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Natural Pigment Production by *Monascus purpureus:* Bioreactor Yield Improvement through Statistical Analysis

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Abstract

Among the ways of pigment production, microbial synthesis has gained more interest for high growth rate, easy extraction and high yield. Pigments are used in the food industry as natural colorants and preservatives; they also have pharmaceutical applications. In this study, fungus *Monascus purpureus* PTCC 5303 was used to produce red, orange and yellow pigments. At first, significant variables were screened based on Plackett-Burman's design. The optimized value of two effective factors, i.e. concentration of yeast extract and K_2HPO_4 by three-level, was more studied by the response surface method (RSM). The most suitable level was 2.75 g/L for yeast extract and 1.5 g/L for K_2HPO_4. Antimicrobial activity of the pigments was shown on Gram-positive food-borne bacteria under optimal conditions. Moreover, pigment production at optimal conditions in a bioreactor was evaluated, and the rate of production of red, orange and yellow pigments was obtained to be 2.05, 1.55 and 0.78 (ODU/ml), respectively.

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1. Introduction

Pigments may be used in many fields such as food production, dyes, cosmetics and pharmaceuticals. There is a global trend toward pigment production using natural substance in recent years. This is due to concerns about the harmful effects of synthetic pigments on humans and the environment. Microorganisms have become a substantial source for pigment production due to high growth rate, cheap culture medium, and easy extraction process; all these offer more advantages for microorganisms than for other biological resources [1, 2].

Monascus purpureus, which belongs to Ascomycetes class and Monascaceae family, is capable of producing many secondary metabolites such as pigments, monacolins, γ-aminobutyric acid, dimerumic acid and so on [3-5]. Pigments of Monascus are combinations of yellow (ankaflavin, monascin), orange (rubropunctatin, monascorubrin) and red (rubropunctamine, monascorubramine) compounds, which possess a range of biological activities [6,7]. Monascus pigments are nontoxic that can be used in the food industry as a natural colorant and potential source for therapeutic applications [8,9].

Monascus-fermented products have been used in food, medicine, and industry for more than thousand years in Asian countries, and several excellent agents with a broad range of applications have been found for them. These products can be applied as food or nutritional supplements with multiple therapeutic profits [10-12].

Solid state fermentation is not proper for large-scale industrial pigment production because of low productivity, high labor cost and control concerns [2]. To overcome these limitations of solid state fermentation, several studies have been conducted on submerged fermentation for pigment production from Monascus [13, 14]. Batch cultivation in shake-flasks and laboratory fermenters have been reported as the most frequently used technique.

Bioreactors provide easier control on the environmental conditions such as temperature, agitation rate, dissolved oxygen, and culture medium pH [15].

Because of predictable influence of medium composition on product yield, designing fermentation medium is a crucial process. Medium composition can be optimized by changing chemical components to maximize the production yield. Statistical approach for screening and optimizing both the process and the culture medium has become a major concern for production of different metabolites [16-18]. Optimization of fermentation conditions and medium components with less number of experiments is performed using the response surface methodology (RSM) [19, 20].

Quantity, quality and composition of produced pigments depend significantly on the type of nutrients including nitrogen and carbon sources, medium pH, temperature, moisture and implemented strains. Hence, before pigment isolation, there are several physical and nutritional parameters to be optimized for achieving maximum growth and pigment production in a submerged fermentation [21-23].

In this research, we selected significant factors for pigment production by Plackett-Burman's experimental design. Then, production of red, orange and yellow pigments was optimized using the RSM. Moreover, for the first time, the correlations between pigment production and the mentioned effective variables were expressed, and the obtained optimum conditions were assessed in bioreactor. Finally, the antimicrobial property of the produced pigments was examined at optimal states.

2. Materials and Methods

2.1. Microorganisms and media

Monascus purpureus PTCC 5303 strain was obtained from Iranian Research Organization for Science and Technology. The strain was maintained on potato dextrose agar (PDA) (Merck) plate at 30°C for 7 days (followed by storage at 4°C), and sub-cultured monthly.

