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PRODUCTION OF ANGKAK THROUGH CO-CULTURE OF *MONASCUS PURPUREUS* AND *MONASCUS RUBER*

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ABSTRACT

Angkak (red mold rice, red yeast rice, Chinese red rice) is a traditional Chinese medicine produced by solid-state fermentation of cooked non-glutinous rice with *Monascus* species. The secondary metabolite of *Monascus* species, monacolin K /lovastatin, has been proven to lower blood lipid levels. In this study, a co-culture of *Monascus purpureus* MTCC 369 and *Monascus ruber* MTCC 1880 was used for angkak production. Four medium parameters screened by Plackett-Burman design were optimized by response surface methodology for highest lovastatin production in angkak during solid-state fermentation by the co-culture. Maximum lovastatin production of 2.84 mg g^{-1} was predicted in solid medium containing 20 g rice and 40 ml liquid nutrients medium (malt extract 9.68 g l^{-1}, dextrose 38.90 g l^{-1}, $MnSO_4.H_2O$ 1.96 g l^{-1}, and $MgSO_4.7H_2O$ 0.730 g l^{-1}) by point prediction tool of Design Expert 7.1 software (Statease Inc. USA).

Key words: Angkak. Co-culture. *Monascus purpureus*. *Monascus ruber*. Lovastatin. Response surface methodology

INTRODUCTION

Angkak (red mold rice, red yeast rice, Chinese red rice) is a traditional Chinese medicine produced by solid-state fermentation of cooked non-glutinous rice (*Orizae sativa* L. Gramineae) with *Monascus purpureus*, *M. ruber, M. anka* and *M. pilosus* (3, 6, 9, 14). The angkak has long been recognized as a folk medicine for improving food digestion and blood circulation and 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) (9, 12). Recent chemical investigation and clinical observation show that angkak contains different secondary metabolites: lovastatin (HMG Co A reductatse inhibitor), also known as monacolin K, which lowers blood lipid levels in animal models and in humans, γ-aminobutyric acid (GABA) which has blood pressure lowering effects, dimerumic acid which is antioxidant, and monascin which has anti-inflammatory effects (6,10,11,24).

A previous study on red mold rice production by *Monascus* species under monoculture conditions showed that secondary metabolites production is greatly affected by fermentation medium, cultivation conditions and types of *Monascus* species used in the fermentation process (1, 5, 13, 21, 23). Increasing concentration of lovastatin in angkak was a prime area of research in order to produce high quality bio-

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nutraceutical or health functional food (4, 11, 22). During process optimization, screening or selection of important parameters influencing fermentation productivity is initially carried out by barrowing method or by Plackett-Burman design, and finally the screened parameters are optimized using different advanced statistical techniques (3, 7, 8, 15). Response surface methodology (RSM) is a widely used technique for optimization of fermentation process parameters. RSM based on Box-Behnken's design has some advantages over other designs like central composite and full factorial design. RSM requires less experiment runs, and is suitable for multiple factor experiments, search for relationships between factors, and for finding the most suitable condition and prediction of response (8, 19).

In nature, solid-state fermentation (SSF) is carried out by mixed or co-cultures of different fungal species. The co-culture of fungi during fermentation may provide help for better biomass and secondary metabolites production. There are several reports of co-culture of fungal species, and mixed cultures have been found to enhance enzyme and organic acid production (2, 16). However, no reports are available on production of lovastatin (monacolin K) by co-culture of different *Monascus* species under SSF condition. Therefore, the objective of this study was to produce finest quality angkak containing maximum amount of lovastatin by co-culture of two *Monascus* species.

Two filamentous fungi, *Monascus purpureus* MTCC 369 and *Monascus ruber* MTCC 1880, were used together as inoculum for production of angkak. The effects of medium parameters on lovastatin production were studied and screened by Plackett-Burman design (PBD) and their optimum levels were determined by response surface methodology (RSM).

MATERIALS AND METHODS

Chemicals and raw materials

Pure lovastatin was a gift from Ranbaxy Laboratories (New Delhi, India). HPLC grade acetonitrile was purchased from Merck (Bombay, India). Microbiological media and chemicals were purchased from Himedia Laboratories (Bombay, India) and long nonglutinous rice was purchased from a local market of New Delhi, India.

Microorganisms

Cultures of *Monascus purpureus* MTCC 369 and *Monascus ruber* MTCC 1880 were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. Fungal cultures were maintained routinely on potato dextrose agar (PDA) medium and subcultured in every 30 d interval (3, 19).

