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Original Article

Optimization of red pigment production by *Monascus purpureus* MTCC 369 under solid-state fermentation using response surface methodology

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Abstract

Monascus pigments are used as a traditional food colorant in orient China, are produced by solid-state fermentation of non-glutinous rice using *Monascus purpureus* MTCC 369. Five nutrient parameters screened using Plackett-Burman experimental design were optimized by central composite design (central rotatory) of response surface methodology for *Monascus* red pigment production under solid-state fermentation. Maximum red pigment production of 12 mg was predicted per gram of rice based solid medium containing 20g pre-soaked rice and 40 ml distilled water containing dextrose 74.59 g/l, peptone 5.54 g/l, NH_4Cl 6.57 g/l, $MnSO_4.H_2O$ 0.58 g/l and malt extract 14.46 g/l using response surface plots and point prediction tool of Design Expert 7.1:3 (Stat-Ease Inc., USA).

Keywords: red pigment, *Monascus purpureus*, optimization, response surface methodology, central composite design

1. Introduction

Pigments that are either natural or synthetic play an important role in the food industry as well as in the pharmaceutical industry, mostly for coloring wine, milk products, candy, and pharmaceutical dosage forms. Pigments are mainly of two types, naturally occurring or synthetic. Synthetic red pigments such as azorubin or tartrazin cause allergic effects (Fabre *et al*., 1993) and C-red has carcinogenic and teratogenic properties (Merlin *et al*., 1987). Thus researchers throughout the globe are intensely looking for safe naturally occurring red pigments. Different *Monascus* sp. namely *Monascus purpureus* (Su *et al*., 2003; Wang *et al*., 2003; Pattanagul *et al*., 2007; Babitha *et al*., 2007a), *M. ruber* (Endo, 1979), *M. paxi* (Manzoni and Rollini, 2002), and *M. anka* (Su *et al*., 2003) are well known to produce various pigments of polyketide origin. *Monascus* pigments consist of orange pigments such as monascorubrin and rubropunctatin, red pigments such as monascorubramine and rubropunctamine and yellow pigments such as ankaflavin and monascin. These pigments are linked to proteins, peptides and amino acids (Blanc *et al*., 1994; Juzlova *et al*., 1996; Hajjaj *et al*., 1999; Wang *et al*., 2004).

Among the biopigments produced by *Monascus*, the red ones are regarded as the most important, because these may be used as substitutes for nitrites in meat products and for synthetic colors such as erythrosine (FD and C red no3). *Monascus* red pigments have been used as food colorant traditionally for hundreds of years predominantly in countries like China, Thailand, Korea and Japan and are produced by using long-established solid-state fermentation method.

Biosynthesis of fermentation products is greatly influenced by fermentation parameters such as fermentation medium and process parameters and requires proper optimization for maximum productivity. Fermentation process optimization is a tedious process due to involvement of multivariable process parameters. In the optimization process, screening of important factors influencing fermentation is

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initially carried out by Plackett-Burman experimental design and selected factors are then optimized by different optimization techniques (Box and Hunter, 1957). Response surface methodology (RSM) is a three dimensional design that gives relationship between one or more measured dependent responses and a number of input (independent) factors. RSM has some advantages that include lower experiment numbers, suitability for multiple factor experiments, and ability to search for relativity between factors, to find the most suitable conditions and to forecast response (Chang *et al*., 2006; Sukkasem *et al*., 2007; Nimnoi and Lumyong, 2011). In this, linear or quadratic effects of experimental variables are used to construct contour plots and a model equation fitting the experimental data. This facilitates the determination of optimum value of factors under investigation and the prediction of response under optimized condition (Chakravarti and Sahai, 2002; Sayyad *et al*., 2007).

The objective of the present study was to optimize the fermentation nutrients for red pigments production by *M. purpureus* MTCC 369 under solid-state fermentation. RSM was used to optimize key nutrients screened by Plackett-Burman experimental design and this article describes the development of new solid-state fermentation medium for maximum production of *Monascus* red pigments.

2. Materials and Methods

2.1 Microorganism

Cultures of *Monascus purpureus* MTCC 369 obtained from Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology, Chandigarh, India. It was maintained on slants of potato dextrose agar (Himedia Mumbai, India) medium at 4°C and subcultured every 30 days.

2.2 Preparation of seed culture

Spore suspension of *Monascus purpureus* MTCC 369 was prepared from actively growing slants in sterile water and diluted and adjusted to a concentration of 5×10^3 spores per ml by hemocytometer. Spore suspension (15%) was inoculated to 250 ml conical flasks containing 50 ml of basal medium (100g dextrose, 10g peptone, 2g KNO_3, 2g $NH_4H_2PO_4$, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$ in 1000 ml distilled water; adjusted to pH 6.0). These cultures were incubated at 30°C for 48 hrs in an orbital shaker incubator (Metrex Scientific Instruments, New Delhi, India) at 110-rpm (Su *et al*., 2003, Sayyad *et al*., 2007). Finally *Monascus* seed / inoculums developed in seed culture medium was added to solid medium for solid-state fermentation.

