Asian Journal of Chemistry; Vol. 24, No. 1 (2012), 335-338

ASIAN JOURNAL OF CHEMISTRY

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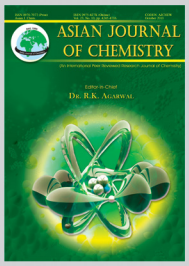

High Yield of Melanin Production by the Strain *Rhizobium* sp. R 593 in Liquid State Fermentation

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(*Received*: 28 December 2010; *Accepted*: 21 September 2011) AJC-10430

In this study, the medium composition and culture conditions for melanin production were investigated by the strain *Rhizobium* sp. R 593. The significant factors such as L-tyrosine, yeast extract and $CuSO_4 \cdot 5H_2O$ in the medium were selected by Plackett-Burman design and then these three factors was further optimized by the steepest ascent and central composite design and analyzed by response surface methodology and the optimum level of the factors were calculated and confirmed by experiments. The values of L-tyrosine, yeast extract, $CuSO_4 \cdot 7H_2O$ were 1.15, 2.62 and 0.027 g/L, respectively and the melanin production reached 3.64 g/L under the optimized medium, which was of 1.8 folds of the initial yield.

Key Words: Melanin, Fermentation, Optimization, *Rhizobium* sp.

INTRODUCTION

Melanins are nature polyphenolic heteropolymers, which produced by many organisms such as fungi, bacteria, plants and animals. The dark-brawn pigments are synthesized by tryosinase (monophenol, L-dopa: oxygen oxidoreductase; EC 1.14.18.1)[1,2]. These pigments are not essential for growth and development of the organisms, but rather they enhance the survival and competitive abilities of species in certain environments[3]. As a kind of nature pigment, melanin could be used as food colours or photoprotection materials[4].

Many *Rhizobium* species could produce melanins under certain conditions[5-7]. It was found that increasing levels of melanin production are observed when rhizobial strains in the presence of L-tyrosine and Cu^{2+} ($CuSO_4$), suggesting that tyrosinase might be involved in the pigment synthesis process.

In this study, a novel strain of *Rhizobium* sp. R 593 (isolated from medicago sativa) was used for melanin production in liquid state fermentation. The Plackett-Burman design (PBD) followed by a central composite design (CCD) of response surface methodology (RSM) were used to optimized the medium composition for melanin production.

EXPERIMENTAL

Strain: *Rhizobium* sp. R 593, isolated from Alfalfa (*Medicago sativa* L.), stored on the slant at 4 ºC before use.

Cuture: Solid slant culture medium (g/L): K_2HPO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, mannitol 10, yeast extract 3, agar 20, pH 7.0.

Seed culture medium (g/L): peptone 5.0, yeast extract 3.0, $CaCl_2 \cdot 2H_2O$ 0.7, pH 7.0. Basal liquid medium (g/L): K_2HPO_4 0.25, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, mannitol 15, yeast extract 3, L-tryosine 1.5, $CuSO_4 \cdot 5H_2O$ 0.03, $FeSO_4 \cdot 7H_2O$ 0.01, pH 7.0.

Plackett-Burman design: The independent variables in the medium composition were K_2HPO_4, $MgSO_4 \cdot 7H_2O$, NaCl, mannitol, yeast extract, L-tryosine, $CuSO_4 \cdot 5H_2O$. A Plackett-Burman design is used for rapid screening multifactor to select the most significant factors[8,9]. The eight factors were estimated using the Plackett-Burman design with a first-order polynomial equation. Each factor was evaluated at low (-1) and high (+1) levels. Eleven variables (including three dummy variables) were screened in 12 experimental runs. Table-1 lists the levels and factors in the Plackett-Burman design. The fitted first-order model is:

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

where Y is the predicted response, β_0 and β_i are constant coefficients and x is the coded independent factors.

Steepest ascent: The steepest ascent is the procedure for moving along the direction of the maximum increase in the response[10]. The direction of the steepest ascent is the direction in which melanin increased rapidly by adjusting the content of the significant factors. The factors selected by Plackett-Burman design were further optimized by this method and the path was according to the first-order model obtained from the Plackett-Burman design. Experiments were carried out along the path until the melanin production did not increase

TABLE-1
LEVELS AND FACTORS IN THE PLAKETT-BURMAN DESIGN

Level	Factor (g/L)							
	K_2HPO_4 (X_1)	L-tyrosine (X_2)	$MgSO_4·7H_2O$ (X_3)	Mannitol (X_4)	Yeast extract (X_5)	$CuSO_4·5H_2O$ (X_6)	NaCl (X_7)	$FeSO_4·7H_2O$ (X_8)
+1	0.3	2	0.3	20	4	0.04	0.15	0.015
-1	0.2	1	0.1	10	2	0.02	0.05	0.005

