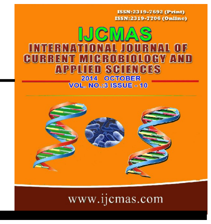

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Original Research Article

Evaluation of pH, carbon source and temperature effect on the pigments production by *Monascus purpureus* in a liquid culture using response surface methodology

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A B S T R A C T

Angkak (red mold rice, red yeast rice, Chinese red rice), a traditional Chinese functional food is used as a natural dietary supplement. The secondary metabolite of Monascus species, pigments are widely used in food and biotechnology as natural colorants, flavors and Preservatives. *Monascus* fungi produce pigments which can be categorized into 3 groups based on color as follows: yellow pigments, orange pigments and red pigments. There are various factors influencing the pigments productions. In this study, the combined effects of pH, temperature and carbon source on pigments production were evaluated in a liquid culture of *Monascus purpureus ATCC 1603*. Response surface design was used to optimize each parameter. The data were analyzed using Minitab 14 software. pH value, carbohydrates concentration and temperature screened using response surface methodology for high pigments production in liquid culture system. The treatments were nine pH, four temperature and eleven carbon source levels of three different carbohydrates (glucose, maltose and sucrose). The highest production of pigments was reached with a pH value of 3, using maltose with 250g.l^{-1} concentration and at temperature of 25 °C.

Keywords

Monascus purpureus; pigments; Response surface methodology; Carbon source; Temperature

Introduction

After the *Monascus* genus was named by van Tieghem (1884) over 100 years ago, the fungus became known to Westerners as contaminants of cereals, starch, silage and other materials (Tzann and Arnold, 1991).

Monascus purpureus, *Monascus ruber* and *Monascus pilosus* are three traditional fermentation fungi used in food for thousands of years in East Asia (Hawksworth and Pitt 1983; Shen Sun *et al.*, 2008). *Monascus* spp. produce valuable metabolites, including red pigments, γ-aminobutyric acid, and monacolin K, which are used as colorants, medicinals, and health supplements. Many studies have focused on better understanding the mechanisms responsible for the biosynthesis of these metabolites (Yoshizaki *et al.,* 2010). *Monascus* fungi produce at least six major related pigments which can be categorized into 3 groups based on color as follows: yellow pigments: monascin ($C_{21} H_{26} O_5$) and ankaflavin ($C_{23} H_{30} O_5$); orange pigments: monascorubrin ($C_{23} H_{26} O_5$) and rubropunctatin ($C_{21} H_{22} O$); and red pigments: monascorubramine ($C_{23} H_{27} NO_4$) and rubropuntamine ($C_{21} H_{23} NO_4$) (Subhasree *et al.,* 2011). Pigments produced by *Monascus spp* have been used as a natural food colorant for fish, bean curd and wine (Tzann and Arnold, 1991). The "Angkak" has long been recognized as a folk medicine for improving food digestion and blood circulation and also for treatment of muscle bruising and dysentery. The manufacturing process for "Angkak" and its therapeutic applications are well documented in the ancient Chinese pharmacopoeia (Ben-Taso-Gum-Mu) (Panda *et al.,* 2009). Carbon source, nitrogen source and pH have been shown to influence pigment production by *Monascus purpureus* (Omamor *et al.,* 2008). Carbon and nitrogen are known to be some of the most essential elements required by fungi for their growth (Omamor *et al.,* 2008). The structure of pigments depends on type of substrate and other specific factors during culture such as pH, temperature and moisture content (Pattanagul *et al.,* 2000; Subhasree *et al.,* 2011). Among the most important variables affecting biotechnological processes, the pH and temperature are environmental conditions with a strong effect on the biosynthesis of metabolites such as pigments, so it is very important to control them in industrial bioprocesses (Mendez *et al.,* 2011). However, not all sources of carbon or nitrogen are good for growth of a particular fungus and some fungi may utilize one source of carbon better than the others (Omamor *et al.,* 2008). Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes (Subhagar *et al.,* 2009). RSM is very useful to test multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time (Shen Sun *et al.,* 2008).

