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## Original Article

# Optimizing Medium Compositions and Bioreactor Conditions to Improve and Cost-effectively Produce *Monascus purpureus* Pigments

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**ABSTRACT**

Bio pigments produced by *Monascus* spp. have potential applications mainly in the food and medical industries. In the present study, a two-step statistical method was used to optimize the production of yellow, orange, and red pigments from *Monascus purpureus*. Eleven independent variables, including four carbon sources (wheat, barley, rice, and potato extracts), two nitrogen sources (ammonium nitrate and urea), nutrient elements (P, K, and microelements), and four bioreactor conditions (temperature, aeration, stirring, and pH) were optimized through Plackett-Burman design (PBD) and response surface method (RSM) methods. The model for each pigment was constructed and validated. With regard to carbon sources, the highest level of pigments was achieved at 2 g/l of rice and 9 g/l barley for yellow pigment, 2 g/l of rice and 18 g/l of barley for orange pigment, and 18 g/l of rice and 18 g/l wheat for red pigments. Temperature and barley extracts triggered the production of yellow pigments. The orange pigment was increased by pH and barley. Rice and wheat have a positive significant influence on red pigments. Aeration, pH, and stirring increased the production of the pigment. Citrinin is a biotoxin produced by *Monascus* during the fermentation process. The concentration of citrinin varied from 0.054 to 0.135 (µg/mL). The lowest amount of citrinin was achieved at 2 L/Min aeration or 6 L/Min stirring. This system is critical for the bioprocess, as it inhibits the citrinin product, and it could be a promising step in increasing pigment yield.

## INTRODUCTION

Nowadays, natural compounds of medicinal plants and medicinal mushrooms used as additives, color intensifiers, and antioxidants, are extracted from natural sources and have gained attention because of their advantages over synthetic colorants, such as good quality, easy large-scale production, and especially a lack of safety concerns [1,2]. One of the natural sources for producing natural pigments is *Monascus spp*., which has a long history of being used to produce fermented products such as therapeutic compounds and food colorants. It is a good way to start the day [1,3]. *M. spp*. produce mainly six pigments, including ankaflavin and monascin (yellow pigments), monascorubrin and rubropunctatin (orange pigments), and rubropunctamine and monascorubramine (red pigments) [4].

The main use of red pigments is as a food coloring and they have been successfully produced on a large scale, but orange and yellow ones are still not produced in an amount suitable for industrial use [1, 3]. It has been estimated that 31% of the global pigment market belongs to natural pigments with a value of US $27.5 billion [5]. The market trend for natural pigments is gradually rising. However, some limitations, especially the high production cost and the concentration of citrinin (biotoxin produced by *Monascus* during the fermentation process), hinder production of bio pigments at industrial levels [1, 3]. This biotoxin is correlated with a difference in detrimental effects and advisory measures for this biotoxin have been set at 0.2 g/mL in Japan and 0.1 g/mL in the Union. European [6].

Finding low-cost carbon and nitrogen sources replacements have been investigated as one of the

ways to produce cost-effective bio pigments [1, 3, 5, 7, 8]. Many studies have mostly investigated carbon sources such as glucose, sucralose, and starch to improve the production of bio pigments in *Monascus spp* [1, 3,9]. Several other studies have reported low-cost agro products, mainly rice, corn, and sweet potatoes, as carbon sources to reduce production costs [7,8,10]. Moreover, the influence of nitrogen sources on pigment production has been shown [11]. A few materials, such as ammonium chloride, ammonium nitrate, and glutamate, have been reported as efficient in pigment production [1,3]. Recently, studies have shifted to using statistical methods to optimize two or rarely more substrates and growth conditions with the aim of improving the co-production of bio pigments. Srivastav, and Yadav [10] in their study on red pigment production using the sweet potato-based medium in submerged fermentation, used statistical methods to select and optimize seven medium variables and reported that sweet potato could be used as a cost-effective substrate for red pigment production in *M. purpureus*. Embaby, Hussein [7] in their study, used three different statistical methods (one variable at a time (OVAT), Plackett-Burman design (PBD), and central composite design (CCD) to optimize the co-production of orange and red pigments. Patrovsky and Sinovska [12] reported that a combination of initial pH and peptone as a nitrogen source improves the co-production of yellow and orange pigments in *M. purpureus*. The objective of the present study was to enhance and optimize the production of bio pigments in *M. purpureus* via submerged fermentation using low-cost carbon and nitrogen sources as well as optimizing bioreactor conditions. In this study, a two-step statistical method was employed. Moreover, citrinin concentration production was evaluated on the optimized medium. To the best of our knowledge, this work is the first study to enhance and optimize the production of three bio pigments (yellow, orange, and red) using cheap carbon and nitrogen sources.

