

# Enhanced melanin pigment production from *Dietzia schimae* NM3 in cheese whey using Box-Behnken design

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## Original article

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# Abstract

Melanin is a natural, dark-brown complex molecular structure pigment formed by the oxidative polymerization of phenolic compounds. Microbial melanin pigment can be used in industrial fields, canned additives and preservatives. Optimization method is used to produce a quick and sufficient product with reliable and cost-effective processes. In this project, four factors (temperature, L-tyrosine, pH and CuSO<sub>4</sub>) affecting in melanin production by *Dietzia schimae* NM3 were optimized by response surface methodology with Box–Behnken design in inexpensive medium (whey powder). The anti-bacterial activity of *D. schimae* melanin was assayed by disk diffusion test. The optimal medium compositions were obtained in whey 5% (w/v), L-tyrosine 2.5 g/l, CuSO<sub>4</sub> 0.013 g/l, pH 10.5, and temperature 32 °C by maximum yield of 790 mg/l melanin pigment. The ANOVA results of RSM showed a significant P-value (0.0001), model F-value (78.84) and probability R<sup>2</sup> (0.98), with insignificant lack of fit (0.091). Melanin also showed antibacterial activity against gram-positive strains such as *Bacillus cereus* (20 mm), *B. subtilis* (18 mm), *Streptococcus pyogenes* (17 mm), *Staphylococcus epidermidis* (18 mm), and *S. aureus* (18 mm), which was comparable with amoxicillin (AMX) and cefotaxime (CTX) as control positives. We realized the ability of *D. schimae* melanin pigment as natural substances to be considered for industrial fields due to its biocompatibility and physicochemical properties.

## Introduction

Melanin pigment is a dark or black brown polymer with an irregular complex structure, a hydrophilic and a negative charge produced by nitrogen oxidation or free nitrogen-containing diphenols and produced through oxidative polymerization of phenolic or indolic compounds in various organisms (Plonka and Grabacka 2006). Melanin biosynthesis begins with a series of enzymatic and non-enzymatic reactions by tyrosinase enzyme (El-Naggar and El-Ewasy 2017). Melanin is produced by a wide variety of microorganisms such as plants, fungi, yeast, algae and bacteria. Melanin have powerful antioxidant, anti-viral and antibacterial properties (Plonka and Grabacka 2006). Melanin are resistant to heat and chemicals (e.g., heavy metals and oxidizing agents) and biochemical agents (e.g., host defense against invasive germs) (Cordero et al. 2017). Melanin possesses anti-UV radiation property by absorbing the electromagnetic spectrum and preventing optical damage in living organisms. Melanin has been used in antifungal drugs (Kurian and Bhat 2014; Venil et al. 2013).

Optimizing the growth conditions for microorganisms, particularly the physicochemical parameters and nutrition in developing every type of pigment production is significant. An optimization method using “one factor at a time” is difficult and time consuming. The production of pigments is under the influence of the physical and chemical conditions governing the production system (El-Naggar and El-Ewasy 2017; Madhusudhan et al. 2014). Response Surface Methodology (RSM) is a set of mathematical methods which determine the relationship between one or more response variables with several independent variables (Surwase et al. 2012). This method has been employed as one of the run design tools (Tarangini and Mishra 2014a). RSM can improve the process through finding the optimal inputs, resolving the problems and the weak points of the process and stabilizing it (El-Batal et al. 2017). This

method uses 3 different designs, the most comprehensive of which is Box-Behnken (Dholakiya et al. 2017). Using this method, the key parameters of pigment production are rapidly optimized and produced (Madhusudhan et al. 2014; Rani et al. 2013; Tarangini and Mishra 2014b).

## Materials And Methods

### Preparation of inoculum and media

*Dietzia schimae* strain NM3(KP207685), which isolated previously as an actinobacterium which resisted to UV radiation, dryness, oxidant agents including hydrogen peroxide and mitomycin C, was used in this research (Gabani and Singh 2013; Zamanian and Etemadifar 2017). *D. schimae* strain NM3 inoculated in TGY medium and incubated at 30 °C overnight until to reach the stationary phase.