The purpose of the present study was to determination of the best conditions for pigments production by *Monascus pupureus* ATCC 1603 using liquid cultured. Interaction between parameters could be identified by response surface design. RSM, an experimental strategy was used for finding the optimum combination of mixed substrate (pH, temperature, glucose, maltose and sucrose) for pigments production by *Monascus purpureus*.

Materials and Methods

Microorganism

Monascus purpureus ATCC 1603 was purchased from the Leibniz Institute DSMZ German Collection of Micr oorganisms and cell cultures (DSMZ, Germa ny). *Monascus purpures* was maintained on the PDA (Merck, Germany) slants at 4 ˚C, and cultured at 37˚C for 10 days were used for inoculum preparation.

Culture conditions and inoculum preparation

Three carbon sources (glucose, maltose and sucrose) were screened. Ten day-old PDA pure cultures of *M. purpureus*. (1 pool standard) were used for inoculation of conical flasks containing the fermentation medium: 4% of glucose, 1% of yeast extract and 0.1% of KH_2PO_4 in 30 mL of distilled water, adjusted to pH 6. These cultures were incubated at 25˚C for 48 h in a shaking incubator at 100 rpm. The basal medium consisted of: 0.15% of NH_4CL, 0.1% of KH_2PO_4, 0.05% of $MgSO_4·7H_2O$, 0.05% of NaCl, 0.01% of $FeSO_4·7H_2O$ and 150 ml of deionized water. The contents of the flasks were mixed and autoclaved at 121˚C at 15 psi for 20min. After inoculation (7.5%, v/v), the fermentation was carried out for 14 days in a shaking incubator at 150 rpm.

Pigment estimation

Pigment concentration was estimated using a spectrophotometer (Cecil 2010UV-visible) set at 400 nm for yellow pigment, 460 for orange pigment and 500 nm for red pigment. The results are expressed as optical density units per gram of dried medium multiplied by dilution factor (Lin and Iizuka, 1982; Lee *et al.*, 2007).

Experimental design

So that the optimum conditions, a response surface design was selected. The involved crucial factors are temperature (X_1), pH (X_2), maltose concentration (X_3), glucose concentration (X_4) and sucrose concentration (X_5). All evaluated factors, and their level at which the experiments were carried out, are given in Table 1.

The low and high levels were coded as -1 and +1, the middle level was coded as 0. A total of 33 runs with 7 central points were generated. The central point of the design arrangement decided on was: temperature 25˚C, concentration of each carbon source (maltose, glucose and sucrose) 125 $g.l^{-1}$ and pH 5.

Response surface methodology

The data were analyzed using Minitab 14 software (Minitab Inc., USA). The quadratic model for predicting the optimal point was express as follow:

$$Y=b_\circ+ b_1 x_1+ b_2 x_2+ b_3 x_3+ b_4 x_4+ b_5 x_5+ b_{11} x_1^2+ b_{22} x_2^2+ b_{33} x_3^2+ b_{44} x_4^2+ b_{55} x_5^2+ b_{12} x_1. x_2+ b_{13} x_1. x_3+ b_{14} x_1. x_4+ b_{15} x_1. x_5 + b_{23} x_2. x_3+ b_{24} x_2. x_4+ b_{25} x_2. x_5+ b_{34} x_3. x_4+ b_{35} x_3. x_5+ b_{45} x_4. x_5$$

where Y is the amount of pigment produced (mg/g dry substrate), and x_1, x_2, x_3, x_4 and x_5 are input variables.

X_1, x_2, x_3, x_4, and x_5 indicate temperature, p H, maltose concentration, glucose concentrat ion and sucrose concentration, respectively. B_0 is a constant and B_1, B_2, B_3, B_4 and B_5 are linear coefficients. B_1, B_2, B_3, B_4, and B_5 ind icate temperature, pH, maltose concentratio, glucose concentration and sucrose concentration, respectively. B_{11}, B_{22}, B_{33}, B_{44} and B_{55} are quadratic coefficients and B_{15}, B_{23}, B_{24}, B_{25}, B_{34}, B_{35} and B_{14}, B_{12}, B_{13}, B_{45} are cross-product coefficients. B_{12}, B_{13}, B_{14}, B_{15}, B_{23}, B_{24}, B_{25}, B_{34}, B_{35}, and B_{45} indicate temperature. pH, temp altose, temperature.glucose, temperature.sucrose, p H.maltose, pH.glucose, pH.sucrose, maltose. glucose, maltose.sucrose, and clucose.sucrose, respectively (Figures 1,2,3).