2.3 Solid-state fermentation

Long-grain, non-glutinous rice was purchased from the local market of New Delhi, India, and was used as base solid substrate for *Monascus* red pigment production under solid-state culture. Initially 20 g of pre-soaked rice (100g of clean and dry rice soaked with 150 ml of distilled water for 8 hrs) was taken in a 250 ml conical flask to which 40 ml of distilled water containing different nutrients (as per the experimental designs, Table 1) was added, pH of the medium was adjusted to 6.0 with 0.1M HCl or NaOH and autoclaved for 20 min at 121°C. After being cooled the rice based solid medium was inoculated with 10% *Monascus* seed and incubated at 30°C, 70% relative humidity for 14 d in a humidity chamber for solid-state fermentation (Su *et al*., 2003).

2.4 Design of experiment

Experimental design for solid-state fermentation was formulated according to central composite design (central rotatory) tool of RSM using software Design Expert 7.1.3 (Stat-Ease Inc., USA) for selected five nutrient parameters (dextrose, peptone, NH_4Cl, $MnSO_4.H_2O$ and malt extract) screened by Plackett-Burman experimental design from nine parameters (dextrose, peptone, glycerin, NaCl, NH_4Cl, Mg $SO_4.7H_2O$, $CaCl_2.2H_2O$, $MnSO_4.H_2O$, malt extract) including two dummy factors (dummy 1 and dummy 2). The various levels of nutrients are summarized in Table 2. Relative effects of two variables on response to red pigment were identified from contour plots. An optimum value of the factors for maximum production of red pigment was determined by point prediction tool of Design Expert 7.1.3 software.

2.5 Extraction and estimation of *Monascus* red pigment

Fermented red "mold" rice (10g) was taken in a 100 ml conical flask, dissolved with 20 ml of acetone and kept in an orbital shaker (100 rpm) for 15 min at 25°C. The upper acetone layer was filtered through Whatman filter paper. The filtrate was centrifuged at 2500 g for 10 min. The resultant supernatant was collected filtered through 0.45 mm syringe filter. Finally clear filtrate was analyzed by spectrophotometer at $_{max}$ of 500 nm (Johns and Stuart, 1991; Lin and Demain, 1992; Babitha *et al*., 2007a; Pattanagul *et al*., 2008) for quantification of red pigment in fermented red "mold" rice with reference to a standard *Monascus* red pigment (YKS natural pigment factory, China) solution in acetone. The quantity of red pigment is expressed in terms of milligram per gram wet fermented red rice.

Table 1. Levels of nutrient parameters used in experiment

Nutrient parameter (g/l)	Levels				
	-2	-1	0	+1	+2
Dextrose	40	60	80	100	120
Peptone	1	3	5	7	9
NH_4Cl	0	3	6	9	12
$MnSO_4.H_2O$	0.0	0.3	0.6	0.9	1.2
Malt extract	5	10	15	20	25

Table 2. Influence of medium variables on pigment production from Plackett-Burman studies

Variable	MeanSquare	Effect	F	% Contribution
Dextrose	0.685	2.869	38.055	13.393
Peptone	0.821	-3.140	45.611	16.042
Glycerine	0.000	-0.103	0.000	0.017
NaCl	0.042	0.715	2.333	0.832
NH_4Cl	1.171	3.375	65.055	22.871
$MgSO_4.7H_2O$	0.182	-1.478	10.111	3.556
Dummy 1	0.000	-0.035	-	0.002
$CaCl_2.2H_2O$	0.356	-2.069	19.777	6.967
$MnSO_4.H_2O$	1.055	3.559	58.6111	20.604
Malt Extract	0.768	3.036	42.666	14.99
Dummy 2	0.036	0.666	-	10.721

3. Results

To identify the concentration of key nutrients influencing *Monascus* red pigment production, a flask method was used. From Plackett-Burman studies, out of total 11 parameters (09 nutritional and 02 dummy parameters) only 5 nutrient parameters greatly influenced the red pigment biosynthesis (Table 2). Five medium components (dextrose, peptone, NH_4Cl, $MnSO_4.H_2O$ and malt extract) screened by Plackett-Burman experimental design were selected for optimization study. An experimental design of 32 runs containing 6 central points was made according to central composite design (central rotatory) of response surface methodology for five selected medium parameters. The individual and interactive effects of these nutrient variables were studied by conducting experimental fermentation run at different levels (Table 1) of all five parameters. The fermentation response was measured in terms of red pigment production in solid medium after the fermentation process was over. The results of experimental data and simulated values are listed in Table 3. Data collected for red pigment concentration in each experimental fermentation run were analyzed using the software Design Expert 7.1.3 and fitted into a multiple nonlinear regression. The resulting model proposes the following equation (in coded factors) for red pigment production,

$$\text{Red pigment (mg/g)} = 12.10 + 0.82B - 1.19A^2 - 1.56B^2 - 1.09C^2 - 1.27D^2 - 1.86E^2$$