### Optimizing Melanin Pigment Production Using RSM (Box-Behnken Design)

In response surface methodology (RSM), the relationship among various factors has been taken into account, and the linear effects and or the quadratic effects of the studied variables have been depicted in 3-D graphs (El-Naggar and El-Ewasy 2017). Concerning the previous experiments by RSM statistical method in actinobacteria, four factors (l-tyrosine, pH, CuSO<sub>4</sub>, and temperature) were the most effective factors in melanin production and bacterial growth (Dholakiya et al. 2017). Expert design 11.1.2.0 software has been applied for the runs design type (box-behnken) and design model (quadratic) (El-Batal et al. 2017; Surwase et al. 2013).

The 27 designed runs were cultured in 100 ml flasks containing 50 ml medium at 160 rpm. To determine the quadratic surface response, the factorial design with four factors and three replicates were done for the precision and accuracy of the results. Expert design 11.1.2.0 was applied for regression analysis and the graphs depiction. The above variables' optimal concentration and value were defined through surveying the contour graphs. The model's statistical analysis has been represented as ANOVA (El-Batal et al. 2017; Surwase et al. 2013).

The multivariate model is as it follows Eq.(Y)1:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j + \epsilon$$

### Melanin Production And Extraction In Whey Media

Five ml of the overnight bacterial culture (equivalent to standard 1 McFarland) inoculated to 1000 ml of the whey medium enriched by 2.5 g/l of L-tyrosine (based on the optimized results) and 0.1% of  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$  salts (to activate the tyrosinase enzyme) (Dholakiya et al. 2017; Rani et al. 2013; Tarangini and Mishra 2014b) (Zaidi et al. 2014). The incubation was carried out at a temperature of 32.5 °C, a rotational speed of 160 rpm and pH 10.5 until black pigmented melanin appeared. To extract melanin, the modified method by Gibson et al. (Gibson and George 1998) was used. The darkly pigmented medium was centrifuged at 3500 rpm for 15 min (by refrigerated Eppendorf 5810R) to separate the bacterial cells (pelleted) from the supernatant. The supernatant was acidified by 3N HCl to pH 3, left at room temperature for 24 hours, and then centrifuged at 3500 rpm for 15 min to separate crude pigment granules (melanin). The mixture was washed by ethanol-acetone [1:1], the supernatant was removed, and the pellet mixed with methanol 70%, then boiled in a water bath for 5 min to avoid the formation of melanoidins, and finally dried in the oven and kept it in refrigerator for test assays.

## Antibacterial Assay With Disk Diffusion Test

The Moeller-Hinton Agar media cultivated with bacterial suspensions equivalent to standard 1 McFarland ( $1.5 \times 10^8$  CFU/ml) from each gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Bacillus cereus*. The antibiotic paper discs containing 20 µl of extracted melanin (equivalent to 50 mg/ml concentration) were placed on the petri dishes under sterile conditions in the biological hood. The plates were incubated for 24 h at 37 °C. The diameter size of the growth inhibition regions around the paper disks were measured. In addition, two antibiotic discs including amoxicillin (AMX), and cefotaxime (CTX) were used as positive antibacterial control and compared with melanin pigment. The zone of inhibition diameters determined and interpreted with the Clinical and Laboratory Standards Institute indicated Criteria, previously approved by National Committee of Laboratory Standards (NCCLS or CLSI) in the US Food and Drug Administration (FDA) (Nosanchuk and Casadevall 2006; Vasanthabharathi et al. 2011).