Preparation of mixed seed cultures

Spore suspensions of *M. purpureus* and *M. ruber* were prepared separately from actively growing slants in sterile water and diluted to a concentration 5.7×10^3 spores ml^{-1}. Spore counting was carried out using a hemocytometer. Spore suspension (15% v/v) (7.5ml) of *M. purpureus* was inoculated to conical flasks containing 50ml 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) and incubated at 30°C for 48 h in a shaker incubator at 110 rpm (19,21). For preparation of *Monascus ruber* seed culture, 15% spore suspension (7.5ml) of *M. ruber* was inoculated to conical flasks containing 50ml of potato dextrose broth (PDB), and incubated at 30°C for 4 days with shaking at 150 rpm (3). Finally the two seed cultures of *M. purpureus* and *M. ruber* were mixed at a ratio of 1:1.

Solid -State Fermentation (SSF)

Long grain, non-glutinous rice was purchased from a local market of New Delhi, India and used as substrate for angkak production under solid-state culture. Initially 20 g of pre-soaked rice was transferred to a 250 ml conical flask to which 40 ml of distilled water containing different nutrients (Table 1) was added. The pH of the medium was adjusted to 6.0 with 0.1M HCl or NaOH and autoclaved for 20min at 121°C. The cooled rice-based medium was inoculated with 5ml of the mixed seed cultures of *M. purpureus* and *M. ruber*, incubated for 14 days at 30°C and 70% relative humidity (21).

Table 1. Concentrations of variables of liquid medium in Plackett-Burman design for solid-state fermentation.

Designation	Variable	Low level (-) Per liter	High level (+) Per liter
X_1	Peptone	5.0g	15.0g
X_2	Glucose	80g	160g
X_3	Glycerin	16ml	32ml
X_4	NaCl	2.0g	8.0g
X_5	NH_4Cl	2.0g	8.0g
D_1	Dummy 1	-	-
X_6	$MgSO_4.7H_2O$	0.1g	0.9g
X_7	$CaCl_2.2H_2O$	0.0g	0.6g
X_8	$MnSO_4.H_2O$	0.0g	1.5g
X_9	Malt extract	2.0g	8.0g
D_2	Dummy 2	-	-

Extraction of lovastatin from angkak

Angkak (1g) was suspended in 5 ml ethyl acetate and kept in a shaker incubator at 180 rpm and 70°C for 1.5 h. The mixture was centrifuged at 3000g for 8 min and 10 ml of 1% (v/v) trifluoroacetic acid was added to 1ml of the supernatant for lactonization of the lovastatin. The mixture was concentrated at 80°C, mixed with 1ml with acetonitrile and filtered through 0.45µm filter for HPLC (High Performance Liquid Chromatography) analysis (21).

Quantitative analysis of lovastatin

For HPLC analysis the procedure of Samiee *et al.* for HPLC, with slight modification, was used. Lovastatin concentration was estimated by HPLC (SHIMADZU, Japan) using a 250 mm x 4.6 mm ID Lichrosper® 100 C_{18} column containing 5µm sized particles and a 20µl loop injector. The mobile phase was acetonitrile:water acidified to the concentration 0.1% (v/v) with *ortho*-phosphoric acid (65:35 v/v). The flow rate was 1.5 ml min^{-1}. Detection was carried out by a UV-detector at 235 nm (18, 19).

Screening of nutrient parameters

Peptone, glucose, glycerine, NaCl, NH_4Cl, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, malt extract, and $MnSO_4.H_2O$ were the nine medium constituents selected for study. The selection of nutrients was done by barrowing methodology (8, 15). The Plackett-Burman experimental design for eleven variables (Table 2) with nine nutritional components (X_1 to X_9) (independent variables) and two dummy variables (D_1 and D_2) were used to evaluate the relative importance of the nutrients for high quality angkak production with high quantity of monacolin K (lovastatin). For each nutrient variable two different concentrations [high (+) and a low (-)] were tested (Table 2). Influence of medium variables on lovastatin production was calculated according to standard analysis procedure of Plackett-Burman experimental design (17).

Table 2. Plackett-Burman experimental design of 12 trials for eleven variables (9 nutrients + 2 dummy) and observed concentration of lovastatin in angkak samples.

