Engineering Rice Based Medium for Production of Lovastatin with *Monascus* Species

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Abstract

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Angkak (red mold rice, red yeast rice, Chinese red rice), a traditional Chinese functional food is produced by solid-state fermentation of cooked non-glutinous rice with *Monascus* species. The secondary metabolite of *Monascus* species, monacolin K (lovastatin), has been proved to lower blood lipid levels. In this study, the best *Monascus purpureus* strain was selected from MTCC 369, 410, 1090 based on lovastatin concentration. Four medium parameters (NH_4Cl, $MgSO_4$, NaCl, $CaCl_2$) screened by Plackett-Burman design from total nine medium variables were optimised by Box-Behnken design of response surface methodology. Maximum lovastatin production of 3.420 mg/g was predicted in the solid medium containing 20 g rice and 40 ml liquid nutrient (NH_4Cl 14.32 g/l, $MgSO_4$ 0.76 g/l, NaCl 14.65 g/l, and $CaCl_2$ 0.54 g/l) by the point prediction tool of Design Expert Ver. 7.1 software at 14^{th} day of fermentation.

Keywords: medium engineering; *Monascus* sp.; angkak; lovastatin

Angkak, a traditional Chinese functional food produced by solid state fermentation of cooked non-glutinous rice with *Monascus* sp., contains different high value secondary metabolites such as lovastatin, γ-aminobutyric acids (GABA), monascodilone, monascorubramine, monascin, ankaflavin, rubropunctatin (Miyake *et al*. 2005; Chiu *et al*. 2006; Lee *et al*. 2006a; Lin *et al*. 2008). One of the well-documented secondary metabolites of angkak is lovastatin, a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (Endo 1980; Chen & Hu 2005; Lee *et al.* 2006b), which is a regulatory and rate limiting enzyme of cholesterol biosynthesis.

Angkak is produced traditionally by fermenting washed and cooked non-glutinous rice combined with polygonum grass juice, red wine mesh, and alum water with *Monascus purpureus* under solid state fermentation. However, to the best of our knowledge no detailed modern medium engineering was studied regarding the influence of different nutrient parameters on the quality of angkak.

Therefore, the objective of this research was to produce by medium engineering finest-quality rice-based nutraceutical containing maximum amount of lovastatin. The initial screening of different important nutrient parameters and their contribution to the production of high quality *angkak* was done using Plackett-Burman tools (Plackett & Burman 1946; Naveena *et al.* 2005; Panda *et al.* 2007; Sayyad *et al.* 2007b) and was further optimised by the quadratic model developed by Box-Behnken's Design (Box & Hunter 1957; Chakravarti & Sahai 2002; Chang *et al.* 2006; Sayyad *et al.* 2007a).

MATERIALS AND METHODS

Microorganisms. The cultures of *Monascus purpureus* MTCC 369, *M. purpureus* MTCC 410 and *M. purpureus* MTCC 1090 were obtained from the Microbial Type Culture Collection & Gene Bank of the Institute of Microbial Technology (IMTECH), Chandigarh, India, were maintained on slants of potato dextrose agar medium at 4°C and subcultured in 30-day intervals (Sayyad *et al.* 2007a, b).

Preparation of seed cultures. Spore suspensions of *M. purpureus* MTCC 369, 410, 1090 were prepared from actively growing slants in sterile water and diluted to a concentration of 5.7×10^3 spores per ml. Spore counting was carried out using a hemocytometer. A total of 15% spore suspension was inoculated into conical flasks containing 50 ml of the basal medium (100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g $NH_4H_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$ in 1000 ml distilled water; adjusted to pH 6.0). These cultures were incubated at 30°C for 48 h in a shaker incubator at 110 rpm (Su *et al.* 2003; Sayyad *et al.* 2007a).