## MATERIALS AND METHODS

### Treatments and Experimental Design

This study was conducted to enhance the cost-effective production of bio pigments from *Monascus purpureus*. The culture was in submerged ferment and 11 parameters were assessed to reach an optimal production process. Wheat, rice, and barley were ground, then dissolved in 50 °C distilled water. The potatoes were cooked in distilled water. All extracts were passed through an 850 µm filter. The extracts were dried in an oven at 70 °C and the dry weight was calculated. The four carbon sources, including wheat, barley, rice, and potato extracts in three levels (2, 10, and 18 g/L) were added to 1000 mL of distilled water. The nitrogen sources (ammonium nitrate and urea) in 1, 3, and 5 g/L were replaced with NaNO3 in the modified Basal medium. The nutrient elements ($KH_2PO_4$ 40%, $MgSO_4.7H_2O$ 40%, KCl 19%, and $FeSO_4.7H_2O$ 1%) were mixed together and then weighed at three levels: 1, 2, and 4 g/L. A simple but effective bioreactor was constructed. The pump in this bioreactor delivers liquid into the tank and equally mixes it with the sterile air outflow of the air pump in one section of the circuit. The bioreactor cultivation conditions (temperature, aeration, stirring, and pH) were applied to the modified media. The Basal media containing various carbon and element sources were mixed and autoclaved (20 min; 115 °C). The media containing various carbon and nitrogen sources and concentrations were put under different temperatures (20, 25, and 30 °C), aeration (1, 1.5, and 2 liters per minute (L/Min), stirring (3, 4.5, and 6 (L/Min) and pH of 3, 5, and 7 for 7 days (Table 1).

A two-step statistical strategy [7] was anticipated to optimize and determine the relationship between yellow, orange, and red pigment production in *M. purpureus* with carbon sources, nitrogen sources, and bioreactor culture conditions. PBD [13] was employed to monitor the linear effect of three parameters using 15 experimental trials designed in a fractional factorial design. A three-level optimization (+1, 0, -1) arranged in the CCD of response surface methodology (RSM) was used to specify the optimal levels of each key parameter deduced from the PBD approach.

### Microorganism and Seed Culture

*M. purpureus* spores were provided by the Department of Horticultural Science and Landscape, Ferdowsi University of Mashhad, Iran. The fungal spores were inoculated on Potato Dextrose Agar (PDA) slants and incubated for 7 days at 30 °C. *M. purpureus* was maintained on PDA at 4 °C for further use. In order to prepare a suspension culture, spore-inoculated PDA was washed using sterile water and added to the seed culture medium. The seed medium was prepared by using a modified Basal medium (2 g

$NaNO_3$, 2 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, and 0.01 g $FeSO_4.7H_2O$) plus various carbon sources (wheat, barley, rice, and potato extract); nitrogen sources (ammonium nitrate and urea); and pH ranging from 3 to 6 in 1000 ml of distilled water. The cultures were incubated at three different temperatures (20, 25, and 30 °C) for 7 days in a rotary shaker at 200 rpm. To quantify the effects of treatments on pigment production, after 7 days, all fermented media were filtered through Whatman paper and separated mycelia were transferred to a 250 Erlenmeyer flask. A solution of ethanol 70% (20 mL with pH of 2) was added to the flask and extraction was performed at 30 °C and 100 rpm for 1 hour using a shaker (WS-50DR). A UV-vis spectrophotometer (2800 UV/VIS UNIC) was used to measure the concentration of yellow, orange, and red pigments. The absorbance at 410 nm for yellow pigments, 470 nm for orange, and 500 nm for red pigments was recorded and the results were expressed as absorbance units at the corresponding wavelength per ml (AU/ml) [8].

### Citrinin Detection

Citrinin analysis was performed using HPLC analysis according to the method described in [14]. The analysis was performed using a Waters 244, column: Shoex $C_{18}$, 4 m, 250 mm 4.0 mm, temperature: 28 °C, injector volume: 20 l. The Fluorescence detector (Waters 470) is set at 331 nm excitation and 500 nm emission wavelength. The mobile phase consisted of methanol/acetonitrile/water (3:3:4 v: v: v). The pH value of the mixture was 2.5, and the flow rate was 0.15 mL/Min. The concentrations of citrinin in samples were calculated by the equation:

$$Cs_{1/4}\ Cp\ As = Ap:$$

In this equation, Cp and Cs are the concentrations of pure citrinin solution and sample solution respectively. Ap and as are the areas of peaks of pure citrinin solution and sample solution respectively.

Statistical analysis and calculations

Each experiment was performed in triplicate. To screen and optimize the production of *Monascus* pigments, the software Design Expert 13 (Stat-Ease Inc., USA) was used. All tables and diagrams were prepared in Microsoft Excel 2010. The effects of the treatments on pigment and citrinin were analyzed by one-way ANOVA, and tests for significant differences were determined by using Student's t-test at $p \leq 0.05$.

## RESULTS

### Screening and Optimizing Medium and Bioreactor Variables

To screen medium variables (carbon, nitrogen, and elements) and bioreactor conditions (temperature, aeration, stirring, and pH), fifteen experiments were performed based on the PBD design. The responses of pigments and dry weight were tabulated. Yellow, orange, and red pigment concentrations ranged from 0.149 to 0.327, 0.286 to 0.501, and 0.415 to 0.912 optical density (OD), respectively. The responses were analyzed by one-way ANOVA and the results, including F-value and p-value, were reported in Table 2. Analysis of PBD concluded significant variables on pigments ($p \leq 0.05$, Table 2). The yellow pigment was significantly affected by stirring, urea, temperature, rice, and barley extracts. Five variables, including pH, ammonium, potato, rice, and barley extracts, significantly affected orange production. Stirring, pH, aeration, ammonium, nutrient elements, rice, and wheat extracts were variables that statistically changed the production of red pigment.