TABLE-2
PLACKETT-BURMAN DESIGN FOR SIGNIFICANT FACTORS EVALUATION IN MELANIN PRODUCTION BY THE STRAIN *Rhizobium* sp. R 593

Run no.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	melanin (g/L)
1	-1	1	1	1	-1	-1	-1	1	-1	-1	1	1.96
2	1	1	1	1	1	1	1	1	1	1	1	1.35
3	1	-1	-1	-1	1	-1	-1	1	-1	1	1	2.18
4	-1	1	-1	-1	1	-1	1	1	1	-1	-1	1.62
5	-1	1	-1	1	1	1	-1	-1	-1	1	-1	1.49
6	-1	-1	1	-1	-1	1	-1	1	1	1	-1	2.43
7	-1	-1	1	-1	1	1	1	-1	-1	-1	1	1.99
8	1	-1	1	1	1	-1	-1	-1	1	-1	-1	2.25
9	-1	-1	-1	1	-1	-1	1	-1	1	1	1	2.29
10	1	-1	-1	1	-1	1	1	1	-1	-1	-1	1.74
11	1	1	-1	-1	-1	1	-1	-1	1	-1	1	1.76
12	1	1	1	-1	-1	-1	1	-1	-1	1	-1	2.28

X_1-K_2HPO_4; X_2- L-tyrosine; X_3- $MgSO_4·7H_2O$; X_4-mannitol; X_5-Yeast extract; X_6- $CuSO_4·5H_2O$; X_7-NaCl; X_8-$FeSO_4·7H_2O$; X_9, X_{10} and X_{11}-dummy variables

any more. The experimental design and results are listed in Table-4.

Central composite design and response surface analysis: A central composite design of response surface methodology was used to optimize the three most significant factors (yeast extract, L-tyrosine, $CuSO_4·5H_2O$) for enhancing melanin production of the strain *Rhizobium* sp. R 593, which were screened by Plackett-Burman design. Three independent factors were investigated at five levels (-1.68, -1, 0, +1, +1.68) (Table-5) and the experimental design was shown in Table-5. The melanin production of the strain *Rhizobium* sp. R 593 was fitted using a second-order polynomial equation and a multiple regression of the data was employed for obtaining an empirical model related to the most significant factors. The general form of the second-order polynomial equation is:

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

where Y is the predicted response, x_i and x_j are independent factors, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

Statistical analysis: Design-expert (Version 7.0, STAT-EASE Inc., Minneapolis, USA) was used for the experimental designs and statistical analysis of the data in this study. The analysis of variance (ANOVA) was used for the statistical parameters estimation.

Melanin extraction and assay: Melanin in the broth was extracted by centrifugation (10,000 g, 30 min) and the content was determined at OD_{400}[11]. The standard curve was established with commercial melanin (Sigma-Aldrich, USA) at OD_{400}.

RESULTS AND DISCUSSION

Morphology: The strain *Rhizobium* sp. R 593 was incubated at 37 ºC for 24 h.

The morphology observation by light microscope revealed that the strain R 593 was short-rod bacterium (0.5-1.0 μm × 1-5 μm), gram-negative, no spore, flagellate and forming round colonies with white colour on the YMA plate. In the later stage of the culture process, the bacterium was a little coarse, node shaped and in homogeneous strain and the strain could produce melanin in liquid fermentation and the broth became black in colour.

Plackett-Burman design: The Plackett-Burman design could effectively screen the significant factors. Twelve runs were employed to estimate the effect of 11 variables (including 3 dummy variables in this study) on melanin production and the results are shown in Table-2.

The first-order model was fitted to the results obtained from the 12 tests analyzed by Design-Expert:

$$Y(g/L) = 1.96 - 0.018X_1 - 0.20X_2 + 0.098X_3 - 0.098X_4 - 0.13X_5 - 0.15X_6 - 0.067X_7 - 0.065X_8 \quad (3)$$

The coefficient R^2 of the model was 0.9649, indicating that about 96.5 % of the variability in the response could be explained by the model. The t-test was used to identify the effect of every factor on the melanin production. In Table-3, it could be concluded that the yeast extract, $CuSO_4·7H_2O$ and L-tyrosine are the most significant factors ($P < 0.05$) and these three factors were selected for further optimization by central composite design.