Selection of Monascus purpureus strain. Long grain, non-glutinous rice was purchased from a local market of New Delhi, India, and was used as the basic solid substrate for angkak production by means of solid-state culture. Initially, 20 g of pre-soaked rice was placed in a 250 ml conical flask to which 40 ml of distilled water was added, pH of the medium was adjusted to 6.0 with 0.1M HCl or NaOH and autoclaved at 121°C for 20 minutes. After being cooled, the rice based medium was inoculated with 5 ml of the seed culture of *M. purpureus* 369, 410, 1090, and incubated at 30°C and 70% relative humidity for 14 days (Su *et al.* 2003).

Solid-state fermentation for angkak production. Long grain, non-glutinous rice (Jagat, Perl 1) was purchased from a local market of New Delhi, India, and was used as the basic solid substrate for angkak production by means of solid-state culture. Initially, 20 g of pre-soaked rice was placed in a 250 ml conical flask to which 40 ml of distilled water containing different nutrients (as per the experimental designs Table 3) was added, pH of the medium was adjusted to 6.0 with 0.1M HCl or NaOH and autoclaved at 121°C for 20 minutes. After being cooled, the rice based medium was inoculated with 5 ml of the seed culture of the selected *M. purpureus* strain and incubated at 30°C and 70% relative humidity for 14 days (Su *et al.* 2003).

Plackett-Burman experimental design. Dextrose, $FeSO_4 \cdot 7H_2O$, NaCl, NH_4Cl, malt extract, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, and $CaCl_2 \cdot 2H_2O$ were the nine medium constituents selected for the study. The Plackett-Burman experimental design for eleven variables (Tables 1 and 2), i.e. nine nutritional components (independent variables) and two dummy variables, were used to evaluate the relative importance of various nutrients for a high quality angkak production providing a high quantity of lovastatin. For each nutrient variable, two different concentrations, i.e. high (+) and a low (–), were tested, dextrose (2 g/l, 8 g/l), $FeSO_4 \cdot 7H_2O$ (0.00 g/l, 0.04 g/l), NaCl (0.1 g/l, 0.4 g/l), NH_4Cl (0.1 g/l, 0.4 g/l), malt extract (0.1 g/l, 0.4 g/l), KH_2PO_4 (0.1 g/l, 0.4 g/l), $MgSO_4 \cdot 7H_2O$ (0.00 g/l, 0.04 g/l), $MnSO_4 \cdot H_2O$ (0.00 g/l, 0.03 g/l), and $CaCl_2 \cdot 2H_2O$ (0.00 g/l, 0.03 g/l). Data analysis was carried out by the standard procedure of Plackett-Burman experimental design (Plackett & Burman 1946; Chauhan *et al.* 2006; Abdel-Fattah *et al.* 2007).

Response surface methodology. Four important nutrient parameters (NH_4Cl, $MgSO_4$, NaCl, $CaCl_2$) screened from Plackett-Burman design were studied at 3 levels for the determination of optimal value for lovastatin production in angkak. An experimental design of 29 runs containing 5 central points (Table 3) was made according to Box-Behnken's response surface design for the selected four parameters using Design Expert 7.1 software (Statease Inc., USA) (Box & Hunter 1957; Chang *et al.* 2002, 2006; Chauhan *et al.* 2006; Abdel-Fattah *et al.* 2007; Sayyad *et al.* 2007a). The relative effects of two variables on lovastatin production were identified from the contour and response surface plot. Optimum values of the parameters for maximum production of lovastatin were determined by the point prediction tool of the software.

Determination of lovastatin concentration in angkak. Matured angkak (5 g) was suspended in 25 ml ethyl acetate and kept in a shaker incubator at 180 rpm and 70°C for 1.5 hours. The mixture was centrifuged at 3000 g for 8 min, 5 ml of the supernatant was collected and treated with 50 ml of 1% trifluoroacetic acid for lovastatin lactonisation. The resultant solution was concentrated at 80°C, diluted to 5 ml with acetonitrile, filtered through 0.45 µm filter and subjected to high performance

Table 1. Plackett-Burman experimental design of 12 trials for eleven variables (9 nutrients + 2 dummy) along with observed concentration of lovastatin in angkak samples

Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Lovastatin (mg/g)
1	+	+	–	+	+	+	–	–	–	+	–	1.999
2	–	+	+	–	+	+	+	–	–	–	+	1.587
3	+	–	+	+	–	+	+	+	–	–	–	0.965
4	–	+	–	+	+	–	+	+	+	–	–	1.312
5	–	–	+	–	+	+	–	+	+	+	–	0.887
6	–	–	–	+	–	+	+	–	+	+	+	1.769
7	+	–	–	–	+	–	+	+	–	+	+	1.590
8	+	+	–	–	–	+	–	+	+	–	+	1.606
9	+	+	+	–	–	–	+	–	+	+	–	1.568
10	–	+	+	+	–	–	–	+	–	+	+	1.479
11	+	–	+	+	+	–	–	–	+	–	+	1.219
12	–	–	–	–	–	–	–	–	–	–	–	1.841

liquid chromatography (HPLC) analysis according to the procedure given by Sayyad *et al.* (2007a) using 250 × 4.6 mm ID Lichrosper®100 C18 column, 5 µm particle size. The mixture of acetonitrile and water (65:35 v/v) acidified with *ortho*-phosphoric acids (0.1%) was used as the mobile phase. The flow rate of the mobile phase was maintained at 1.5 ml/min and the detection was carried out at 235 nm with a UV detector (Samiee *et al.* 2003; Sayyad *et al.* 2007a).

RESULTS

Selection of *Monascus purpureus* strain for angkak production

The highest amount of lovastatin per g of angkak after fermentation for 14 days was produced by *M. purpureus* MTCC 369 followed by *M. purpureus* MTCC 1090 and *M. purpureus* MTCC 410 with 1.703 mg, 0.365 mg, and 0.183 mg, respectively.

Table 2 Influence of medium variables on lovastatin production in angkak samples

Designation	Variable	ΣH	ΣL	Mean square	Effect	F value	Contribution (%)
X_1	Dextrose	8.947	8.875	0.000	0.012	0.000	00.034
X_2	NaCl	9.551	8.271	0.136	0.213	6.800	10.981
X_3	NH_4Cl	7.705	10.117	0.484	–0.402	24.20	38.994
X_4	KH_2PO_4	8.743	9.079	0.009	–0.056	0.450	00.756
X_5	Malt extract	8.594	9.228	0.033	–0.105	1.650	02.694
X_6	Dummy 1	8.813	9.009	0.003	–0.032	0.150	00.241
X_7	$FeSO_4 \cdot 7H_2O$	8.791	9.031	0.004	–0.040	0.200	00.386
X_8	$MgSO_4 \cdot 7H_2O$	7.839	9.983	0.383	–0.357	19.15	30.810
X_9	$CaCl_2 \cdot 2H_2O$	8.361	9.461	0.109	–0.183	5.45	08.110
X_{10}	$MnSO_4 \cdot H_2O$	9.292	8.530	0.048	0.127	2.40	03.891
X_{11}	Dummy 2	9.250	8.572	0.038	0.113	1.90	03.056

Plackett-Burman experimental design

Maximum lovastatin production was found in the experimental trial 1, whereas, minimum in trial 5 (Table 1) under solid-state fermentation using *M. purpureus* MTCC 369. Among the nine nutrient components used in the study, NH_4Cl, $MgSO_4$, and NaCl contributed in a large extent to lovastatin production. Sucrose, KH_2PO_4, and $FeSO_4$ had a small impact, while malt extract, $MnSO_4$, and $CaCl_2$ contributed moderately. Sucrose, NaCl, and $MnSO_4$ influenced the production only in higher concentrations, whereas the rest were effective at lower levels (Table 2).