**Table 1** Treatments are used to enhance the co-production of pigments in *M. purpureus.*

| Treatments | | Cs 1 (g/L) | Ns 2 (g/L) | Ne 3 g/L) | Temperature (°C) | Stirring (L/Min) | pH | Aeration (L/Min) |
|---|---|---|---|---|---|---|---|---|
| Materials | | 4 We 4, Be 5, Pe 6, Re 7 | 2 An 8, Urea | 1 Moe 9 | 1 Cm 10 | 1 Water jet | 1 HCL-NAOH | 1 - |
| Levels | +1 | 18 | 5 | 4 | 30 | 6 | 7 | 2 |
| | 0 | 10 | 3 | 2 | 25 | 4.5 | 5 | 1.5 |
| | -1 | 2 | 1 | 1 | 20 | 3 | 3 | 1 |

[1] Carbon sources, [2] Nitrogen sources, [3] Nutrient elements, [4] Wheat extract, [5] Barley extract, [6] Potato extract, [7] Rice extract, [8] Ammonium nitrate, [9] Mixed of elements ($KH_2PO_4$ 40%, $MgSO_4.7H_2O$ 40%, KCl 19%, and $FeSO_4.7H_2O$ 1%), [10] Culture media

A Pareto chart (Fig. 1) shows the order of variables for yellow, orange, and red pigments. The statistical significance of models was determined using the F-value. The results showed a statistically significant F-value of 25.29, 42.41, and 19.91 for yellow, orange, and red pigments, respectively.

Yellow pigment (OD) =0.2314-0.0362A+0.0223E+0.0320G+0.0222J+0.0207L
Orange pigment (OD) =0.3917+0.0252B+0.0163D-0.0262H+0.0455J+0.0318L
Red pigment (OD) =0.6055+0.0208A+0.0240B+0.0373C-0.0317D-0.0322F+0.0848J+0.0713K

In the models, A: stirring (L/Min), B: pH, C: aeration (L/Min), D: ammonium (g/L), E: urea (g/L), F: element (g/L), G: temp (°C), H: potato (g/L), J: rice (g/L), K: wheat (g/L), and L: barley (g/L).
From the PBD experiment, the variables with significant effects (Table 2) and based on their order of importance (Fig. 1) were selected for the optimization process by RSM. RSM was applied to determine an optimal level of variables screened from PBD. Fifteen experiments according to CCD of RSM were conducted and, a model for yellow, orange and red pigments was constructed.

## Effect of Variables on Yellow, Orange, and Red Pigment

As illustrated in Figure 2 (a), the 3D surface plots were used to study the interactions of the variables in the production of pigments and to confirm the predicted optimal levels. Temperature and barley extracts triggered the production of yellow pigments ($p \leq 0.05$) while other carbon sources (wheat and potato) did not show a significant effect. Urea was the only nitrogen source that increased yellow pigment. With regard to bioreactor conditions, the temperature increased the production of yellow pigments. However, stirring showed a negative effect and remarkably decreased yellow pigment in *M. purpureus*. The highest significant level of yellow pigment, 0.334 OD, was achieved at 3 L/Min of stirring, 30 °C of temperature, 3 g/l of urea, 2 g/l of rice, 9 g/l of barley, pH of 5, 1.5 L/Min of aeration, 1 g/l of ammonium, 0.5 g/l of the nutrient element. ANOVA analysis for optimizing the yellow pigment production of *Monascus purpureus* by CCD is shown in (Table 3). The following is the equation for optimizing yellow pigment production with CCD.

yellow= $0.2539+0.0137A+0.0280B+0.0189C+0.0316AB-0.0108AC-0.0290BC-0.0019A^2+0.0088B^2-0.0362C^2$

In the models, A: temp (°C), B: urea (g/L), and C: barley (g/L).
Two carbon sources (rice, and barley) increased the production of orange pigment ($p \leq 0.05$) and wheat did not show a statistically significant effect. None of the nitrogen sources showed a significant effect. The orange pigment was increased by pH and barley ($p \leq 0.05$) while other bioreactor conditions (stirring and temperature) did not have a significant effect. The highest significant level of orange pigment (0.4864 OD) was achieved at 2 g/l of rice and 18 g/l of barely, pH of 7, and 1.4 L/Min of aeration Figure 2(b). The optimal levels of stirring, ammonium, urea, nutrient element, and temperature were 1.4 L/Min, 1 g/l, 1.64 g/l, 0.47 g/l, and 25 °C, respectively. ANOVA analysis for optimizing the orange pigment production of *Monascus purpureus* by CCD is shown in (Table 4). The following is the equation for optimizing orange pigment production with CCD.

orange= $0.2977+0.0502A+0.0004B+0.0194C-0.0314AB+0.0095AC-0.0201BC-0.0286A^2+0.0462B^2-0.0072C^2$

In the models, A: pH, B rice (g/L), and C: barley (g/L).
Rice and wheat have a positive significant influence on red pigments, while barley and potato did not have a statistically significant effect ($p \leq 0.05$). Aeration, pH, and stirring increased the production of the pigment. However, ammonium nitrate as a nitrogen source and nutrient element had a significant negative effect and decreased red pigment production ($p \leq 0.05$). The highest level of red pigments (0.7357 OD) was observed at 18 g/l of rice and 18 g/l of wheat, 4 L/Min of aeration, 1 g/l of nutrient elements, pH of 5, 6 L/Min of stirring, and 30° of temperature Figure 2 (c). ANOVA analysis for optimizing the red pigment production of *Monascus purpureus* by CCD is shown in (Table 5). The following is the equation for optimizing red pigment production with CCD.