Results and Discussion

Among the five factors used in the response surface design, maltose, glucose, sucrose, p H and temperature were used for pigment production. Table 2 shows the results of experimental data and simulated values. Multiple regression analysis of the response surface design for the pigments production:

Table.1 Levels of variable used in response surface design

		Coded-variable level		
variable	symbol	-1	0	1
Temprature(˚C)	X_1	25	32.5	40
pH value	X_2	3	5	7
Maltose concentration($g.l^{-1}$)	X_3	0	125	250
Glucose concentration($g.l^{-1}$)	X_4	0	125	250
Sucrose concentration($g.l^{-1}$)	X_5	0	125	250

Table.2 Response surface design with actual and predicted pigments production

The analysis of variance of regression for pigment production was summarized in Table 3. The r^2 values of Red pigment level, Orange pigment level, and Yellow pigment level were 0.95, 0.95,

Run	Block	Code levels					Pigment production (UA400)		Pigment production (UA460)		Pigment production (UA500)	
		X_1	X_2	X_3	X_4	X_5	Actual	predicted	Actual	predicted	Actual	predicted
1	1	1	-1	-1	-1	-1	2.562	2.498	2.982	2.869	2.547	2.454
2	1	-1	-1	-1	-1	1	0.131	0.165	0.070	0.052	0.040	0.011
3	1	-1	-1	-1	1	-1	0.162	0.234	0.086	0.098	0.056	0.050
4	1	1	-1	-1	1	1	0.097	0.191	0.047	0.101	0.043	0.060
5	1	-1	-1	1	-1	-1	3.980	3.870	3.300	3.188	1.488	1.425
6	1	1	-1	1	-1	1	0.302	0.214	0.141	0.071	0.095	0.055
7	1	1	-1	1	1	-1	0.543	0.493	0.285	0.245	0.183	0.165
8	1	-1	-1	1	1	1	0.362	0.411	0.228	0.283	0.121	0.167
9	1	-1	1	-1	-1	-1	1.174	1.161	0.719	0.689	0.405	0.378
10	1	1	1	-1	-1	1	0.158	0.167	0.068	0.081	0.057	0.053
11	1	1	1	-1	1	-1	0.382	0.429	0.203	0.245	0.144	0.163
12	1	-1	1	-1	1	1	0.326	0.472	0.153	0.291	0.106	0.189
13	1	1	1	1	-1	-1	0.220	0.085	0.128	0.046	0.088	0.050
14	1	-1	1	1	-1	1	0.117	0.080	0.063	0.077	0.044	0.070
15	1	-1	1	1	1	-1	0.452	0.453	0.262	0.305	0.172	0.220
16	1	1	1	1	1	1	0.515	0.539	0.236	0.321	0.155	0.226
17	1	0	0	0	0	0	0.674	0.642	0.288	0.274	0.184	0.170
18	1	0	0	0	0	0	0.724	0.642	0.359	0.274	0.231	0.170
19	1	0	0	0	0	0	0.868	0.642	0.345	0.274	0.201	0.170
20	1	0	0	0	0	0	0.731	0.642	0.321	0.274	0.187	0.170
21	1	0	0	0	0	0	0.352	0.642	0.136	0.274	0.098	0.170
22	1	0	0	0	0	0	0.480	0.642	0.185	0.274	0.111	0.170
23	2	0	0	0	0	-1	0.595	0.847	0.336	0.616	0.223	0.401
24	2	0	0	0	0	1	0.205	- 0.026	0.086	- 0.185	0.062	- 0.108
25	2	0	0	0	-1	0	0.427	0.830	0.158	0.556	0.092	0.359
26	2	0	0	0	1	0	0.586	0.203	0.297	- 0.092	0.212	- 0.048
27	2	0	0	-1	0	0	0.833	0.508	0.316	0.219	0.157	0.197
28	2	0	0	1	0	0	0.266	0.611	0.126	0.232	0.108	0.075
29	2	0	-1	0	0	0	0.782	0.845	0.314	0.547	0.161	0.346
30	2	0	1	0	0	0	0.301	0.259	0.164	-0.060	0.144	- 0.034
31	2	-1	0	0	0	0	0.833	0.691	0.482	0.380	0.207	0.129
32	2	1	0	0	0	0	0.250	0.412	0.143	0.254	0.133	0.218
33	2	0	0	0	0	0	0.605	0.503	0.235	0.189	0.152	0.116

