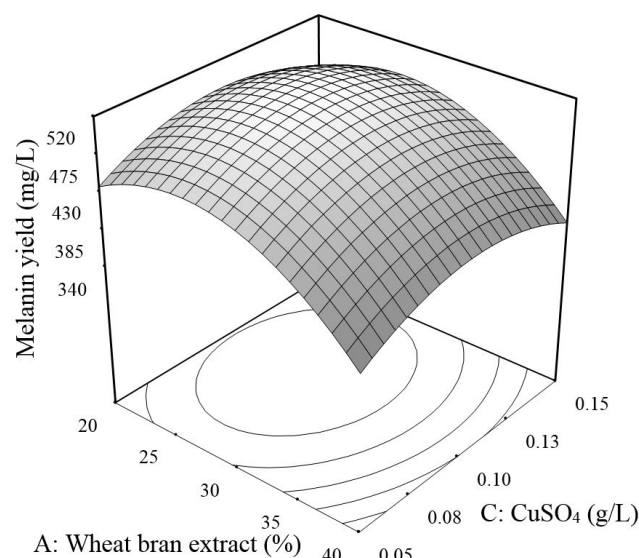
Figure 1 indicated effect of l-tyrosine and wheat bran extract on melanin yield in *A. auricula* fermentation medium when the concentration of CuSO₄ was maintained at 0.1 g/L. l-Tyrosine and wheat bran extract had very significant (p < 0.01) linear and quadratic impact on melanin yield. However, l-tyrosine

Table 2. Experimental design for optimization of melanin production.

Run	A	B	C	Melanin yield (mg/L)
1	0	0	0	513.17
2	1	1	0	384.89
3	-1	1	0	461.66
4	0	-1	-1	362.93
5	1	0	1	356.65
6	0	0	0	502.24
7	-1	-1	0	411.31
8	-1	0	-1	451.01
9	0	0	0	505.14
10	-1	0	1	447.02
11	1	0	-1	358.54
12	0	0	0	504.68
13	0	1	1	409.25
14	0	-1	1	466.63
15	0	0	0	516.97
16	1	-1	0	344.50
17	0	1	-1	453.67

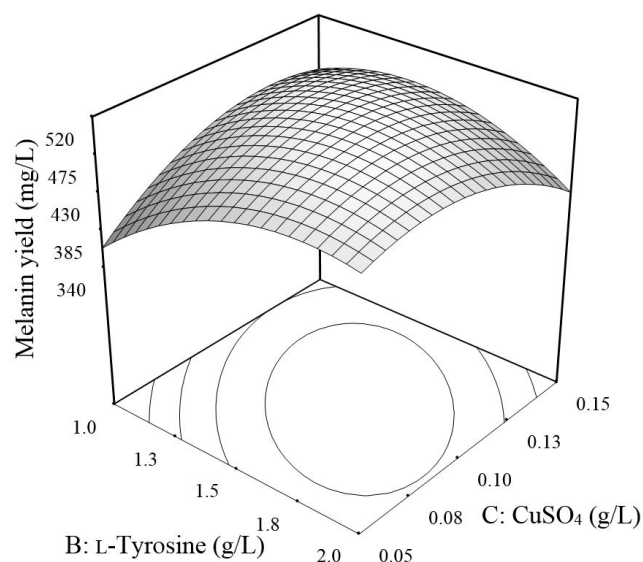
Table 3. ANOVA of quadratic polynomial model.

Source	SS	DF	MS	F-value	P>F	
Model	57193.62	7	8170.52	55.73	< 0.0001	Significant
A	13319.57	1	13319.57	90.85	< 0.0001	Significant
B	1925.41	1	1925.41	13.13	0.0055	Significant
C	356.44	1	356.44	2.43	0.1534	Not significant
A ²	17156.83	1	17156.83	117.03	< 0.0001	Significant
B ²	8158.53	1	8158.53	55.65	< 0.0001	Significant
C ²	7182.29	1	7182.29	48.99	< 0.0001	Significant
BC	5484.88	1	5484.88	37.41	0.0002	Significant
Lack of Fit	1160.85	5	232.17	5.86	0.0558	Not significant
Cor. Total	58513.08	16	R ² = 0.9775			