Red= $0.5943+0.0224A+0.0919B+0.0938C+0.0078AB-0.0174AC-0.0022BC-0.0062A^20.0366B^2-0.0083C^2$

In the models, A: aeration (L/Min), B: wheat (g/L), and C: rice (g/L).

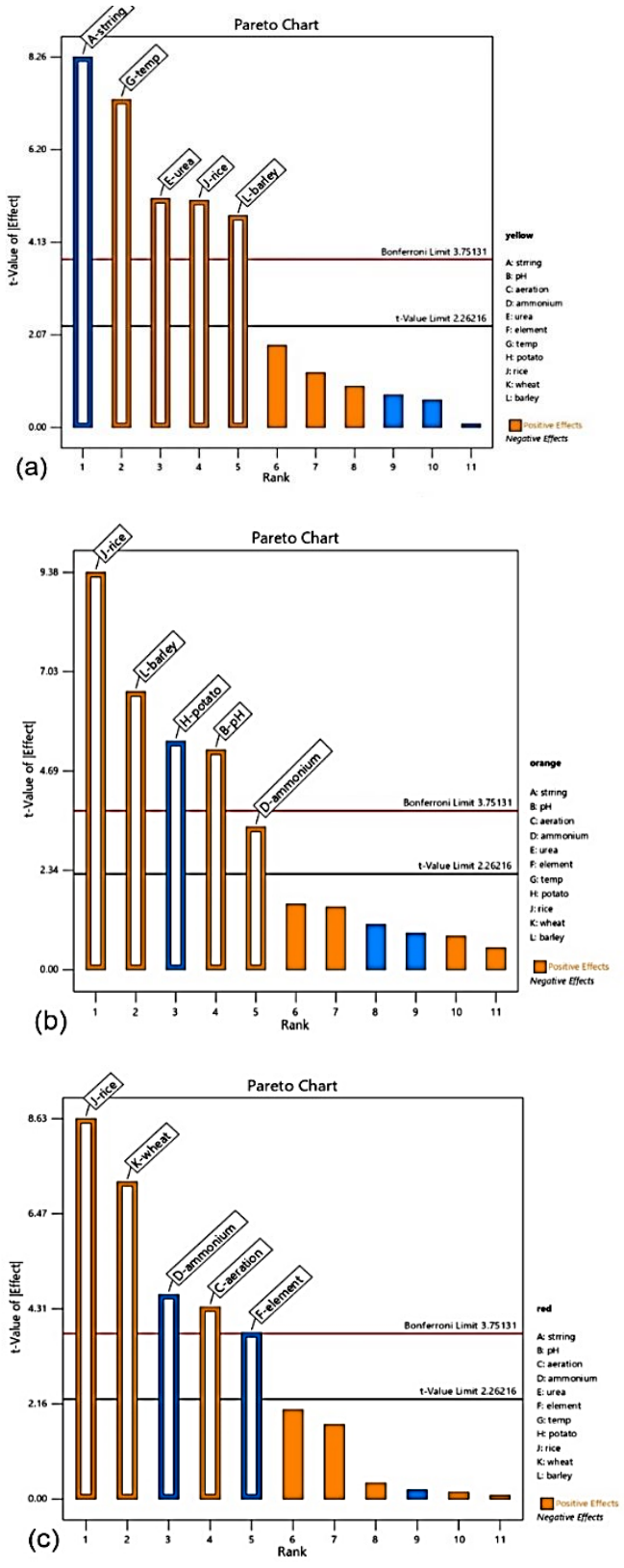


**Fig. 1** Pareto chart (Analysis of PBD) displaying the order of significant medium and bioreactor variables for (a) yellow; (b) orange: and (c) red; pigment production by *M.purpureus.*