Steepest ascent: Three factors (the yeast extract, $CuSO_4·7H_2O$ and L-tyrosine) were selected for optimization by steepest ascent in the experiment. The path of steepest ascent was determined to obtain the proper direction of these variables based on the center of the Plackett-Burman design. The experimental design and results are shown in Table-4. It could be concluded that the highest response was 3.54g/L when the factors were: yeast extract 2.6g/L, $CuSO_4·7H_2O$ 0.22 g/L and L-tyrosine 1.1 g/L. And then this point was chosen for further optimization by central composite design.

TABLE-3
STATISTICAL ANALYSIS OF THE MODEL

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P>F
Model	1.31	8	0.16	10.32	0.0404*
X_1	0.004	1	0.004	0.25	0.6491
X_2	0.49	1	0.49	30.71	0.0116*
X_3	0.12	1	0.12	7.30	0.0736
X_4	0.12	1	0.12	7.30	0.0736
X_5	0.21	1	0.21	13.09	0.0363*
X_6	0.28	1	0.28	17.37	0.0251*
X_7	0.053	1	0.053	3.36	0.1643
X_8	0.050	1	0.050	3.19	0.1720

*Statistically significant at 95 % of confidence level

TABLE-4
EXPERIMENTAL DESIGN AND RESULTS OF THE STEEPEST ASCENT PATH

Run	L-tyrosine (g/L)	Yeast extract (g/L)	$CuSO_4 \cdot 5H_2O$ (g/L)	Melanin (g/L)
Origin	1.5	3.0	0.03	2.02
1	1.4	2.9	0.028	2.41
2	1.3	2.8	0.026	2.84
3	1.2	2.7	0.024	3.27
4	1.1	2.6	0.022	3.54
5	1.0	2.5	0.020	3.31
6	0.9	2.4	0.018	2.87

Central composite design and response surface analysis: The response surface methodology is used when the optimal region for the experiment has been identified. According to the results of the Plackett-Burmant design and the steepest ascent, central composite design was performed to find the optimal points of the three selected factors and the experimental design and results were shown in Tables-5-7. And the second-order polynomial equation was obtained from central composite design:

$$Y(g/L)=3.57 + 0.083A - 0.030B - 0.006C - 0.054AB - 0.19AC - 0.13BC - 0.21A^2 - 0.17B^2 - 0.22C^2 \quad (4)$$

where A, B and C are the coded factors of L-tyrosine, yeast extract and $CuSO_4 \cdot 7H_2O$, respectively.

TABLE-5
LEVELS OF THE FACTORS TESTED IN THE CENTRAL COMPOSITE DESIGN

Factor	Level (g/L)				
	-1.68	-1	0	+1	+1.68
L-tyrosine (A)	0.76	0.9	1.1	1.3	1.44
Yeast extract (B)	2.26	2.4	2.6	2.8	2.94
$CuSO_4 \cdot 7H_2O$ (C)	0.014	0.017	0.022	0.027	0.030

The coefficient of determination R^2 was 0.9486, indicating that about 95 % of the variability in the response could be explained by this model. The statistical significance of the model was estimated by F-test. The F-value of 20.49 suggested the model was significant and there was only a 0.01 % chance that the model F-value could occur due to noise. The P-value was also very low ($P < 0.0001$) indicating the significance of the model. Values of $P > F$ less than 0.01 suggested that the model terms were very significant. The coefficient of variation (CV) indicates that the degree of precision with which the treatments are compared. A low coefficient of variation means a higher reliability of the experiment. The value of coefficient of variation (3.28 %) indicated the experiments were highly reliable. The lack of fit F-value of 0.5064 implies the lack of fit was not significant relative to the pure error. There is a 50.64 % chance that a lack of fit F-value this large could accure due to noise. The response surface curves are shown in Figs. 1-3. Every figure demonstrates the effect of two factors while the other factors were fixed at zero level. The predicted optimal coded values of the three factors were: A = 0.26, B = 0.09, C = 0.10, respectively and the values of L-tyrosine, yeast extract, $CuSO_4 \cdot 7H_2O$ were 1.15, 2.62 and 0.027 g/L, respectively. The maxmum melanin production was 3.58 g/L, about 1.8 folds of the initial yield.