Where A, B, C, D, and E represent dextrose, peptone, NH_4Cl, $MnSO_4.H_2O$ and malt extract respectively in gram per liter of liquid medium added to solid rice medium. In the given equation B, A^2, B^2, C^2, D^2, E^2 are significant model terms (p-values greater than 0.1000 indicates the model terms are not significant).

This model resulted in ten response surface graphs. A few response surface plots of calculated model for red pigment production are shown in Figure 1 (a-d). The analysis of variance of regression for red pigment production by *Monascus purpureus* MTCC 369 under solid-state fermentation is summarized in Table 4. All the response surfaces / contour were analyzed to determine the optimized value of the each nutrient factors by point prediction tool of Design Expert 7.1.3 software (Stat-Ease Inc., USA) for maximum red pigment production. The optimum values of dextrose, peptone, NH_4Cl, $MnSO_4.H_2O$ and malt extract were determined at 74.59 g/l, 5.54 g/l, 6.57 g/l, 0.58 g/l and 14.46 g/l respectively. Solid-state fermentation under optimum values predicts 12.43 mg red pigment production per gram of pre- soaked rice based solid medium. These optimized values of nutrient parameters were validated by further fermentation study in duplicate and an average of 11.80 mg/g red pigment production was obtained with 95.59% validity of the predicted model with optical density (OD) of 27.4 per gram of wet fermented red rice at 500 nm.

4. Discussion

Amalgamation of Plackett-Burman studies and response surface methodology proved to be a powerful tool for screening and optimization of fermentation medium parameters for red pigment biosynthesis by *M. purpureus* MTCC 369 under solid-state fermentation. Dextrose was found to be useful carbon source followed by glycerin. Among the entire nitrogen sources screened, NH_4Cl was found to be best, followed by peptone and malt extract, and contributed highly towards red pigment synthesis. This is possibly due to the growth of *M. purpureus* being largely dependent on one type of nitrogen source (Miyake *et al*., 2006). Moreover, NH_4Cl contributes highly towards the biosynthesis of lovastatin (Sayyad *et al*., 2007). This suggests that secondary metabolite synthesis by *M. purpureus* MTCC 369 depends on NH_4Cl concentration. In fungal nutrition magnesium and calcium are noted as macronutrients and manganese, iron, copper and zinc as micronutrient but in the case of red pigment biosyn-

Table 3. Central Composite Design with results (actual and predicted)

Std Run	Dextrose (g/l)	Peptone (g/l)	NH_4Cl (g/l)	$MnSO_4.H_2O$ (g/l)	Malt Extract (g/l)	Red Pigment (mg/l)	
						Actual	Predicted
1	60.00	3.00	3.00	0.30	20.00	4.00	4.56
2	100.00	3.00	3.00	0.30	10.00	3.16	3.62
3	60.00	7.00	3.00	0.30	10.00	7.88	7.57
4	100.00	7.00	3.00	0.30	20.00	3.30	3.40
5	60.00	3.00	9.00	0.30	10.00	6.62	6.93
6	100.00	3.00	9.00	0.30	20.00	3.48	4.20
7	60.00	7.00	9.00	0.30	20.00	7.09	7.04
8	100.00	7.00	9.00	0.30	10.00	4.58	4.43
9	60.00	3.00	3.00	0.90	10.00	4.29	5.06
10	100.00	3.00	3.00	0.90	20.00	2.46	3.65
11	60.00	7.00	3.00	0.90	20.00	3.97	4.38
12	100.00	7.00	3.00	0.90	10.00	5.38	5.70
13	60.00	3.00	9.00	0.90	20.00	1.82	2.85
14	100.00	3.00	9.00	0.90	10.00	2.59	3.53
15	60.00	7.00	9.00	0.90	10.00	6.94	7.10
16	100.00	7.00	9.00	0.90	20.00	7.28	7.85
17	40.00	5.00	6.00	0.60	15.00	9.02	8.46
18	120.00	5.00	6.00	0.60	15.00	7.38	6.18
19	80.00	1.00	6.00	0.60	15.00	6.32	4.21
20	80.00	9.00	6.00	0.60	15.00	7.12	7.47
21	80.00	5.00	0.00	0.60	15.00	7.84	6.97
22	80.00	5.00	12.00	0.60	15.00	9.35	8.47
23	80.00	5.00	6.00	0.00	15.00	7.17	7.22
24	80.00	5.00	6.00	1.20	15.00	8.61	6.81
25	80.00	5.00	6.00	0.60	5.00	5.79	5.42
26	80.00	5.00	6.00	0.60	25.00	5.31	3.92
27	80.00	5.00	6.00	0.60	15.00	13.72	12.10
28	80.00	5.00	6.00	0.60	15.00	12.00	12.10
29	80.00	5.00	6.00	0.60	15.00	12.23	12.10
30	80.00	5.00	6.00	0.60	15.00	11.32	12.10
31	80.00	5.00	6.00	0.60	15.00	10.23	12.10
32	80.00	5.00	6.00	0.60	15.00	11.32	12.10