The experiments were carried out in 100 ml Erlenmeyer flasks based on the statistical design. Each flask was inoculated with small fragments of vegetative cells obtained from the 7 days old PDA plate, and incubated at 30°C for 7 days on a rotary shaker (130 rpm) in a dark place.

For antimicrobial activity assessment, the bacterial strains consisting of *Bacillus cereus* (PTCC1247) and *Staphylococcus aureus* (PTCC1431), obtained from Iranian Research Organization for Science and Technology, were cultured at 37°C and nutrient agar.

2.2. Pigments measurement

To measure the amount of pigment, mycelia were separated from the fermented broth by a filtration membrane. Cell-free culture filtrate was then centrifuged at 7511×g for 15min, and the supernatant was decanted. Finally, extracellular pigment concentration was determined by spectrophotometer, and results were expressed in terms of specific absorbance related with each concentration [24].

2.3. Statistical analysis

Plackett-Burman's (PB) experimental design was done for screening and considering the role of various nutrients in pigment production (Table 1) [25]. Glucose (Sigma) was added at a constant amount of 30 g/L to the media.

Table 1- Variables concentrations at different levels using Plackett-Burman experimental design

Code	Variable	Low level (-1) (g/L)	Central level (0) (g/L)	High level (+1) (g/L)
A	MSG	1	3	5
B	casein	1	3	5
C	Yeast extract	1	3	5
D	K_2HPO_4	1	1.5	2
E	$MgSO_4$	0.5	1	1.5
F	Vitamin B_1	0.01	0.03	0.05

Production of red, orange and yellow pigments was implemented using response surface methodology (RSM) to optimize the concentration of two effective factors, i.e. concentration of yeast extract and K_2HPO_4 at three levels (Table 2). All experiments design was considered in Minitab software (version 16). The statistical significance of examined parameters was evaluated by P-value. Parameters with p-value less than 0.05 would be statistically significant.

Table 2- Variable levels for optimization of pigments production

Variable	Code	Level (-1) (g/L)	Level (0) (g/L)	Level (+1) (g/L)
Yeast extract	C	0.25	1.5	2.75
K_2HPO_4	D	0.3	0.9	1.5

2.4 Pigments extraction

Mycelium was harvested after an incubation period, which is then followed by supernatant filtration using a Whatman's filter paper. Then two volumes of 95% (v/v) ethanol were added to the exhausted culture broth according to the below procedure [26]:

Firstly, approximately 60% of the required solvent volume was diluted, a resulting mixture was then centrifuged at1533×g rpm for 15 min, followed by a rotary shaker incubation at 180 rpm, 30°C for 30 min. The pellet was re-suspended in the remaining volume of ethanol, and centrifuged at 1533×g for 5 min. The supernatant was collected and filtered through a Whatman's filter paper. The extracted pigments were concentrated in a rotary device with pH adjusted to 7. Pigments powder was obtained by freeze dryer device for investigation of the antibacterial activity.

2.5. Antibacterial activity assay

Micro-dilution method on Gram positive foodborne bacteria, Bacillus cereus and Staphylococcus aurous, was utilized for evaluating the antibacterial activity of the produced pigments [27]. The bacterial strains were cultured overnight at 37°C in Muller Hinton agar (MHA). The pigments' stock solutions and dilution series, were arranged from 1 to 40 mg/ml, and then transferred into 96 well microlitre plates in addition to Muller Hinton broth (MHB) and standardized microorganism suspensions, containing 108 (cfu/ml). After 24 h incubation at 37°C, a well without turbidity was assigned as Minimum inhibitory concentration (MIC) (mg/ml) amount. Finally, the inhibition of bacterial growth was evaluated by measuring absorbance at 630 nm using ELISA reader [27].