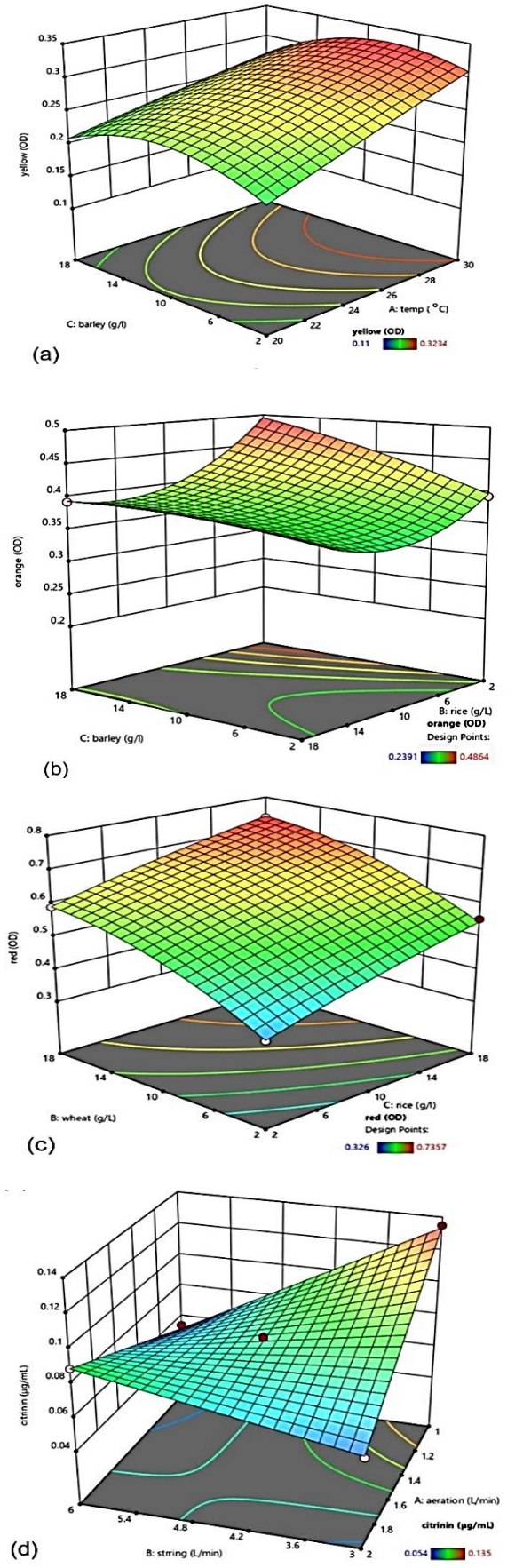


**Fig. 2** The 3D surface plots for the variables in relation to *Monascus* pigment production. (a) yellow; (b) orange; (c) red; (d) The 3D surface plots for the variables in relation to *Monascus* citrinin production.

**Table 2** ANOVA analysis for Screening of yellow, orange, and red pigments produced by *M. purpureus* by PBD

| | Source | SoS [1] | df | MS [2] | F-value | p-value |
|---|---|---|---|---|---|---|
| yellow | Model | 0.0450 | 5 | 0.0090 | 25.29 | < 0.0001** |
| | A-stirring | 0.0157 | 1 | 0.0157 | 44.11 | < 0.0001 |
| | E-urea | 0.0060 | 1 | 0.0060 | 16.82 | 0.0027 |
| | G-temp | 0.0123 | 1 | 0.0123 | 34.54 | 0.0002 |
| | J-rice | 0.0059 | 1 | 0.0059 | 16.57 | 0.0028 |
| | L-barley | 0.0051 | 1 | 0.0051 | 14.40 | 0.0042 |
| | Residual | 0.0032 | 9 | 0.0004 | - | - |
| | LoF 4 | 0.0029 | 7 | 0.0004 | 2.48 | 0.3171ns |
| | Pure Error | 0.0003 | 2 | 0.0002 | - | - |
| | $R^2$ | 0.9336 | - | - | - | - |
| | AP 5 | 15.1440 | - | - | - | - |
| orange | Model | 0.0560 | 5 | 0.0112 | 42.41 | < 0.0001** |
| | B-pH | 0.0076 | 1 | 0.0076 | 28.77 | 0.0005 |
| | D-a 3 | 0.0032 | 1 | 0.0032 | 12.12 | 0.0069 |
| | H-potato | 0.0082 | 1 | 0.0082 | 31.10 | 0.0003 |
| | J-rice | 0.0248 | 1 | 0.0248 | 94.04 | < 0.0001 |
| | L-barley | 0.0122 | 1 | 0.0122 | 46.03 | < 0.0001 |
| | Residual | 0.0024 | 9 | 0.0003 | - | - |
| | LoF 4 | 0.0022 | 7 | 0.0003 | 3.31 | 0.2513 ns |
| | Pure Error | 0.0002 | 2 | 0.0001 | - | - |
| | $R^2$ | 0.9593 | - | - | - | - |
| | AP 5 | 20.1369 | - | - | - | - |
| red | Model | 0.2007 | 7 | 0.0287 | 19.91 | 0.0009** |
| | A-stirring | 0.0052 | 1 | 0.0052 | 3.62 | 0.1059 |
| | B-ph | 0.0069 | 1 | 0.0069 | 4.80 | 0.0710 |
| | C-airing | 0.0167 | 1 | 0.0167 | 11.61 | 0.0144 |
| | D-a 3 | 0.0120 | 1 | 0.0120 | 8.36 | 0.0277 |
| | F-element | 0.0124 | 1 | 0.0124 | 8.62 | 0.0261 |
| | J-rice | 0.0864 | 1 | 0.0864 | 59.96 | 0.0002 |
| | K-wheat | 0.0611 | 1 | 0.0611 | 42.40 | 0.0006 |
| | Curvature | 0.0011 | 1 | 0.0011 | 0.7703 | 0.4139 |
| | Residual | 0.0086 | 6 | 0.0014 | - | - |
| | LoF 4 | 0.0035 | 4 | 0.0009 | 0.3464 | 0.8325 ns |
| | Pure Error | 0.0051 | 2 | 0.0026 | - | - |
| | $R^2$ | 0.9727 | - | - | - | - |
| | AP 5 | 21.1674 | - | - | - | - |