Response surface analysis

To identify the concentrations of the key nutrients (NH_4Cl, $MgSO_4$, NaCl, $CaCl_2$) influencing lovastatin production in angkak under solid-state fermentation, the response surface methodology (RSM) for medium optimisation was applied. The individual and interactive effects of these nutrient variables for

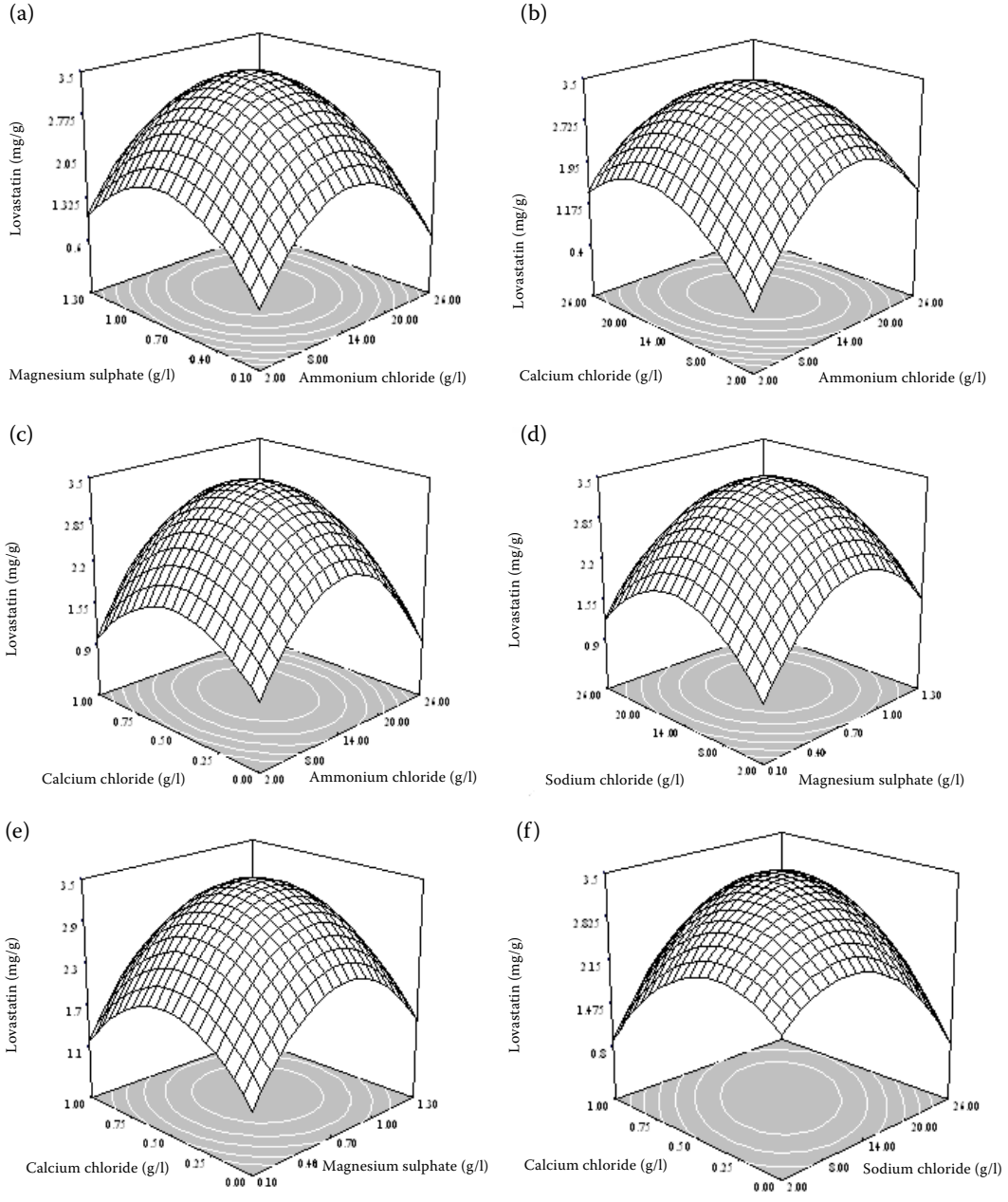

Figure 1. Response surface plots showing relative effects of different medium parameters on lovastatin production during fermentation

Table 3. Box–Behnken's design of RSM with results (actual and predicted) for monoculture system.