## Results

### Disk Diffusion Test

The antibacterial activity of extracted melanin from *Dietzia* was assessed via disk diffusion test and the results indicated that the isolated melanin could prevent the bacterial growth in used gram-positive strains including *B. cereus* (20 mm), *S. pyogenes* (17 mm), *B. subtilis* (18 mm), *S. epidermidis* (18 mm), *S. aureus* (18 mm). The results showed that melanin has the same activity against gram-positive bacteria in compare to antibiotics (AMX and CTX). These results suggested that the extracted melanin from *D. schimae* can be used as a bacterial growth inhibitor in food products (canning factory).

### RSM based Optimization of Melanin Production by *Dietzia schimae* NM3

*D. schimae* NM3, inoculated in whey medium along with L-tyrosine as substrate. The dark brown pigment melanin was diffused after 3–4 days in whey broth medium. The optimization process showed appropriate result than to nutrient broth melanin production (450 mg/L). Maximum melanin production was 790 mg/L in large-scale fermentation (one liter) whey medium as an inexpensive medium.

The statistical methods can be considered as the part of the primary stages of every study. They pursue the goal to focus on the critical variables and to discover the most effective ones in the study. Through this method, it is merely viable to gain the proper concentration for each factor separately. Benefitting from this statistical design, the number of the runs declines and all quadratic regression model's coefficients and the interactions can be estimated. The most remarkable issue in this research refers to the factors' main effects and interactions. Thus, the response surface's statistical design was picked up. The different interactions of each factors of the test variables were representing as Various response surfaces.

This table has three replicates at the central point. The resulted model was showed in Table 1, in which the melanin yield  $Y$  is a function of the independent variables value.

Table 1  
Experimental design for optimization of melanin production (layout and results)

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Std	Run	A:pH	B:Temperature	C:L-tyrosine	D:CuSO4	Melanin Yield
			°C	g/L	g/L	mg/L
2	1	12.0	29	2.5	0.013	140
24	2	10.5	35	2.5	0.020	550
17	3	9.0	32	1	0.013	280
10	4	12.0	32	2.5	0.005	80
26	5	10.5	32	2.5	0.013	790
11	6	9.0	32	2.5	0.020	300
5	7	10.5	32	1	0.005	280
6	8	10.5	32	4	0.005	300
21	9	10.5	29	2.5	0.005	220
15	10	10.5	29	4	0.013	350
18	11	12.0	32	1	0.013	80
19	12	9.0	32	4	0.013	320
23	13	10.5	29	2.5	0.020	320
16	14	10.5	35	4	0.013	600
4	15	12.0	35	2.5	0.013	290
3	16	9.0	35	2.5	0.013	280
20	17	12.0	32	4	0.013	310
27	18	10.5	32	2.5	0.013	770
13	19	10.5	29	1	0.013	320
22	20	10.5	35	2.5	0.005	260
14	21	10.5	35	1	0.013	400
9	22	9.0	32	2.5	0.005	220
25	23	10.5	32	2.5	0.013	780
7	24	10.5	32	1	0.020	400

		Factor 1	Factor 2	Factor 3	Factor 4	Response 1
1	25	9.0	29	2.5	0.013	250
12	26	12.0	32	2.5	0.020	280
8	27	10.5	32	4	0.020	550

For this purpose, the yield of melanin production was taken as the dependent variable or response Y. As the results of mentioned method indicated a quadratic polynomial model is employed for predicting the response as Final Equation in Terms of Coded Factors from Box-Behnken experiment Eq. (1).

Yield = + 780

$$-39.17 A + 65 B + 55.83 C + 86.67 D + 30 AB + 47.50 AC + 30 AD + 42.50 BC + 47.50 BD + 32.50 CD - 343.75 A^2 - 200 B^2 - 173.75 C^2 - 227.50 D^2$$

As spotted in Eq. (1), the coefficient of the one factor, pH is negative. Their negative effect on response function suggests that higher values of this parameter leads to lower melanin yield.  $CuSO_4$  possesses the max effect out of these four parameters.