Table.3 ANOVA for response surface design during Pigments production by *M. purpureus*

Source	Df[a]	Sum of square		
		Red pigment (A500/g)	Orange pigment (A460/g)	Yellow pigment (A400/g)
Regression	20	6.83131	15.4670	16.7207
Linear	5	2.66557	6.5030	7.1447
Square	5	0.15818	0.3738	0.0559
Interaction	10	4.00757	8.5903	9.5201
Residual	11	0.33192	0.7292	0.9788
Lack of fit	6	0.31810	0.6876	0.8018
Pure error	5	0.01382	0.0416	0.1770
Variability explain(r^2)		0.955	0.956	0.945

[a] df: degree of freedom.

Figure.1 Response surface plots showing relative effects of two parameters on yellow, orange and red pigments production while keeping others at constant levels

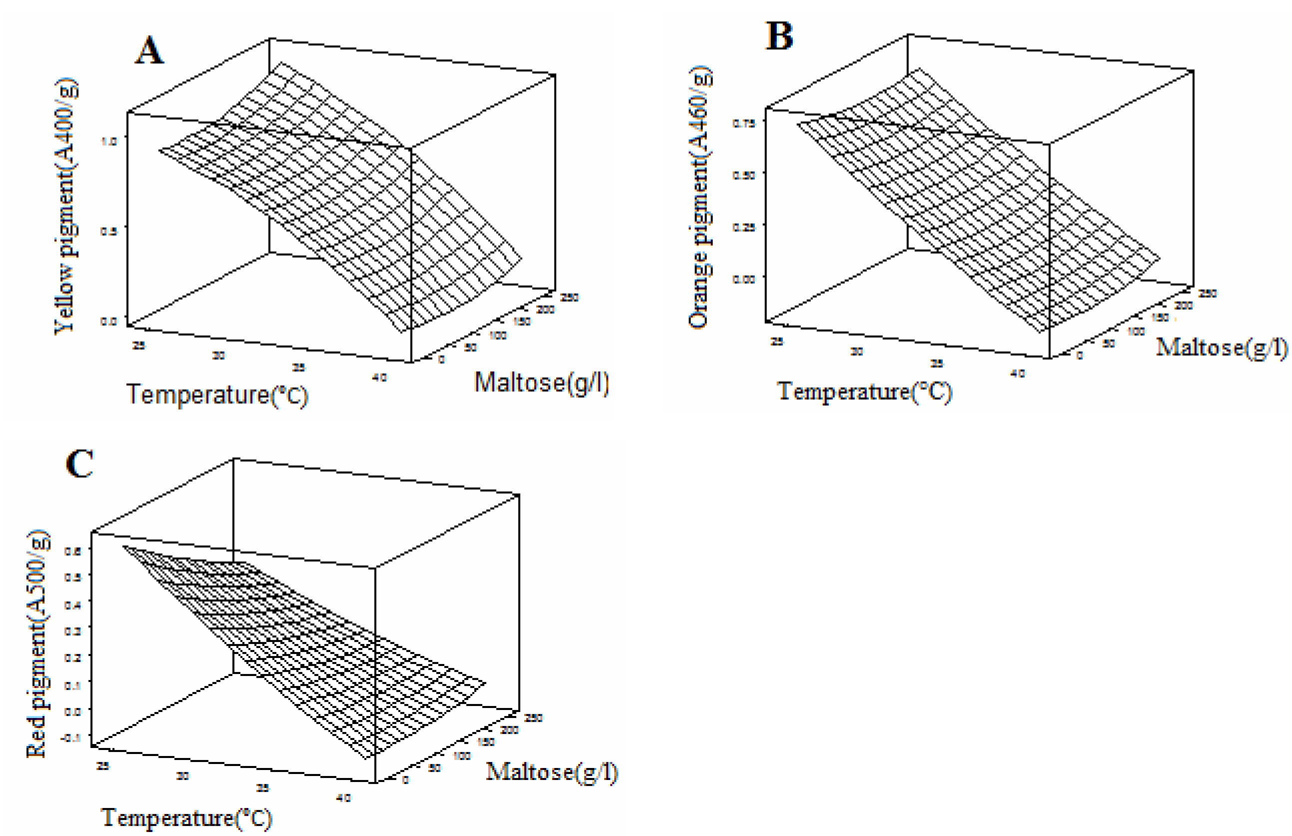


Figure.2 Response surface plots showing relative effects of two parameters on yellow, orange and red pigments production while keeping others at constant levels