[1] Sum of Squares, [2] Mean Square, [3] D-ammonium, [4] Lack of Fit, [5] Adeq Precision, ** significant, ns not significant

**Table 3** ANOVA analysis for optimizing yellow pigment production of *M. purpureus* by CCD

| Source | SoS [1] | df | MS [2] | F-value | p-value |
|---|---|---|---|---|---|
| Model | 0.0547 | 9 | 0.0061 | 121.97 | < 0.0001** |
| A-temp | 0.0026 | 1 | 0.0026 | 51.30 | < 0.0001 |
| B-urea | 0.0107 | 1 | 0.0107 | 214.73 | < 0.0001 |
| C-barley | 0.0049 | 1 | 0.0049 | 98.24 | < 0.0001 |
| AB | 0.0080 | 1 | 0.0080 | 160.90 | < 0.0001 |
| AC | 0.0009 | 1 | 0.0009 | 18.74 | 0.0015 |
| BC | 0.0067 | 1 | 0.0067 | 134.62 | < 0.0001 |
| $A^2$ | 0.0001 | 1 | 0.0001 | 1.06 | 0.3264 |
| $B^2$ | 0.0009 | 1 | 0.0009 | 18.76 | 0.0015 |
| $C^2$ | 0.0189 | 1 | 0.0189 | 379.45 | < 0.0001 |
| Residual | 0.0005 | 10 | 0.0000 | - | - |
| LoF [3] | 0.0004 | 5 | 0.0001 | 2.93 | 0.1312 ns |
| PE 4 | 0.0001 | 5 | 0.0000 | - | - |
| $R^2$ | 0.9910 | - | - | - | - |
| AP 5 | 40.8987 | - | - | - | - |

[1] Sum of Squares, [2] Mean Square, [3] Lack of Fit, [4] Pure Error, [5] Adeq Precision, ** significant, ns not significant

**Table 4** ANOVA analysis for optimizing orange pigment production of *M. purpureus* by CCD

| Source | SoS [1] | df | MS [2] | F-value | p-value |
|---|---|---|---|---|---|
| Model | 0.0934 | 9 | 0.0104 | 63.20 | < 0.0001** |
| A-pH | 0.0344 | 1 | 0.0344 | 209.42 | < 0.0001 |
| B-rice | 0.000002 | 1 | 0.000002 | 0.0159 | 0.9022 |
| C-barley | 0.0052 | 1 | 0.0052 | 31.44 | 0.0002 |
| AB | 0.0079 | 1 | 0.0079 | 47.99 | < 0.0001 |
| AC | 0.0007 | 1 | 0.0007 | 4.36 | 0.0633 |
| BC | 0.0032 | 1 | 0.0032 | 19.75 | 0.0012 |
| $A^2$ | 0.0118 | 1 | 0.0118 | 71.74 | < 0.0001 |
| $B^2$ | 0.0308 | 1 | 0.0308 | 187.43 | < 0.0001 |
| $C^2$ | 0.0007 | 1 | 0.0007 | 4.51 | 0.0596 |
| Residual | 0.0016 | 10 | 0.0002 | - | - |
| LoF [3] | 0.0006 | 5 | 0.0001 | 0.6460 | 0.6784 ns |
| PE [4] | 0.0010 | 5 | 0.0002 | - | - |
| $R^2$ | 0.9827 | - | - | - | - |
| AP [5] | 27.6767 | - | - | - | - |

[1] Sum of Squares, [2] Mean Square, [3] Lack of Fit, [4] Pure Error, [5] Adeq Precision, ** significant, ns not significant

**Table 5** ANOVA analysis for optimizing red pigment production of *M. purpureus* by CCD

| Source | SoS [1] | df | MS [2] | F-value | p-value |
|---|---|---|---|---|---|
| Model | 0.2652 | 9 | 0.0295 | 407.47 | < 0.0001** |
| A-aeration | 0.0069 | 1 | 0.0069 | 94.93 | < 0.0001 |
| B-wheat | 0.1155 | 1 | 0.1155 | 1596.84 | < 0.0001 |
| C-rice | 0.1203 | 1 | 0.1203 | 1663.30 | < 0.0001 |
| AB | 0.0005 | 1 | 0.0005 | 6.67 | 0.0273 |
| AC | 0.0024 | 1 | 0.0024 | 33.36 | 0.0002 |
| BC | 0.0000 | 1 | 0.0000 | 0.5126 | 0.4904 |
| $A^2$ | 0.0006 | 1 | 0.0006 | 7.62 | 0.0201 |
| $B^2$ | 0.0193 | 1 | 0.0193 | 266.43 | < 0.0001 |
| $C^2$ | 0.0010 | 1 | 0.0010 | 13.57 | 0.0042 |
| Residual | 0.0007 | 10 | 0.0001 | - | - |
| LoF [3] | 0.0005 | 5 | 0.0001 | 2.09 | 0.2193 ns |
| PE [4] | 0.0002 | 5 | 0.0000 | - | - |
| $R^2$ | 0.9973 | - | - | - | - |
| AP [5] | 69.2558 | - | - | - | - |