Eq. (1) was statistically evaluated through F-test and ANOVA of surface response quadratic model. F parameter is the data standard deviation from the mean. F-value is 78.84 typically very high. P-value < 0.0001 refers to the model being meaningful. F-value for this model box- behnken indicates that the model is fully meaningful.

Lack of fit (0.0919) estimation can be a good reason for the model data accuracy. Virtually, this parameter being insignificant is favorable and means that the model is able to predict the enzyme activity under diverse circumstances of the above-mentioned four independent factors combination. This parameters value for the regression of Eq. (1) was not meaningful Table 2.

Table 2

Analysis of variance (ANOVA) and regression for the fitted quadratic model of melanin production.

Source	Sum of Squares	df	Mean Square	F-value	p-value	significance
<b>Model</b>	9.635E + 05	14	68823.21	78.84	< 0.0001	significant
A: pH	18408.33	1	18408.33	21.09	0.0006	
B: Temperature	50700.00	1	50700.00	58.08	< 0.0001	
C: L-tyrosine	37408.33	1	37408.33	42.85	< 0.0001	
D: CuSO <sub>4</sub>	90133.33	1	90133.33	103.26	< 0.0001	
AB	3600.00	1	3600.00	4.12	0.0650	
AC	9025.00	1	9025.00	10.34	0.0074	
AD	3600.00	1	3600.00	4.12	0.0650	
BC	7225.00	1	7225.00	8.28	0.0139	
BD	9025.00	1	9025.00	10.34	0.0074	
CD	4225.00	1	4225.00	4.84	0.0481	
A <sup>2</sup>	6.302E + 05	1	6.302E + 05	721.96	< 0.0001	
B <sup>2</sup>	2.133E + 05	1	2.133E + 05	244.39	< 0.0001	
C <sup>2</sup>	1.610E + 05	1	1.610E + 05	184.45	< 0.0001	
D <sup>2</sup>	2.760E + 05	1	2.760E + 05	316.22	< 0.0001	
<b>Residual</b>	10475.00	12	872.92			
Lack of Fit	10275.00	10	1027.50	10.27	0.0919	not significant
Pure Error	200.00	2	100.00			
<b>Cor Total</b>	9.740E + 05	26				

As the result of the model analysis, Std.Dev was 29.55 therefore the model are accurate and change is justifiable R-squared (0.989). This parameter value for melanin yield 790 mg/l denotes the model's accuracy. The closer R-squared to 1, the better relationship exists between the lab (Adjusted R<sup>2</sup>= 0.97) and the estimated results (Predicted R<sup>2</sup>= 0.938).

Predicted and actual charts show that our data are normal. The more linear the data is and the nearer the middle line the data has a normal distribution. Vertical or curved lines indicate abnormal scattering. In this study, the dispersion is normal. Chart shows actual test data to match the expected is high Fig. 1.

The Fig. 2 is the scatter chart showing the relationship of the variables. The horizontal column (x) shows the coded value and the vertical column (y) shows the value of the product. The left side shows the lowest and the right side shows the highest value. From left to right the value of the variable increases. As the amount of the variable increases, the amount of product goes up to a point and then declines. The variable factor pH (A) had the most drop in Fig. 2.

The three-dimensional graphs are shown the overall impact of the factors relationship. In Fig. 3a two factors of temperature at 32 °C and copper 0.013 g/L are constant and two factors of acidity and tyrosine are measured by their interaction. Blue shows the lowest product and red shows the highest product. Interactions between L-tyrosine and pH improved the yield of melanin by increasing the pH from 9 to 11 and decreasing L-tyrosine to 2.2–2.8 g/L.

The interactions of copper and temperature was shown in Fig. 3b. Two factors of acidity at 10.5 and L-tyrosine 2.5 g/L are constant and two factors of temperature and copper were measured by their interactions. It was indicated that by increasing of temperature to 32 °C and Cu to 0.013 g/L, the melanin production was increased.