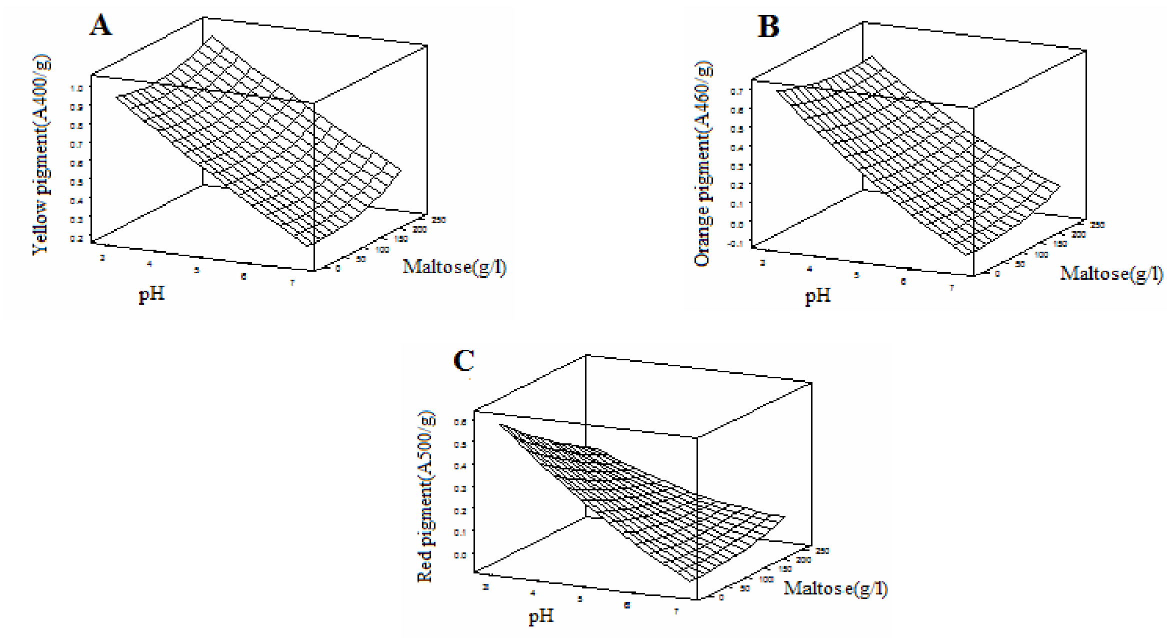
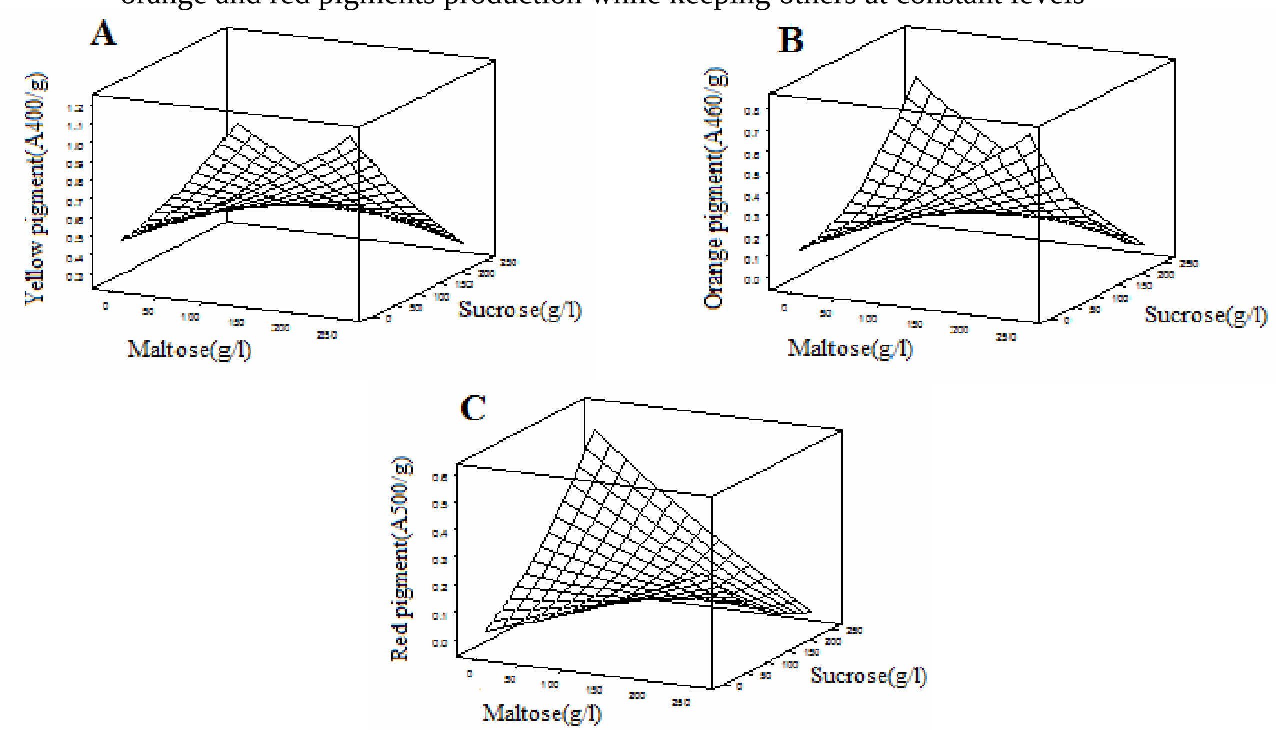