Trial	X_1	X_2	X_3	X_4	X_5	D_1	X_6	X_7	X_8	X	D_2	Mean lovastatin (mg g^{-1})
1	+	+	-	+	+	+	-	-	-	+	-	1.060
2	-	+	+	-	+	+	+	-	-	-	+	0.563
3	+	-	+	+	-	+	+	+	-	-	-	0.764
4	-	+	-	+	+	-	+	+	+	-	-	0.450
5	-	-	+	-	+	+	-	+	+	+	-	1.880
6	-	-	-	+	-	+	+	-	+	+	+	1.990
7	+	-	-	-	+	-	+	+	-	+	+	1.780
8	+	+	-	-	-	+	-	+	+	-	+	0.764
9	+	+	+	-	-	-	+	-	+	+	-	1.760
10	-	+	+	+	-	-	-	+	-	+	+	1.080
11	+	-	+	+	+	-	-	-	+	-	+	0.984
12	-	-	-	-	-	-	-	-	-	-	-	0.534

Response surface methodology experimental design

In Plackett-Burman experimental design, various carbon, nitrogen and micronutrients were evaluated for their influence in the production of lovastatin during solid-state fermentation by mixed cultures of *M. purpureus* and *M. ruber.* Preliminary data of Plackett-Burman experimental design indicated that lovastatin production was greatly influenced by malt extract, dextrose, $MnSO_4.H_2O$ and $MgSO_4.7H_2O$ (Table 3). Therefore these four medium parameters were chosen for further optimization by response surface methodology (3, 19, 20). The various levels of nutrients are summarized in Table 4. An experimental design of 29 runs containing 3 central points (Table 5) was made according to Box-Behnken's response surface design for selected four parameters using Design Expert 7.1 software of Statease Inc. USA. The response was measured in terms of lovastatin concentration in produced angkak. An optimum value of the factors for maximum production of lovastatin was determined by the point prediction tool of the software.

Table 3. Influence of medium variables on lovastatin production in angkak samples

Designation	Variable	∑H	∑L	Mean square	Effect	F Value	% Contribution	P value
X_1	Peptone	7.112	6.497	0.034	0.102	1.214	00.968	
X_2	Dextrose	5.677	7.932	0.421	-0.375	15.035	11.900	
X_3	Glycerine	7.031	6.578	0.016	0.075	0.571	00.454	
X_4	NaCl	6.328	7.281	0.076	-0.158	2.714	02.160	0.072
X_5	NH_4Cl	6.717	6.892	0.002	-0.029	0.071	00.061	
X_6	$MgSO_4.7H_2O$	7.307	6.302	0.085	0.167	3.035	02.400	
X_7	$CaCl_2.2H_2O$	6.718	6.891	0.002	-0.288	0.071	00.076	
X_8	$MnSO_4.H_2O$	7.828	5.781	0.345	0.341	12.321	09.710	
X_9	Malt extract	9.550	4.059	2.510	0.915	89.642	70.700	

Table 4. Levels of nutrient parameters used in Box-Behnken's response surface design

Nutrient parameter ($g\ l^{-1}$)	Levels		
	-1	0	+1
Malt extract	07.00	10.00	13.00
Dextrose	20.00	40.00	60.00
$MnSO_4.H_2O$	01.00	01.50	02.00
$MgSO4.7H_2O$	00.50	01.00	01.50

Table 5. Box-Behnken's design with actual and predicted lovastatin concentrations

Run	Malt extract ($g\ l^{-1}$) Code A	Dextrose ($g\ l^{-1}$) Code B	$MnSO_4.H_2O$ ($g\ l^{-1}$) Code C	$MgSO_4.7H_2O$ ($g\ l^{-1}$) Code D	Lovastatin ($mg\ g^{-1}$)	
					Actual	Predicted
1	7	20	1.5	1	1.58	1.44
2	13	20	1.5	1	0.612	0.27
3	7	60	1.5	1	0.756	0.784
4	13	60	1.5	1	0.986	0.812
5	10	40	1	0.5	2.68	2.27
6	10	40	2	0.5	2.45	1.93
7	10	40	1	1.5	0.764	0.966
8	10	40	2	1.5	0.875	0.97
9	7	40	1.5	0.5	2.58	2.49
10	13	40	1.5	0.5	0.765	1.01
11	7	40	1.5	1.5	0.657	0.446
12	13	40	1.5	1.5	0.654	0.78
13	10	20	1	1	0.435	0.668
14	10	60	1	1	1.78	1.86
15	10	20	2	1	1.78	1.75
16	10	60	2	1	0.645	0.447
17	7	40	1	1	1.76	1.78
18	13	40	1	1	0.784	0.667
19	7	40	2	1	0.674	1.07
20	13	40	2	1	0.783	1.04
21	10	20	1.5	0.5	1.56	1.95
22	10	60	1.5	0.5	1.24	1.62
23	10	20	1.5	1.5	0.645	0.543
24	10	60	1.5	1.5	0.873	0.763
25	10	40	1.5	1	2.65	2.63
26	10	40	1.5	1	2.56	2.63
27	10	40	1.5	1	2.67	2.63
28	10	40	1.5	1	2.65	2.63
29	10	40	1.5	1	2.63	2.63

RESULTS

Among the nine nutrient components used in the Plackett-Burman Experimental Design, malt extract, dextrose, $MnSO_4.H_2O$, and $MgSO_4.7H_2O$ had contributed to a large extent for lovastatin production. Peptone, glycerine, $CaCl_2.2H_2O$, and NH_4Cl had little impact, while NaCl contributed moderately in production of lovastatin in angkak under the co-culture system.