2.6. Fermenter

Fermentation was carried out in a 5-liter stirred tank fermenter (Sibtech, Iran) in the obtained optimal conditions. Fungus was cultured at 30°C, stirring speed 300 rpm, and aeration rate 60% v/v for 7 days at optimal conditions (Minitab 16; $p<0.05$) [18].

3. Results and Discussion

3.1. Screening of nutrient variables

Overall, 16 experiments were designed and performed according to the Plackett-Burman's method (Table 3). Among the considered parameters, a negative effect on pigment production was observed for six medium variables. Yeast extract (nitrogen source) and K_2HPO_4 (phosphate source) were considered important for production of red, orange and yellow pigments. As it was expected, phosphate and nitrogen sources had vital roles for metabolite production and growth**.**

Table 3- Screening of variables data by Plackett-Burman design

experiment	MSG	case in	Yeast extract	K_2HPO_4	$MgSO_4$	Vitamin B_1	Red pigment (ODU/ml)	Orange pigment (ODU/ml)	Yellow pigment (ODU/ml)
1	1	-1	1	-1	-1	-1	0.44	0.64	**0.56**
2	1	1	-1	1	-1	-1	1.14	1.18	**1.30**
3	-1	1	1	-1	1	-1	0.50	0.74	**0.76**
4	1	-1	1	1	-1	1	0.76	0.62	**0.92**
5	1	1	-1	1	1	-1	0.37	0.51	**0.22**
6	1	1	1	-1	1	1	0.39	0.62	**0.58**
7	-1	1	1	1	-1	1	0.55	0.89	**0.40**
8	-1	-1	1	1	1	-1	0.18	0.31	**0.74**
9	-1	-1	-1	1	1	1	0.76	0.81	**0.94**
10	1	-1	-1	-1	1	1	3.30	2.45	**1.00**
11	-1	1	-1	-1	-1	1	0.86	0.90	**1.10**
12	-1	-1	-1	-1	-1	-1	6.30	4.69	**2.87**
13	0	0	0	0	0	0	0.74	0.62	**0.92**
14	0	0	0	0	0	0	0.42	0.59	**0.46**
15	0	0	0	0	0	0	0.45	0.65	**0.50**
16	0	0	0	0	0	0	0.42	0.62	**0.52**

3.2. Optimization of pigment production

Pigment production was optimized using central composite design (CCD). As shown in Table 4, the results of 13 experimental runs were obtained by three-level two-factor fractional CCD.

Table 4- Optimization of variables data based on response surface design

Experiment	Yeast extract	K_2HPO_4	Red Pigment (ODU/ml)	Orange Pigment (ODU/ml)	Yellow Pigment (ODU/ml)
1	-1	-1	2.50	2.20	2.50
2	1	-1	4.60	3.10	1.80
3	-1	1	4.15	2.60	2.50
4	1	1	5.90	2.60	2.10
5	-1	0	3.70	2.40	2.45
6	1	0	8.20	5.00	1.90
7	0	-1	0.88	0.73	0.98
8	0	1	7.10	3.70	3.15
9	0	0	1.50	1.00	0.75
10	0	0	1.30	0.95	0.70
11	0	0	1.50	1.00	0.70
12	0	0	1.40	0.95	0.75
13	0	0	1.50	1.00	0.75

Based on the research results, the linear effects of yeast extract and K_2HPO_4, as well as the square effect of yeast extract ($p<0.05$) for red pigment production were statistically significant. A second order

polynomial model (Eq. 1) was used to express red pigment production, and R^2 value for red pigment production was obtained as 0.75:

Red pigment (ODU/ml) (R_1) $=2.21+1.39A+1.53B+2.63A^2$ *(1)*

A significant growth in red pigment production was observed by increasing the concentrations of yeast extract and K_2HPO_4 (Figure 1).