[1] Sum of Squares, [2] Mean Square, [3] Lack of Fit, [4] Pure Error, [5] Adeq Precision, ** significant, ns not significant

**Table 6** ANOVA analysis for optimizing citrinin production of *M. purpureus* by CCD

| Source | SoS 1 | df | MS 2 | F-value | p-value |
|---|---|---|---|---|---|
| Model | 0.0051 | 3 | 0.0017 | 338.53 | < 0.0001** |
| A-aeration | 0.0006 | 1 | 0.0006 | 118.38 | < 0.0001 |
| B-stirring | 0.0015 | 1 | 0.0015 | 301.14 | < 0.0001 |
| AB | 0.0030 | 1 | 0.0030 | 596.05 | < 0.0001 |
| Residual | 0.0000 | 9 | 4.983E-06 | - | - |
| LoF 3 | 0.0000 | 5 | 4.730E-06 | 0.8924 | 0.5595 ns |
| PE 4 | 0.0000 | 4 | 5.300E-06 | - | - |
| R ² | 0.9912 | - | - | - | - |
| AP 5 | 66.1347 | - | - | - | - |

[1] Sum of Squares, [2] Mean Square, [3] Lack of Fit, [4] Pure Error,[5] Adeq Precision, ** significant, ns not significant

## Effect of the Optimized Medium on Citrinin Production

The results showed that the concentration of citrinin varied from 0.054 to 0.135 (μg/mL). The lowest amount of citrinin was achieved at 2 L/Min aeration and 3 L/Min stirring or 1 L/Min aeration and 6 L/Min stirring Figure 2 (d). ANOVA analysis for optimizing citrinin production of *M. purpureus* by CCD is shown in (Table 6).

## DISCUSSION

In this study, 11 independent variables, including four carbon sources in three levels (wheat, barley, potato, and rice), two nitrogen sources in three levels (ammonium nitrate and urea), nutrient elements, and four bioreactor conditions (temperature, stirring, pH, and aeration) were evaluated to find the best conditions for co-production of bio pigments in *M. purpureus* using statistical methods. Then, the significant variables selected from multiple linear regression analyses of PBD were optimized using RSM. The aim was to optimize and enhance the co-production of yellow, orange, and red pigments using cost-effective materials.

There is only one report that showed the effect of wheat substrates on the production of *Monascus* pigments. The results showed that high glucose and amino acid concentrations in complex wheat flour media considerably favored the production of red dyes over orange or yellow colorings [15]. We could not find a study reporting the effect of barley on the production of *Monascus* pigments but there are some reports that confirm the medium or substrate elements affect the growth and quality of medicinal mushrooms [16]. The concentration of both the carbon source (mostly in the form of glucose by hydrolysis of starch) and the nitrogen source in the medium (bran, soluble wheat proteins, and gluten) were directly correlated with biomass and pigment synthesis in the majority of *Monascus* cultivations. In the context of studying the effect of potato extract as a carbon source, a study by Srivastav, and Yadav [10] showed that sweet potatoes can be utilized as a low-cost substrate for red pigment production. Rice straw has previously been investigated as a cost-effective substrate for producing *M.* pigments [17]. Only one study showed that ammonium nitrate could be used as a nitrogen source to produce red pigment in *Monascus* [1] and no study reported urea as a nitrogen source for bio pigment production. Recently, the optimization of *Monascus* pigments co-production has been the subject of several studies. Corn cob and glycerol [7,18], rice straw [19], nutrient-rich brewer's spent grain-derived hydrolysate [20] and sugarcane bagasse hydrolysate [21] were mostly used as low-cost substrates to reduce the cost of bio pigment production in *M. purpureus*.

Herein, *Monascus* pigment production was subjected to a two-step statistical method (PBD and CCD of RSM) in order to reduce the capital cost of bio pigment production, maximizing the co-production of *Monascus* pigments. It has been indicated that statistical approaches are a reliable method for enhancing and optimizing the yield of various biological processes [7, 22]. In this regard, the optimal level of each carbon and nitrogen source influencing the co-production of *Monascus* yellow, orange, and red pigments were determined to reduce production costs.

With regard to carbon sources, the highest level of pigments was achieved at 2 g/l of rice and 9 g/l barley for yellow pigment, 2 g/l of rice and 18 g/l of barley for orange pigment, and 18 g/l of rice and 18 g/l wheat for red pigments. 2 g/l of potato negatively affected orange pigment but did not show a

significant effect on yellow and red pigments (Fig. 2). 18 g/L of rice showed a significant effect on yellow, orange, and red pigments, while barley affected only yellow and orange. Wheat only showed a significant effect on red pigment production.

*Monascus* is traditionally cultured on rice through solid-state fermentation to generate a fermenting meal known as red rice, which is widely regarded as an essential element in Asian meals and treatments. Rice extract enhanced pigment production more than barley and wheat extract. This might be attributed to the nature of *M. purpureus* hydrolytic enzymes (cellulases and xylanases) in degrading starch polysaccharides (cellulose and hemicellulose) in rice than in wheat and barley. [15]. The bioavailability of cellulose and hemicellulose in rice extract could facilitate fungal access and simple production of sugar from polysaccharides, and in return, the fast production of fungal pigments. Barley has a rough texture and a tight lignin matrix [23] compared to wheat, and it could limit the accessibility of cellulose and hemicellulose from barley to fungal. Hence, the release of sugars would be reduced and, as a result, the growth of *M. purpureus* and its pigment production would be decreased [8]. The potato has a low level of carbohydrates in the form of starch (7.8 g/100g) [24] and a less hydrophobic nature compared to rice, wheat, and barley [25]. In the meantime, rice, barley, and wheat are some of the crops largely available worldwide, and this adds more advantages to the usage of these extracts in the process of *Monascus* pigment production.