Run	NH_4Cl (g/l) Code – A	$MgSO_4.7H_2O$ (g/l) Code – B	NaCl (g/l) Code – C	$CaCl_2.2H_2O$ (g/l) Code – D	Lovastatin (mg/g)	
					actual	predicted
1	2.0(–1)	0.10(–1)	14.00(0)	0.50(0)	0.450	0.64
2	26.00(+1)	0.10(–1)	14.00(0)	0.50(0)	0.737	0.68
3	2.00(–1)	1.30(+1)	14.00(0)	0.50(0)	1.147	1.02
4	26.00(+1)	1.30(+1)	14.00(0)	0.50(0)	1.610	1.25
5	14.00(0)	0.70(0)	2.00(–1)	0.00(–1)	1.320	2.03
6	14.00(0)	0.70(0)	26.00(+1)	0.00(–1)	0.780	0.84
7	14.00(0)	0.70(0)	2.00(–1)	1.00(+1)	1.132	0.89
8	14.00(0)	0.70(0)	26.00(+1)	1.00(+1)	3.204	2.32
9	2.00(–1)	0.70(0)	14.00(0)	0.00(–1)	1.185	1.08
10	26.00(+1)	0.70(0)	14.00(0)	0.00(–1)	1.000	0.95
11	2.00(–1)	0.70(0)	14.00(0)	1.00(+1)	0.500	0.99
12	26.00(+1)	0.70(0)	14.00(0)	1.00(+1)	0.831	1.38
13	14.00(0)	0.10(–1)	2.00(–1)	0.50(0)	0.755	0.94
14	14.00(0)	1.30(+1)	2.00(–1)	0.50(0)	1.617	1.57
15	14.00(0)	0.10(–1)	26.00(+1)	0.50(0)	0.738	1.22
16	14.00(0)	1.30(+1)	26.00(+1)	0.50(0)	1.286	1.54
17	2.00(–1)	0.70(0)	2.00(–1)	0.50(0)	0.843	0.45
18	26.00(+1)	0.70(0)	2.00(–1)	0.50(0)	1.650	1.44
19	2.00(–1)	0.70(0)	26.00(+1)	0.50(0)	1.470	1.42
20	26.00(+1)	0.70(0)	26.00(+1)	0.50(0)	0.570	0.70
21	14.00(0)	0.10(–1)	14.00(0)	0.00(–1)	1.753	1.17
22	14.00(0)	1.30(+1)	14.00(0)	0.00(–1)	1.527	1.49
23	14.00(0)	0.10(–1)	14.00(0)	1.00(+1)	1.400	1.18
24	14.00(0)	1.30(+1)	14.00(0)	1.00(+1)	1.500	1.82
25	14.00(0)	0.70(0)	14.00(0)	0.50(0)	3.400	3.40
26	14.00(0)	0.70(0)	14.00(0)	0.50(0)	3.403	3.40
27	14.00(0)	0.70(0)	14.00(0)	0.50(0)	3.403	3.40
28	14.00(0)	0.70(0)	14.00(0)	0.50(0)	3.403	3.40
29	14.00(0)	0.70(0)	14.00(0)	0.50(0)	3.403	3.40

lovastatin production were studied by conducting the fermentation runs according to Box-Behnken design of RSM at randomly selected different levels (Table 3) of all four parameters. The data collected for lovastatin concentration in angkak in each run were analysed using the software Design Expert 7.1 and fitted into a multiple nonlinear regression model proposes the following equation (in the coded factor) for lovastatin production in angkak.