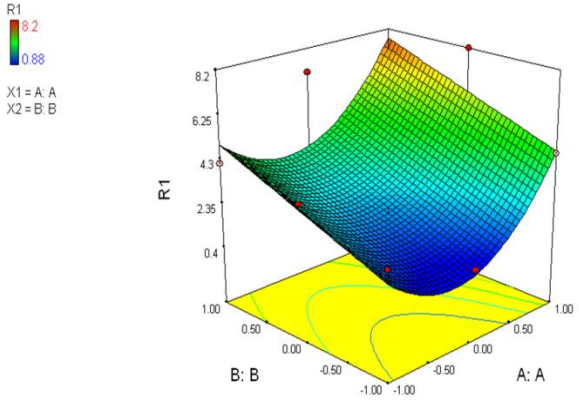

Figure 1. Three-dimensional influence of yeast extract (A) and K_2HPO_4 concentrations (B) on red pigment production

Figure 2 shows comparison between the calculated and experimental values of absorption at 500 nm for red pigment production.

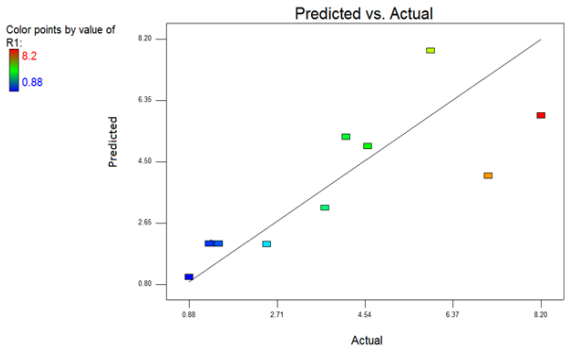

Figure 2. Comparison between calculated and experimental values of absorption at 500 nm (red pigment production)

According to P-values, the square effect of yeast extract ($P<0.05$) was found to be more significant rather than other medium variables for orange pigment production. A second order polynomial model (Eq. 2) was fitted by the regression coefficients for orange pigment production, and R^2 value for orange pigment production obtained as 0.71:

Orange pigment (ODU/ml) $(R_2)=1.35+1.64A$ *(2)*

The maximum production of orange pigment was observed at higher amounts of yeast extract and K_2HPO_4.

The linear effect of K_2HPO_4, and the square effect of yeast extract and K_2HPO_4 ($P< 0.05$) were found to be significant among the other variables of the medium for yellow pigment production. A second order polynomial model (Eq. 3) was obtained by using the regression coefficients, and R^2 value for yellow pigment production was 0.78:

Yellow pigment (ODU/ml) (R_3) $=0.92+0.41B+0.81A^2+0.70B^2$ *(3)*

By increasing the yeast extract and K_2HPO_4 concentrations at optimum amount, yellow pigment production was intensified (Figure 3). Figure 4 shows comparison between the calculated and experimental values of absorption at 400 nm for yellow pigment production.

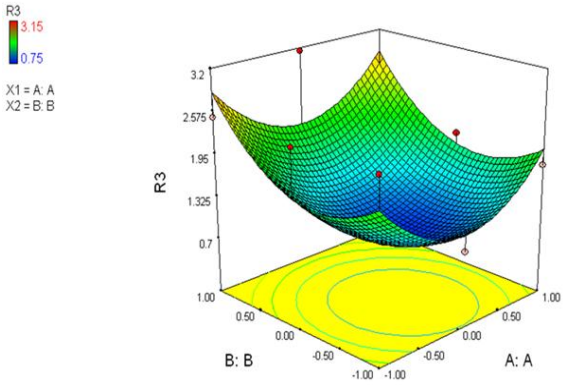

Figure 3. Three-dimensional influence of yeast extract (A) and k_2HPO_4 concentrations (B) on the yellow pigment production