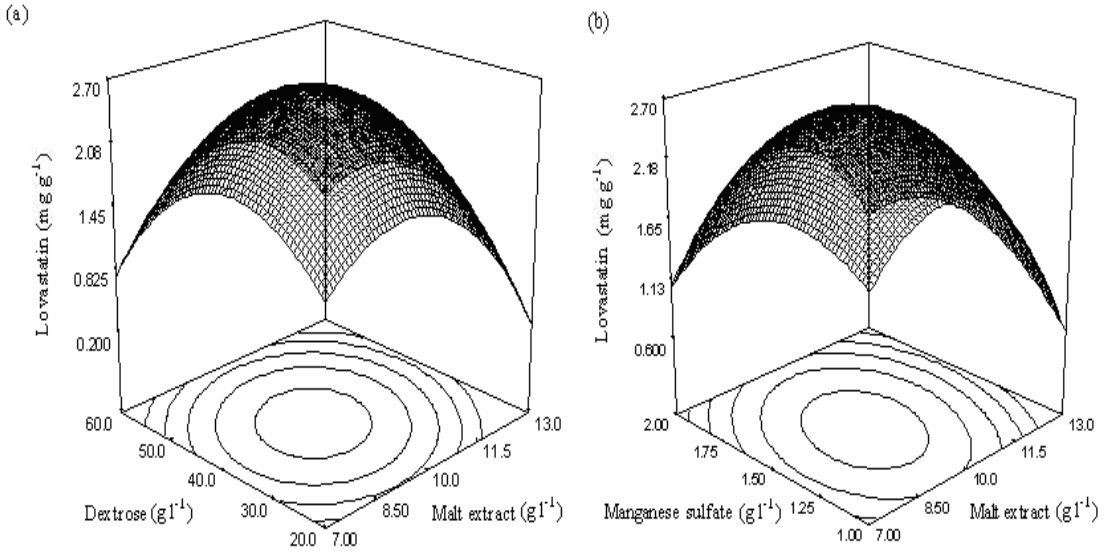
The predicted and experimental lovastatin concentrations obtained from Box-Behnken's response surface design in each run are show in Table 5. Results were analyzed using the software Design Expert 7.1 and fitted into a multiple nonlinear regression model, resulting in the following equation for lovastatin production:

Lovastatin (mg g^{-1}) = +2.63-0.285A-0.0275B-0.0832C-0.568D+0.299AB+0.272AC +0.452AD -0.622BC +0.137BD +0.0853CD -0.924A^2 -0.884B^2 -0.569C^2 -0.529D^2

This model resulted in six response graphs and a few representative surface graphs with contours of nonlinear regression model for lovastatin production, shown in Fig. 1a, 1b, 1c and 1d. Analysis of variance of regression is summarized in Table 6. The optimum values of medium parameters for maximum lovastatin production were malt extract 9.68 g l^{-1}, dextrose 38.90 g l^{-1}, $MnSO_4.H_2O$ 1.96 g l^{-1}, and $MgSO_4.7H_2O$ 0.730 g l^{-1}. These values predicted 2.84 mg g^{-1} of lovastatin production in angkak under co-culture of *M. purpureus* and *M. ruber*. These optimized values of nutrient parameters were validated by solid-state fermentation and an average 2.75 mg g^{-1} of lovastatin production in angkak was obtained, indicating a 96.83 % validity of the predicted model.