**Figure 1.** Response surface and contour plots indicating effect of l-tyrosine and wheat bran extract on melanin yield in *A. auricula* fermentation medium. The concentration of CuSO₄ was maintained at 0.1 g/L.**Figure 2.** Response surface and contour plots indicating effect of CuSO₄ and wheat bran extract on melanin yield in *A. auricula* fermentation medium. The concentration of l-tyrosine was maintained at 1.5 g/L.

and wheat bran extract had not significant ($p > 0.05$) interaction (Table 3). When the concentration of wheat bran extract was fixed, melanin yield increased when the concentration of l-tyrosine was close to 1.6 g/L, and then remained basically unchanged. At a constant concentration of l-tyrosine, melanin yield increased with increase of the concentration of wheat bran extract from 20% to 27% but rapidly reduced when the concentration of wheat bran extract was further enhanced. This showed wheat bran extract was a principal factor affecting the melanin production. Wheat bran was a low-cost agricultural by-product and its use could decrease medium cost (Xu et al., 2005). However, the yield of melanin gradually reduced when the concentration of wheat bran extract was above 27%. It was possibly because of higher concentration of sugar, which could prevent microbial growth and melanin synthesis (Wang et al., 2008). To avoid the decrease of melanin yield, wheat bran extract concentration should not exceed 27%.

Figure 2 indicated effect of CuSO₄ and wheat bran extract on melanin yield in *A. auricula* fermentation medium when the concentration of l-tyrosine was maintained at 1.5 g/L. CuSO₄ had

**Figure 3.** Response surface and contour plots indicating effect of l-tyrosine and CuSO₄ on melanin yield in *A. auricula* fermentation medium. The concentration of wheat bran extract was maintained at 30%.

very significant ($p < 0.01$) quadratic impact on melanin yield. However, CuSO_4 and wheat bran extract had not significant ($p > 0.05$) interaction (Table 3). Melanin yield gradually enhanced when the concentration of CuSO_4 enhanced from 0.05 to 0.11 g/L, thereafter it decreased when the concentration of CuSO_4 was above 0.11 g/L. At a constant concentration of CuSO_4 , Melanin yield sharply enhanced with wheat bran extract addition at the beginning, but decreased rapidly when the concentration of wheat bran extract was raised from 27% to 40%, which suggested that wheat bran extract had great impact on melanin production.

Figure 3 indicated effect of l-tyrosine and CuSO_4 on melanin yield in *A. auricula* fermentation medium when the concentration of wheat bran extract was maintained at 30%. l-Tyrosine and CuSO_4 had very significant ($p < 0.01$) interaction (Table 3). At a constant concentration of CuSO_4 , increase of l-tyrosine concentration enhanced melanin yield but it gradually reduced later. When the concentration of l-tyrosine was about 1.6 g/L, melanin yield attained maximum value, implying that overmuch l-tyrosine might reduce melanin yield. This result was similar to that previously reported by Chandel & Azmi (2009). When the concentration of l-tyrosine was fixed, melanin yield gradually enhanced with increase of CuSO_4 concentration and the optimum concentration was approximately 0.11 g/L. Copper was an essential constituent of tyrosinase which could catalyze multiple oxidation reaction of polyphenols to melanin (Claus & Decker, 2006). Therefore, adding CuSO_4 to fermentation medium might stimulate tyrosinase activity and promote melanin synthesis (Santos & Stephanopoulos, 2008).

3.3 Medium optimization and model verification

According to test results of RSM, the optimum medium composition for obtaining the maximum yield of melanin were 26.80% wheat bran extract, 1.59 g/L l-tyrosine, and 0.11 g/L CuSO_4 . Model verification was carried out according to the method previously (Derringer & Suich, 1980). Under the optimum conditions, the highest melanin yield (519.54 mg/L) was obtained and this observed value was not significant ($p > 0.05$) different from the predicted value (516.33 mg/L). These results suggested that the developed model was very valid in the present study.

4 Conclusions

Effect of medium composition on melanin production by *A. auricula* was investigated. Wheat bran extract, l-tyrosine, and CuSO_4 were chosen to optimize medium composition by Box-Behnken experiment design. ANOVA analysis in RSM showed that the developed model might be used to optimize medium composition. The optimal combination (wheat bran extract 26.80%, l-tyrosine 1.59 g/L, and CuSO_4 0.11 g/L) of medium components was obtained and the maximum melanin yield was 519.54 mg/L. These results could provide a reference to develop the low-cost culture medium for melanin production.

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