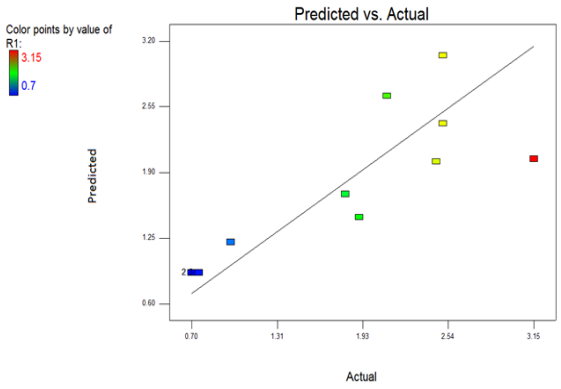

Figure 4. Comparison between calculated and experimental values of absorption at 400 nm (yellow pigment production)

The optimal levels of the medium variables were as follows: yeast extract (2.75 g/L) and K_2HPO_4 (1.5 g/L) with the maximum predicted responses of 7.76 ODU/ml for red, 2.98 ODU/ml for orange, and 2.85 ODU/ml for yellow pigments. Table 5 summarizes the optimum conditions for production of pigments.

Table 5- Optimum conditions for pigments production

Pigment	Yeast extract (g/L)	K_2HPO_4 (g/L)	Optimal response (ODU/ml)
Red (500 nm)	**2.75**	**1.5**	**7.76**
Orange (460 nm)	**2.75**	**1.5**	**2.98**
Yellow (400)	**2.75**	**1.5**	**2.85**

Sharmila *et al*. evaluated the effect of medium compositions on red pigment production by *Monascus purpureus* at submerged culture, using potato powder as a low cost carbon source. Concentrations of potato powder, K_2HPO_4, $ZnSO_47H_2O$ and Monosodium glutamate were optimized by the RSM. The results showed the optimal level of potato powder, K_2HPO_4, $ZnSO_47H_2O$, and MSG was 2.50 % (w/v), 0.480% (w/v), 0.0013% (w/v) and 0.60% (w/v), respectively, and maximum pigment production was reached to 7.18 ODU/ml[28]. Another study performed by Chatterjee *et al.* optimized the growth of a red pigment producing fungus *Monascus purpureus* by changing the physical and nutritional parameters. The maximum yield was recorded at 30°C, pH 6.0, 20g/L glucose and 0.3% MSG, in complete darkness [29].

3.3. Antibacterial activity of pigments

The micro-dilution method was used for assessing the pigments' antibacterial activities against bacterial food-borne pathogens. Antibacterial activities were expressed as MIC values (Table 6). The antibacterial activity of pigments extracted from the optimized medium on selected pathogenic bacteria showed greater inhibitory effects on *Bacillus cereus*. The antibacterial activity against *B. cereus* and *S. aureus* was found to be MIC=5 mg/ml, and MIC=20 mg/ml, respectively.

Kim *et al.* used micro-dilution method for investigating the antimicrobial activities of *Monascus* pigments (MP) on pathogenic bacteria. They suggested a higher antimicrobial activity for amino acid derivatives of *Monascus* compared to the original MPs, resulting from higher surface adsorption and oxygen transfer limitation. MP hydrophobic amino acid derivatives have shown to inhibit pathogenic bacteria more than hydrophilic ones [30,31]. According to the procedure of Vendruscolo *et al.*, the antimicrobial activity of natural pigments produced by *Monascus ruber* CCT 3802 on foodborne bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis*, in submerged fermentation, was reported to be in range of 10 to 20mg/mL [14].