Regarding nitrogen sources, this study showed that 3 g/l of urea triggers yellow pigment production, while ammonium nitrate did not show a significant effect on yellow pigment production. However, ammonium nitrate triggered the production of orange pigment at 5 g/l and red pigment at 1 g/l. Previous studies have revealed the influence of different substrates, including ammonium nitrate, on the production of *Monascus* pigments [1]. However, there was no available study reporting the effect of urea as a nitrogen source on *Monascus* pigment production. Lin and Demain [26] reported that ammonium nitrate supports poor pigment production by *Monascus purpureus* and they showed that the poor ability of ammonium nitrate to donate nitrogen is the main reason for the low production of pigment. It has been shown that ammonium salts, especially ammonium nitrate, inhibit some key enzymes for *Monascus* pigment synthesis and their inhibitory effect on red pigments is greater than on orange and yellow pigments [27]. There are also some reports that confirm the effect of macro and microelements on secondary productions production [28, 29].

The results of the study showed that nutrient elements (NPK) did not have a significant effect on yellow and orange pigments. However, nutrient elements significantly reduced red pigment production. This study could not show the reason behind the negative effect of elements at three levels on bio pigment production. No report is available about its application as a substrate in *Monascus spp* to compare with the results of this study.

The temperature of the production medium is one of the main factors for the optimal production of pigments by *M. purpureus* [1,12,19,20]. Several studies have already shown that different isolates of *Monascus spp* grow best under an optimum temperature range of 30 °C to 37 °C [18]. These findings support the results of this study (Table 2). Alternatively, the optimum acidity of the culture medium is the point of discrepancy. A study on red pigment production by *M. purpureus* CCT3802 revealed that acidity promotes fungus growth and red pigment production [30]. Another study reported that the highest amount of red and orange pigments were achieved at an acidity of 4.5 to 5 [7].

It is widely known that *Monascus* fungus may grow in a broad pH range (2.5 to 8.0), with the optimum between 4.0 and 7.0 [31]. The sort of pigments that are created varies on how acidic the culture media is; at low pH, yellow pigment dominates, whereas, at high pH, red pigment dominates [32]. As shown in Figure 2, the highest pigments, especially yellow ones, were produced when the pH was five. At a pH of 5, created by adding HCL, the amount of yellow pigment was more than orange, and orange pigment was produced more than red pigment. However, at a pH of 7, orange and red pigment production was more than yellow pigment. Studies have shown that the discrepancy could be attributed to the strain of Monascus, carbon, and nitrogen sources.

Regarding stirring speed, Lv, Zhang [5] reported the highest yield of the *Monascus* yellow pigment at an agitation speed of 180 rpm and the highest level of red pigment was at 4 L/Min of stirring. Moreover, Embaby, Hussein [7] showed that aeration in terms of 150 rpm promotes the co-production of orange and red pigments in *M. purpureus*. Here, 3-4 L/Min of

aeration promoted the production of bio pigments, whereas stirring at 3-4 L/Min resulted in the highest number of red pigments.

This study results showed that citrinin production declined at 18 g/l of barely, 18 g/l of rice, 1 g/L of urea, pH of 3, 30°C temperature, 2 L/M of aeration, 3 L/M of stirring, 0.5 g/l of the element, and 0.5 g/l of ammonium nitrate. Potatoes, wheat, and urea were the most effective variables in increasing citrinin. In the past decades, many studies have focused on the manipulation of the composition of growth media or fermentation conditions to decrease the production of mycotoxins such as citrinin [12, 22, 33]. Then, Jirasatid, Nopharatana [22] reported that in the medium containing 2% glycerol, 0.14% methionine, and 0.01% sodium nitrate at 25°C for 16 days of cultivation, the highest amount of yellow pigment and the minimum citrinin concentration of 0.26 mg/kg (36 times lower) could be achieved. Kang, Zhang [33] found that an ideal nitrogen source can be used to control citrinin biosynthesis and showed that when $(NH4)_2SO_4$ or MSG are used as nitrogen sources, citrinin production is reduced to the lowest level. The findings of these studies are comparable to our work and support the results. Herein, present data indicated that aeration and stirring as substrate under optimized conditions could reduce the production of citrinin compared to the basal medium (Fig. 2).

## CONCLUSION

This study used low-cost carbon and nitrogen sources for the production of yellow, orange, and red pigments by *M. purpureus*. Meanwhile, the best concentration and bioreactor conditions were determined using a low-cost and effective method. The low level of citrinin concentration in the optimized medium and bioreactor conditions confirmed the results. The data presented in this study could be used to produce cost-effective *Monascus* pigments on a semi-industrial scale from *M. purpureus*.

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## Conflict of Interest

The authors have no conflict of interest to declare in the present study.

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