thesis by *M. purpureus*, micronutrient like manganese contribution was higher than some of the macronutrients like calcium and magnesium. This may be due to manganese, acting as cofactor for different enzymes required for pigment biosynthesis (Yu *et al*., 1997). Among all the screened nutrient parameters peptone and NH_4Cl were significant positive factors, whereas dextrose, $MnSO_4.H_2O$ and malt extract were significant negative factors.

The proposed model equation illustrates the interaction between two factors. From the quadratic equation it was found that NH_4Cl, $MnSO_4.H_2O$ and malt extract interacted positively with dextrose and peptone, while NH_4Cl and $MnSO_4.H_2O$ showed positive interaction with malt extract and dextrose and NH_4Cl showed negative interaction with peptone and $MnSO_4.H_2O$ respectively. The relative effect of medium components on red pigment production while keeping other parameter constant is depicted in the contour (two dimensional) and response surface (three dimensional) graphs. Remarkably from experimental runs it was found that the optimum level of nutrients remained around the initial search level with minor deviation.

The fungus *Monascus purpureus* MTCC 369 produced a maximum of 11.80 mg of red pigment with OD of 27.4 (per gram of red rice) of optimized solid fermentation media, 20g pre-soaked rice and 40 ml distilled water containing dextrose 74.59 g/l, peptone 5.54 g/l, NH_4Cl 6.57 g/l, $MnSO_4.H_2O$ 0.58 g/l and malt extract 14.46 g/l. The produced red pigment level in fermented red rice is higher than the red pigment produced using corn cob, adlay, jackfruit seed, corn meal (unsupplemented with glucose) as substrates which are 25.42 OD Units,

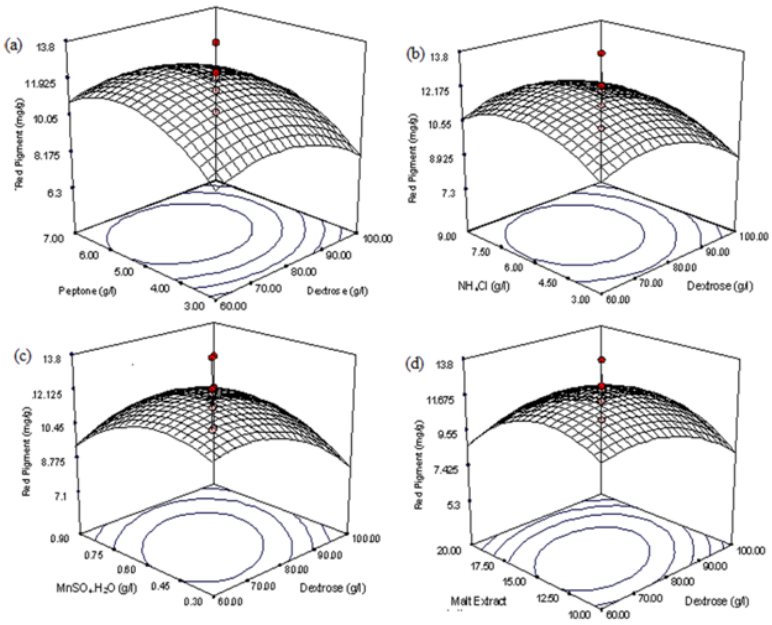

Figure 1. Response surface graph showing relative effect of two nutrient parameters on red pigment production while keeping others at constant level (a, b, c and d).

Table 4. Analysis of variance and summary of calculated model for red pigment production

Regression	
Sum of squares	278.92
df	20
Mean square	13.95
F- value	5.8
p- value	0.0023
Residuals	
Sum of squares	18.13
df	6
Mean square	3.02
Correlation coefficient (R^2)	0.9958

3.43 Units, 25 OD Units and 19.4 OD Units per gram of fermented substrate respectively (Babitha *et al.,* 2007b; Pattanagul *et al.,* 2008; Velmurugan *et al.,* 2011; Nimnoi and Lumyong, 2011). Our study proves that the rice supplemented with glucose is the best medium for red pigment production by *M. purpureus* MTCC 369 under solid- state fermentation conditions.

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