Figure.**3** Response surface plots showing relative effects of two parameters on yellow, orange and red pigments production while keeping others at constant levels



Yellow Pigment production (A400/g): $2.749 - 0.699 x_1 - 2.758 x_2 + 0.841 x_3 - 4.658 x_4 - 1.697 x_5 - 0.487 x_1^2 + 0.072 x_2^2 + 0.297 x_3^2 + 0.255 x_4^2 + 0.255 x_5^2 + 5.857 x_1 . x_2 - 0.277 x_1 . x_3 + 4.394 x_1 . x_4 + 1.841 x_1 . x_5 + 0.261 x_2 . x_3 + 4.875 x_2 . x_4 + 2.007 x_2 . x_5 - 2.490 x_3 . x_4 - 3.970 x_3 . x_5 + 0.282 x_4 . x_5$

Orange pigment production (A460/g): $3.646 - 1.432 x_1 - 3.171 x_2 + 0.290 x_3 - 5.627 x_4 - 0.639 x_5 + 0.158 x_1^2 + 0.258 x_2^2 + 0.219 x_3^2 + 0.328 x_4^2 + 0.775 x_5^2 + 6.423 x_1 . x_2 + 0.335 x_1 . x_3 + 5.219 x_1 . x_4 + 0.725 x_1 . x_5 + 0.708 x_2 . x_3 + 5.557 x_2 . x_4 + 0.850 x_2 . x_5 - 1.187 x_3 . x_4 - 5.180 x_3 . x_5 - 0.323 x_4 . x_5$

Red pigment production (A500/g): $3.560 - 1.465 x_1 - 3.069 x_2 - 1.712 x_3 - 5.497 x_4 + 1.786 x_5 + 0.273 x_1^2 + 0.359 x_2^2 + 0.183 x_3^2 + 0.363 x_4^2 + 0.521 x_5^2 + 5.990 x_1 . x_2 + 2 x_1 . x_3 + 5.077 x_1 . x_4 - 1.154 x_1 . x_5 + 2.326 x_2 . x_3 + 5.397 x_2 . x_4 - 1.065 x_2 . x_5 + 0.786 x_3 . x_4 - 5.020 x_3 . x_5 - 2.081 x_4 . x_5$

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