Table 6. Analysis of variance of the calculated model for lovastatin production

Regression	
Sum of squares	17.8
df	14
Mean squares	1.27
F Value	12.3
P	< 0.0001
Residual	
Sum of squares	1.45
df	14
Mean squares	0.103
Lack of fit test	
Sum of squares	1.44
df	10
Mean squares	0.144
F Value	81.4
P Value	0.000354
Correlation coefficient (r^2)	0.925
Coefficient of variation (CV %)	22.5
Adequate precision value	10.2 (> 4)



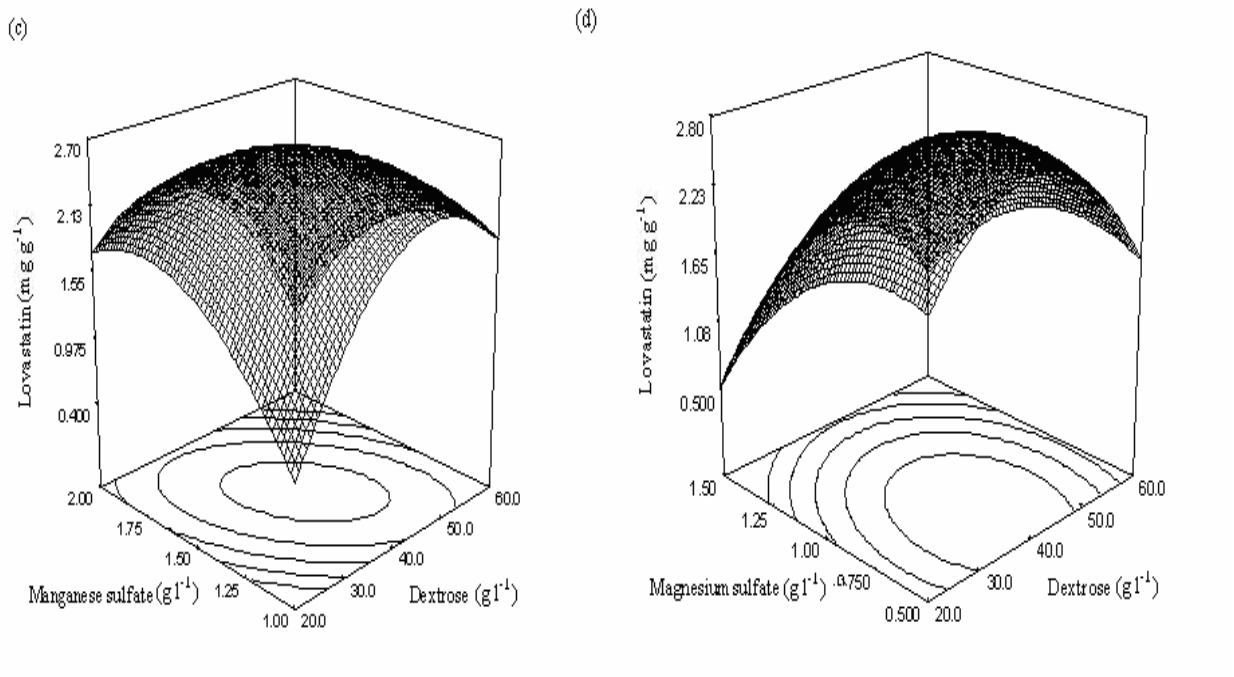


Figure 1. (a-d) Response surface plots showing relative effects of two nutrient parameters on lovastatin production while keeping other two parameters at constant levels.

DISCUSSION

Plackett-Burman experimental design and response surface methodology are powerful tools for screening and optimization of medium parameters for lovastatin production under solid-state fermentation by co-culture of *M. purpureus* and *M. ruber.*

Malt extract, dextrose, $MnSO_4.H_2O$, and $MgSO_4.7H_2O$ influenced negatively lovastatin production. The model equation indicated that all medium parameters except dextrose and $MnSO_4.H_2O$ interacted positively with each other in lovastatin production. The simultaneous effects of two medium parameters on lovastatin production while the other two parameters were kept at optimized levels was depicted in the contours and surface graphs. As shown in Figure 1, reducing malt extract, dextrose, $MgSO_4.7H_2O$ concentrations below the original search levels and increasing $MnSO_4.H_2O$ concentration above the original levels stimulated production of lovastatin under co-culture of *M. purpureus* and *M. ruber.*

Co-culture of the two *Monascus* species resulted in production of 2.75 mg of lovastatin per gram of optimized solid medium in angkak, which is much higher then the lovastatin or monacolin K concentration obtained under mono culture of *Monascus pilosus* M12-69, *Monascus purpureus* NTU 601, 301 or *Monascus purpureus* BCRC 31499, 31504, 31530, 31540, 32966, 32807, 32808, 32809 on different substrates (4). However the lovastatin / monacolin K production in dioscorea by *Monascus purpureus* NTU 601 was found to be equivalent to lovastatin produced by co-culture of *Monascus* species in rice (11). This suggests that co-culture or mixed cultures of different *Monascus* species might result in high quantity of lovastatin or monacolin K in angkak.

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