The interactions of copper and pH (Fig. 3c) indicated an increase in melanin production in the pH range of about 9 to 11 with a decrease in copper concentration to 0.013 g/L. The interactions of L-tyrosine and CuSO<sub>4</sub> (Fig. 3d), indicated an increase in melanin production depended on L-tyrosine 2.5 g/L than to copper. pH and temperature (Fig. 3e) indicated temperature and acidity factors have an equal effect on melanin production. the interaction of two factor temperature and L-tyrosine (Fig. 3f), indicated the effect of factors action on constant acidity on melanin production. According to this image, the increase in temperature does not increase the production of melanin, but shows the increase in tyrosine up to 2.5 g/L effectively.

Melanin showed maximum production at fixed pH of 10.5, temperature (32 °C), and L-tyrosine 2.5 g/L. In the other hand, CuSO<sub>4</sub> (0.013 g/L) was so effective than temperature, and L-tyrosine considered highly important factor in melanin production (Table 2).

The optimal medium compositions were obtained in whey 5% (v/v), L-tyrosine 2.5 g/L, CuSO<sub>4</sub> 0.013 g/L, pH 10.5, at temperature 32 °C by maximum yield of 790 mg/L melanin pigment production.

These interactions indicated design experiment increased the growth *Dietzia schimae* NM3 and melanin production in whey medium are significantly.

## Discussion:

Melanins are important pigment that have many applicable in medicine, cosmetic and other fields. In several studies, the intracellular and extracellular melanin in the bacteria has been reported such as *Pseudomonas aeruginosa*, *Shewanella colwelliana*, *Vibrio cholera*, *Alteromonas nigrifaciens*, *Cellulophaga tyrosinoydans* showed yellow pigment and pheomelanin. *Streptomyces* and *Marinomonas*

*mediterranea* used tyrosinase enzyme to produce melanin from L-tyrosine (Kurian and Bhat 2014). This metabolite (melanin) protects the bacterial cell against environmental stress conditions (Geng et al. 2008). *Dietzia schimae* NM3 contains lots of enzymes for removing heavy metals that enzyme or its gene activating or raising under the influence of the food stuff and the physiological conditions (Gharibzahedi et al. 2014). The produced pigment and its yield level relies on the bacterial growth and activation of the pigment (melanin) producing enzyme. (Fairhead and Thony-Meyer 2012).

The melanin pigment has exhibited a significant antimicrobial activity against gram-positive and gram-negative bacteria. The diameter of the zone of inhibition (DI) against gram positive bacteria was obtained between 18–20 mm in our study, but other study, melanin extracted from *Pseudomonas balearica* strain U7 showed antibacterial activity against *E. coli* (DI = 30 mm). Moreover, melanin induced antibacterial activity against phytopathogenic bacteria, i.e., *E. chrysanthemi* and *E. carotovora* has been examined (11–14 mm) (Zerrad et al. 2014).

Vasanthabharathi reported Actinomycetes produced melanin as maximum 22 mm from DI against *E. coli*. Their extract also showed strong activity against *S. aureus*, *P. mirabilis*, *V. cholerae*, *S. typhi*, *S. paratyphi* and *K. oxytoca*. Actinomycetes sp. showed significant antibacterial activity against 14 pathogenic organisms in zone of inhibition (20–22 mm) (Vasanthabharathi et al. 2011). Melanin has been employed in various drugs (Nosanchuk and Casadevall 2006). The results demonstrated that in near future melanin can be applied as biological controlling agent.

In order to lower the cost of lots of runs within shorter time span, RSM statistical methods (Box-Behnken (BBD), a quadratic design based on three-dimensional), are employed (El-Batal et al. 2017; Surwase et al. 2013).

Optimized melanin production by *Auricularia auricula* showed 519.54 mg/L yield. (Kurian and Bhat 2014; Manivasagan et al. 2013), and 0.3 gm/100 ml by *Azotobacter vinelandii* (Maru and Gadre 2016). Optimized melanin production by *Streptomyces glaucescens* NEAE-H was obtained 31.650 µg/0.1 ml by Plackett–Burman design (El-Naggar and El-Ewasy 2017). In this study, *D. schimae* NM3 produced 790 mg/L melanin pigment.