TABLE-6
THE CENTRAL COMPOSITE DESIGN OF RSM FOR MELANIN PRODUCTION OF *Rhizobium* sp. R 593

Run no.	A	B	C	Melanin (g/L)	
				Observed	Predicted
1	1.68	0	0	3.08	3.12
2	-1.68	0	0	2.97	2.84
3	0	1.68	0	3.09	3.04
4	0	-1.68	0	3.16	3.14
5	0	0	1.68	2.94	2.94
6	0	0	-1.68	3.02	2.96
7	0	0	0	3.54	3.57
8	0	0	0	3.68	3.57
9	0	0	0	3.41	3.57
10	0	0	0	3.51	3.57
11	0	0	0	3.68	3.57
12	0	0	0	3.56	3.57
13	-1	-1	-1	2.52	2.56
14	-1	-1	1	3.09	3.18
15	-1	1	-1	2.74	2.86
16	-1	1	1	2.94	2.97
17	1	-1	-1	3.17	3.20
18	1	-1	1	3.13	3.07
19	1	1	-1	3.31	3.30
20	1	1	1	2.63	2.64

TABLE-7
ANALYSIS OF VARIANCE AND REGRESSION ANALYSIS FOR THE CELLULASE PRODUCTION

Source	Sum of squares	Degree of freedom	Mean square	F-value	P>F
Model	1.98	9	0.22	20.49	< 0.0001
Residual	0.11	10	0.011		
Lack of fit	0.053	5	0.011	0.98	0.5064
Pure error	0.054	5	0.011		
Corrected total	2.09	19			

Validation of the optimized medium: In order to confirm the optimized medium, three additional experiments in shake flasks were carried out using the predicted medium and the melanin production was of 3.64 g/L, which agreed well with the predicted response. And the results implied that the central composite design of response surface methodology was effective for melanin production by *Rhizobium* sp. R 593 in liquid state fermentation.

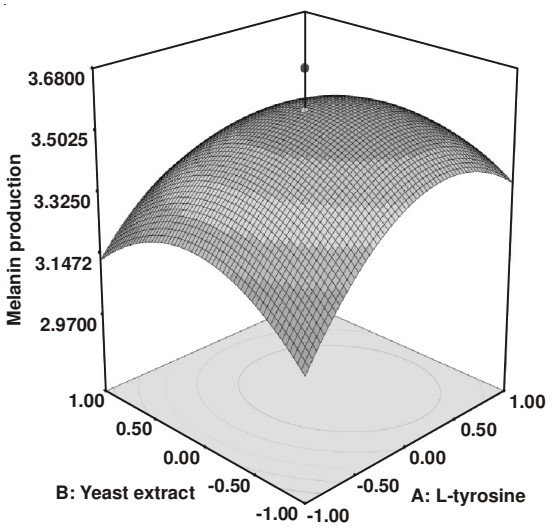

Fig. 1. Response surface curve for melanin production showing the interaction between L-tyrosine and yeast extract.

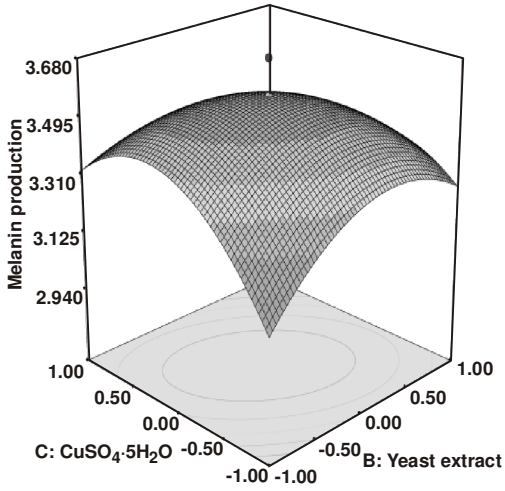

Fig. 3. Response surface curve for melanin production showing the interaction between yeast extract and $CuSO_4 \cdot 5H_2O$

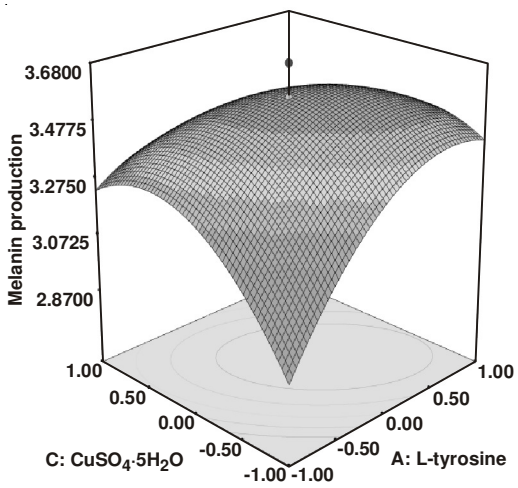

Fig. 2. Response surface curve for melanin production showing the interaction between L-tyrosine and $CuSO_4 \cdot 5H_2O$

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