Table 6 - Antibacterial activity of pigments by micro dilution method

pathogens	Gram reaction	Strain source	MIC (mg/ml)
Bacillus cereus	+	PTCC* 1247	**5**
Staphylococcus aurous	+	PTCC*1431	**20**

PTCC: Persian type culture collection

3.4. Investigation of fermentation conditions

Applying the effective factors determined in the optimization step, the pigment production was considered in a laboratory fermenter. The concentration values of 2.05, 1.55 and 0.78 (ODU/ml) were obtained for red, orange and yellow pigments, respectively. The optimum medium compositions and environmental conditions were investigated in submerged flask cultures by Lee *et al.*, in order to produce *Monascus* red pigments. 30 $g.l^{-1}$ of glucose and 1.5 $g.l^{-1}$ of MSG were determined as optimum carbon and nitrogen sources, respectively. Under the optimum culture conditions, the maximum specific productivity of red pigments in batch fermentation was observed to be 32.5 OD_{500} g DCW^{-1} h^{-1} [32]. Table 7 shows comparison between the present study and other studies.

Table 7- Comparison between present and other studies

Reference	Aim	Microorganisms	System	Statistical Analysis	Yield	Antibacterial Effect
Present study 2014	**Optimization of pigments production Red, orange, Yellow)**	***Monascus purpureus (PTCC 5303)* and *B.cereus* *S.aureus***	**Flask, 5 liter Fermenter**	**RSM**	**7.76 ODU/ml (Red, (500 nm 2.98 ODU/ml (Orange, 460 nm) 2.85 ODU/ml (Yellow, 400 nm)**	**Microdilution, *B. cereus* MIC: 5 mg/mL *S. aureus* MIC: 20 mg/mL**

Sharmila et al 2013	**Optimization of pigment production (Red)**	***Monascus purpureus (MTCC 369)***	**Flask**	**RSM**	**7.18 ODU/ml (Red, 500 nm)**	----------
Vendruscolo et al 2013	**Investigation of Antibacterial activity of pigments (Red, Orange)**	***Monascus ruber* (*CCT 3802*) and *S.aureus* *E.coli* *S.enteritidis***	**4 liter Fermenter**	--------	---------	**Microdilution, 10 to 20 mg/mL.**
Mukherjee et al 2011	**Optimization of Pigment production (Red)**	***Monascus purpureus* (*NFCCI 1756*) and *Bacillus* *Salmonella* *E.coli***	**Flask**	--------	**64 U/g dry cell mass**	**Disk diffusion, Inhibition the growth of Gram positive bacteria**
Chatterjee et al 2009	**Optimization of Pigment production (Red)**	***Monascus purpureus* (*MTCC 1090*) and *S.aureus* *B.subtilis* *E.coli* *S.typhi***	**Flask**	--------	**72 U/g Dry Cell mass**	**Disk diffusion, Inhibition the growth of Gram positive bacteria**
Kim et al 2006 a	**Investigation of Antibacterial activity of Pigment (Red)**	***Monascus sp* (*KCCM 10093*) and *Bacillus* *S.aureus* *Salmonella* *E.coli***	**Flask**	--------	-------	**Microdilution, 8 to 32 mg/mL.**
Lee et al 2001	**Optimization of Pigment production (Red)**	***Monascus purpureus (ATCC 16365)***	**Flask, 3 liter Fermenter**	--------	**32.5 OD500 g DCW-1 h^{-1}**	----------
Chen et al 1993	**Pigments production (Red, Orange, Yellow)**	***Monascus purpureus* (*192F*)**	**Flask Fermenter**	--------	--------	----------

4. Conclusion

In this study, screening of nutritional variables and optimization of effective variables were carried out by experimental design. Yeast extract (as nitrogen source) and K_2HPO_4 (as phosphate source) were found to be effective factors for production of red, orange and yellow pigments. We showed that pigment production would be optimized at high concentrations of yeast extract and K_2HPO_4. The optimum production of all red, orange and yellow pigments was observed at the optimal concentrations obtained for the two variables. Pigments were found to inhibit the growth of Gram-positive pathogenic bacteria. Pigment production was carried out in the fermenter, and the amount of red, orange and yellow pigments was determined to be 2.05, 1.55 and 0.78 (ODU/ml), respectively.

5. Acknowledgment

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6. Conflict of interest

The authors declare that there is no conflict of interest respect to present article.with the others.

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