According to the results of RSM, maximum yield of melanin was obtained 790 mg/L under the optimal medium and culture conditions. Under the usual conditions, the highest melanin yield was obtained as 519.54 mg/L (Zou and Hou 2017). RSM was proven more effective in improving production than the classical 'one factor at a time' method (Surwase et al. 2012).

The predicted yield of melanin by optimal levels of the variable generated by the model was in close correlation with experimental value, which signifies the RSM methodology over traditional optimization approach. In addition, the increased melanin production was observed with the parameters optimized using RSM than the initially used conditions (Surwase et al. 2013). These results suggested that the developed model was very valid in the present study. Results showed that quality of model was adequately good and might describe real relationship among medium components.

# Abbreviations

RSM: response surface methodology; ANOVA: analysis of variance

# Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

All authors gave their consent for publication.

## Availability of data and material

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

SE: Designed and performed experiments, analyzed data and co-wrote the paper.; ZE: Supervised the research and co-wrote the manuscript read and approved the final manuscript.

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## Figures

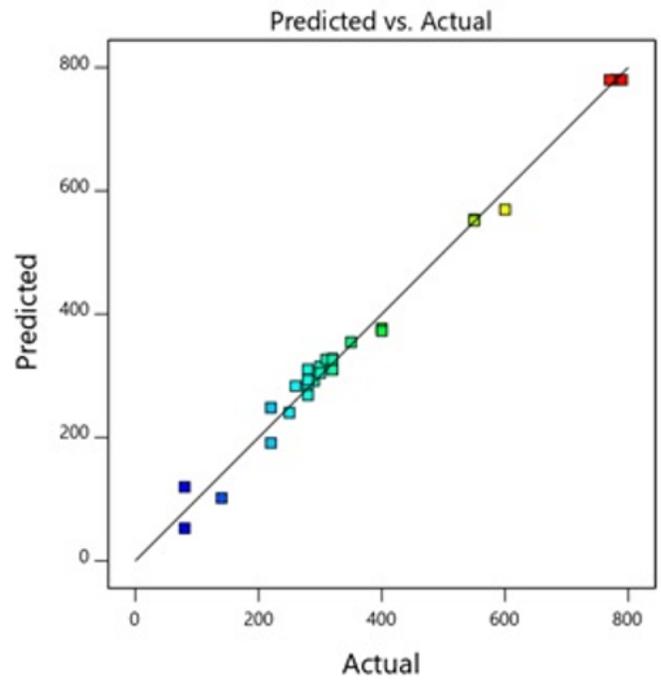
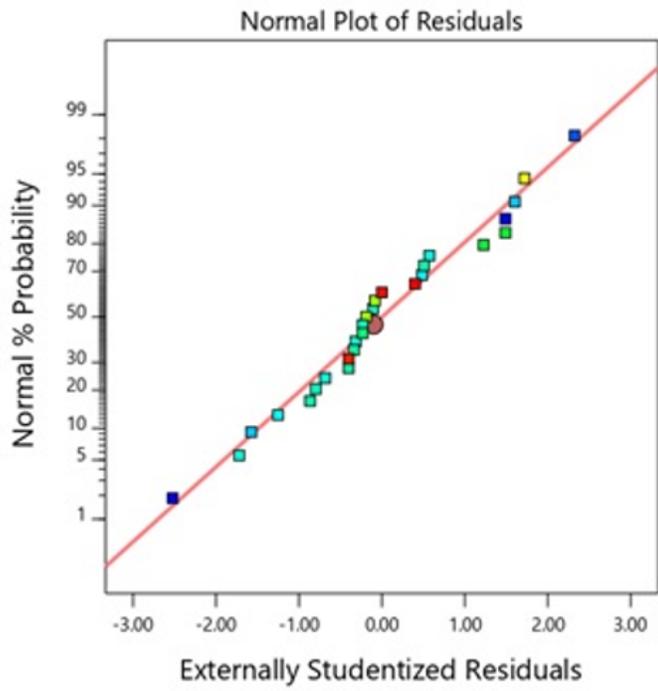
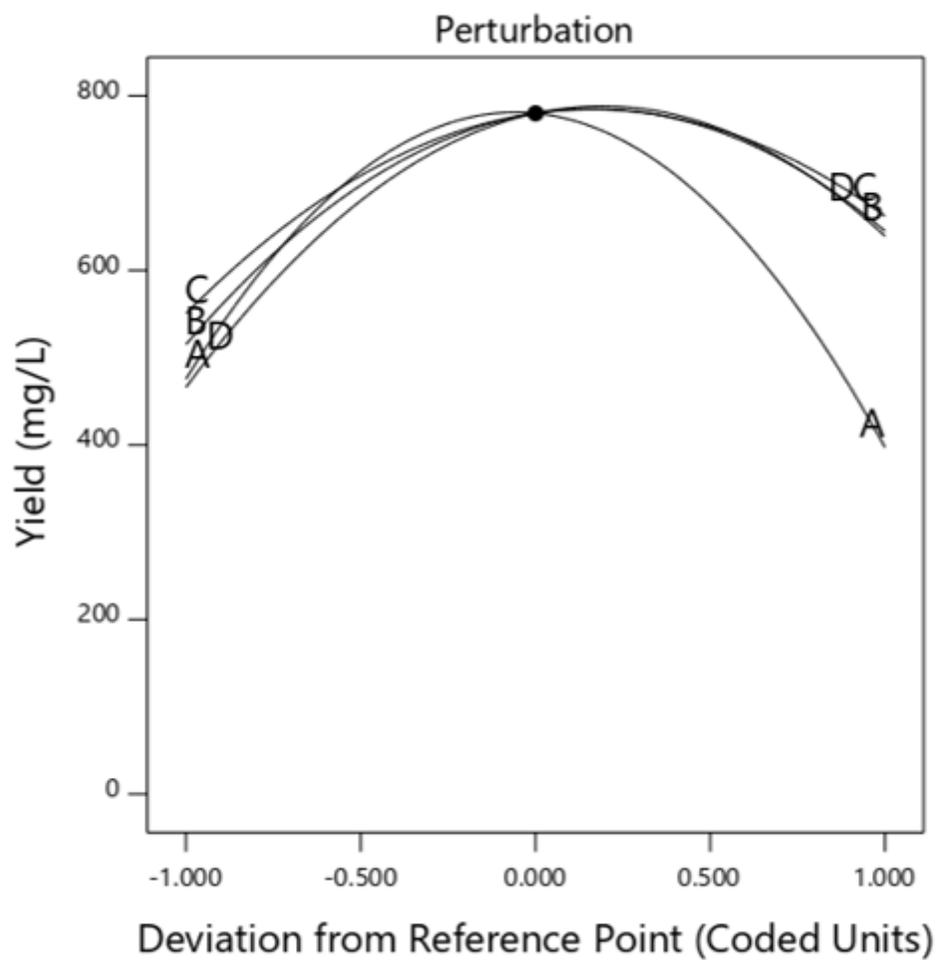