Multiple nonlinear regression model

$$\text{Lovastatin (mg/g)} = 3.40 + 0.067A + 0.24B + + 0.061C + 0.083D + 0.044AB - 0.43AC + + 0.13AD - 0.079BC + 0.081\,BD + 0.65CD - -1.41A^2 - 1.09B^2 - 0.99\,C^2 - 0.89\,D^2$$

where:
A – NH_4Cl; B – $MgSO_4$
C – NaCl; D – $CaCl_2$

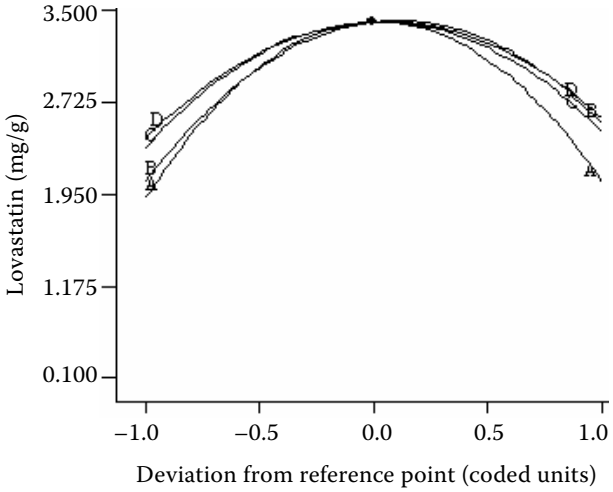

Figure 2. Perturbation plot showing influence of different medium parameters A, B, C, D (in coded) on lovastatin production in angkak by *Monascus purpureus* MTCC 369

This model resulted in six response surfaces with contours in Figure 1 and one perturbation plot. The influence of different parameters on lovastatin production form the reference point is shown in Figure 2. The analysis of variance of regression for lovastatin production is summarised in Table 4. All the surfaces/contour response could be analysed for determining the optimised value of the factors, but it was difficult to analyse all these simultaneously. Hence, the point prediction of the design expert software was used to determine the optimum values of the factors for maximum lovastatin production. Finally, the optimum values of NH_4Cl 14.32 g/l, $MgSO_4 \cdot 7H_2O$ 0.76 g/l, NaCl 14.65 g/l, and $CaCl_2 \cdot 2H_2O$ 0.54 g/l were determined. These values predict 3.420 mg/g of lovastatin production in angkak. These optimised values of the nutrient parameters were validated by solid-state fermentation and an average of 3.406 mg/g of lovastatin production in *angkak* was obtained. This shows 99.59% validity of the predicted model.

Table 4. Analysis of variance of calculated model and residual

Regression	
Sum of squares	24.54
df	14
Mean squares	1.75
F value	7.85
P value	0.0002 (less than 0.0500 indicate model terms are significant)
Residual	
Sum of squares	3.13
df	14
Mean squares	0.22
Correlation coefficient (R^2)	0.8870
Coefficient of variation (*CV*%)	29.78

DISCUSSION

Monascus is a food fungus that has been widely used as an enzymatic agent to make wines and other fermented food products. Angkak (red mold rice, red yeast rice, Chinese red rice), a traditional Chinese medicine is produced from solid-state fermentation of cooked non-glutinous rice (*Oryza sativa* L. Gramineae) with *Monascus purpureus*, *M. ruber, M. anka*, and *M. pilosus* (Endo 1979; Kohama *et al.* 1987; Chang *et al.* 2002; Miyake *et al.* 2006). In the present study, angkak production was achieved by different *Monascus* strains such as *Monascus purpureus* MTCC 369, *M. purpureus* MTCC 1090, and *M. purpureus* MTCC 410. Out of all the strains tested, *M. purpureus* MTCC 369 produces high quality angkak containing maximum amount of lovastatin (1.703 mg/g) on rice-based medium (unsupplemented with other nutrients) on the 14th day of fermentation.