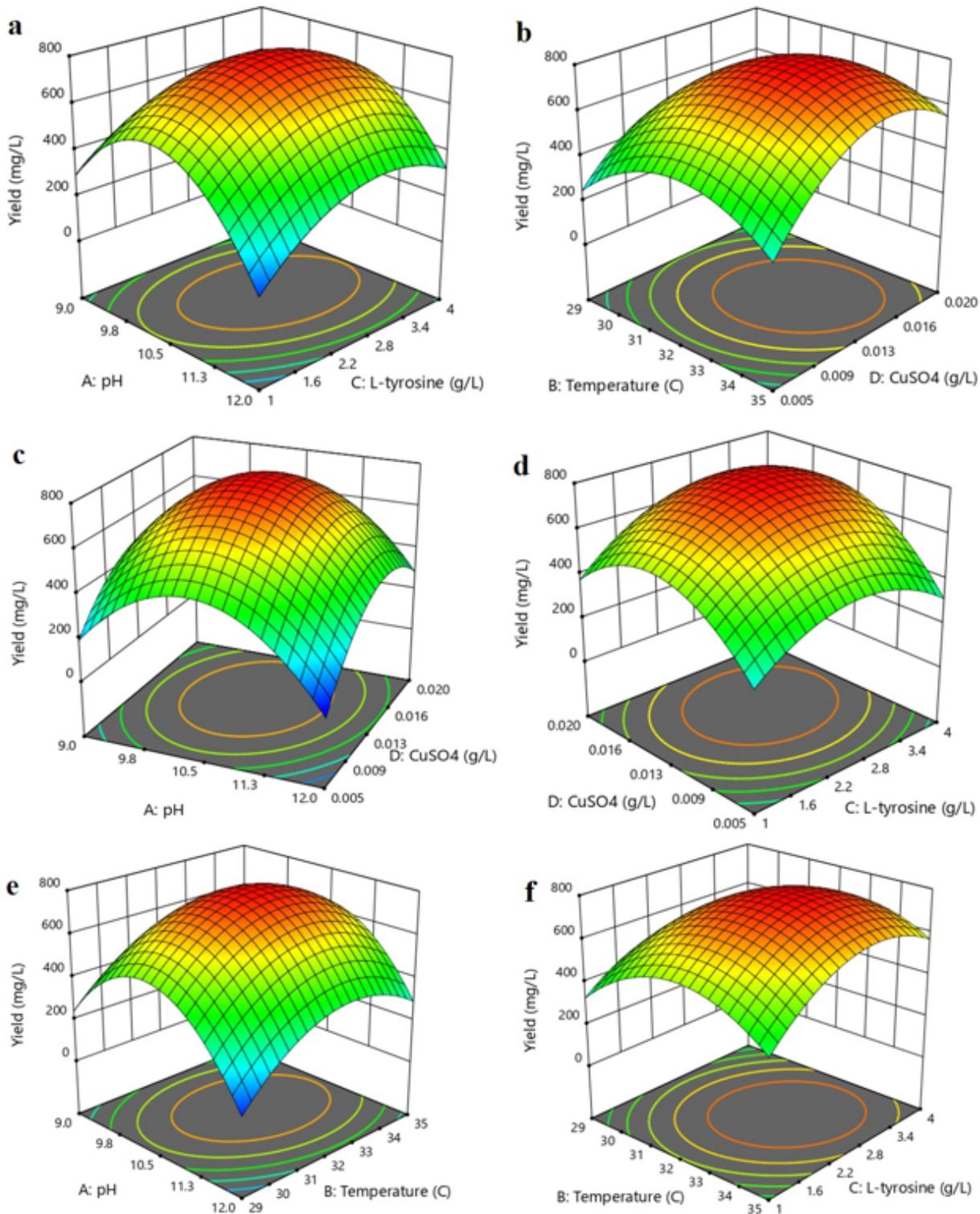
Figure 1

Residual diagnostics of crossed model for melanin production: predicted (left) and actual (right)



**Figure 2**

The graph shows melanin effect for RSM method in four factors include: pH (A), temperature (B), L-tyrosine (C), and CuSO<sub>4</sub> (D).



**Figure 3**

Three-dimensional response surface curve showing the interactions of pH and L-tyrosine (a), temperature and CuSO<sub>4</sub> (b), pH and CuSO<sub>4</sub> (c), L-tyrosine and CuSO<sub>4</sub> (d), pH and temperature (e), temperature and L-tyrosine (f), on melanin production by *Dietzia schimae* NM3 in whey medium.