The screening of nutrients for the highest lovastatin accumulation in angkak was carried out by Plackett-Burman experimental design (Plackett & Burman 1946; Sayyad *et al.* 2007b). The goals of screening were the investigation of the nutrients under study influencing lovastatin production and selection of the important nutrients on the basis of their effects for further optimisation. It was found that NH_4Cl, $MgSO_4$, and NaCl contributed in a large extent to lovastatin production. Dextrose, KH_2PO_4, $FeSO_4$ had a small impact, while malt extract, $MnSO_4$, and $CaCl_2$ contributed moderately. In fungal nutrition, magnesium and calcium are noted as macronutrients, whereas manganese, iron, copper, and zinc as micronutrients (Yu *et al.* 1997). But here manganese and calcium contributed moderately for lovastatin production. Among these factors, NH_4Cl, $MgSO_4$, NaCl, and $CaCl_2$ were found to be significant for lovastatin production by solid-state fermentation with *M. purpureus* MTCC 369.

The Plackett-Burman experimental design is a preliminary technique for a rapid illustration of the effects of various medium constituents (Kennedy & Krouse 1999). It tests each variable at two levels only; hence it can not give an exact idea regarding the optimum level of the constituent required in the medium. Therefore, further optimisation of the selected nutrients for lovastatin production is necessary. In most of the cases, the medium parameters screened by Plackett-Burman experimental design are optimised by Response surface methodology (RSM). This is a three-factorial design that reveals the relationships between one or more measured dependent responses with a number of input (independent) factors. RSM has some advantages that include fewer experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable condition and forecast response (Chang *et al.* 2006; Popa *et al*. 2007). In this, quadratic effects of experimental variables construct contour plots and a model equation fitting the experimental data. This facilitates the determination of optimum values of the factors under investigation and prediction of response under optimised conditions (Chakravarti & Sahai 2002).

In the present study, four important nutrients, NH_4Cl, $MgSO_4$, NaCl, and $CaCl_2$ screened by Plackett-Burman experimental design were optimised by Box-Behnken design of RSM with the help of Design Expert 7.1 software. The nutrient variables studied had different effects on lovastatin production. All the nutrient parameters were positively significant factors. The proposed model equation illustrates the interaction between two factors. From the quadratic model equation it was found that $CaCl_2$ interacted positively with NH_4Cl, $MgSO_4$, and NaCl, whereas NH_4Cl interacted positively with $MgSO_4$, and NaCl interacted negatively with NH_4Cl and $MgSO_4$ with respect to lovastatin production. The relative effects of the medium components on lovastatin production were depicted in the perturbation plot (Figure 2). The optimum values of NH_4Cl 14.32 g/l, $MgSO_4$ 0.76 g/l, NaCl 14.65 g/l, and $CaCl_2$ 0.54 g/l were determined by the point prediction tool of the software. These values predicted 3.420 mg/g with 99.59% validity of the predicted model on lovastatin accumulation in angkak.

A two time increase in lovastatin concentration in angkak produced from rice medium (unsupplemented with other nutrients) under optimised fermentation conditions resulted in lovastatin concentration much higher then that obtained under monoculture of *Monascus pilosus* M12-69 (Chen & Hu 2005), *Monascus purpureus* NTU 601, 301 and *M. purpureus* BCRC 31499, 31504, 31530, 31540, 32966, 32807, 32808, 32809 in rice (Lee *et al.* 2006a). But the concentration of citrinin, a mycotoxin causing hepatonephrotoxic effects, needs to be controlled during angkak production by genetic mutation of the fungal strain, by adding nutrients like fatty acids to the fermentation medium (Hajjaj *et al.* 2000), or by replacing rice with other solid substrate like dioscorea (Lee *et al.* 2006a) which might change the citrinin concentration in angkak.

The present research shows that lovastatin concentration can be increased in angkak by engineering rice-based medium. Moreover, downstreaming of pure lovastatin from fermented medium is not required as fermented rice can be consumed directly after sterilisation and this can produce multiple therapeutic benefits, i.e. blood pressure lowering effects due to the presence of γ-aminobutaric acid (GABA) (Kohama *et al.* 1987), antiinflammatory effects due to the presence of monascin (Lee *et al.* 2006a), anticancer effects due to the presence of ankaflavin (Su *et al.* 2005), antioxidant effects due to the presence of a free radical scavenger dimerumic acid (Taira *et al.* 2002) including the lowering of serum lipids levels.

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