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ORIGINAL PAPER

Optimization of Fermentation Parameters for Higher Lovastatin Production in Red Mold Rice through Co-culture of *Monascus purpureus* and *Monascus ruber*

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Abstract *Monascus*, fermented rice (red mold rice), has been found to reduce the serum total cholesterol and triglyceride due to presence of lovastatin. Lovastatin acts as an inhibitor of 3-hydroxy-3-methyl glutaryl coenzyme A reductase. Coculture of *Monascus purpureus* MTCC 369 and *Monascus ruber* MTCC 1880 was used to produce red mold rice by solid-state fermentation. Optimization of different fermentation process parameters such as temperature, fermentation time, inoculum volume, and pH of the solid medium was carried out by Box–Behnken's factorial design of response surface methodology to maximize lovastatin concentration in red mold rice. Maximum lovastatin production of 2.83 mg/g was predicted at 14th day in solid medium under optimized process condition.

Keywords Coculture · *Monascus purpureus* · *Monascus ruber* · Lovastatin · Response surface methodology · Solid-state fermentation

Introduction

Lovastatin (mevinolin and monacolin K), a hypocholestromic agent, competitively inhibit the rate-limiting enzyme 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the reduction of HMG-CoA to mevalonate during cholesterol biosynthesis (Alberts et al. 1980; Hajjaj et al. 2001). This natural statin was the first fungal secondary metabolite to obtain approval from the US Food and Drug Administration in August 1987 (Tobert 2003; Demain 1999; Manzoni and Rollini 2002). Lovastatin is produced by *Monascus pilosus*, *Aspergillus terreus*, *Monascus ruber*, *Monascus purpureus*, and *Penicillium* species (Hajjaj et al. 2001; Miyake et al. 2005, 2006; Chang et al. 2002). However, some hyperproducing strains of *A. terreus* produces high amount of lovastatin under submerged fermentation (Porcel et al. 2007), but the liquid medium containing lovastatin produced by *A. terreus* is not suitable for consumption directly by human beings since it is not coming under the "generally regarded as safe" designation. Therefore, complex chromatographic and solvent extraction procedures were followed to downstream the lovastatin from the fermented broth.

M. ruber and *M. purpureus* are nonpathogenic fungi and used frequently by Chinese for the production of red mold rice (Kohama et al. 1987; Chen and Hu 2005; Chiu et al. 2006; Lee et al. 2006a). There are several reports on the production of lovastatin and red mold rice by using monocultures of *Monascus* species (Lee et al. 2006b; Chiu et al. 2006; Miyake et al. 2005; Su et al. 2003). In nature, solid substrate fermentation is carried out by mixed cultures of different fungal species. The coculture of fungi during fermentation may provide help for better biomass and secondary metabolite productions; moreover, it helps in proper utilization of substrate. There are several reports of coculture of fungal species found to enhance enzyme, organic acid production, and microbial bioconversion reaction (Banerjee et al. 2005; Pandey et al. 1999; Temudo et al. 2007). Unfortunately, no study has been carried out

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for production of lovastatin by coculture or mixed culture of *Monascus* species under solid-state fermentation.

Therefore, the objective of this research was to produce the finest-quality rice-based nutraceutical-containing maximum amount of a hypocholestromic agent (lovastatin). Two filamentous fungi, *M. purpureus* MTCC 369 and *M. ruber* MTCC 1880, were used together as inocula for the production of the nutraceutical under solid-state fermentation. As the fermentation process is highly regulated by different fermentation process conditions, interactions of parameters and their optimum levels were determined by response surface methodology (RSM).

Materials and Methods

Microorganisms

Fungal cultures of *M. purpureus* MTCC 369 and *M. ruber* MTCC 1880 were obtained from the Institute of Microbial Technology, Chandigarh, India. Fungal cultures were maintained routinely on a potato dextrose agar medium containing agar (1.5%), diced potatoes (30%), and glucose (2%) and subcultured in every 30-day interval (Sayyad et al. 2007; Chang et al. 2002).

Preparation of Mixed Seed Cultures

Spore suspensions of *M. purpureus* and *M. ruber* was prepared separately from actively growing slants in sterile water and diluted to a concentration 5.7×10^3 spores per milliliter. Spore counting was carried out using a hemocytometer. Spore suspension (7.5 ml) of *M. purpureus* was inoculated to conical flasks containing 50 ml basal medium (100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g $NH_4H_2PO_4$, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2$ in 1,000 ml distilled water; adjusted to pH 6.0) and incubated at 30 °C for 48 h in a shaker incubator at 110 rpm (Sayyad et al. 2007; Su et al. 2003). Spore suspension (7.5 ml) of *M. ruber* was inoculated to conical flasks containing 50 ml of potato dextrose broth, incubated at 30 °C for 4 days with shaking at 150 rpm (Chang et al. 2002). Finally both the seed cultures of *M. purpureus* and *M. ruber* were mixed at a ratio of 1:1.

Solid-state Fermentation

Long-grain, nonglutinous rice was purchased from the local market of New Delhi, India, and was used as a base solid substrate for red mold rice production under solid-state culture. Initially, 20 g of presoaked rice was taken in a 250-ml conical flask to which 40 ml of distilled water containing different optimized nutrients (malt extract 9.68 g/l, dextrose 38.90 g/l, $MnSO_4.H_2O$ 1.96 g/l, and $MgSO_4.7H_2O$ 0.730 g/l) obtained by a Plackett–Burman design and RSM were added, and the pH of the medium was adjusted as per the experimental design with 0.1 M HCl or NaOH and autoclaved for 20 min at 121 °C. After being cooled, the rice-based medium was inoculated with mixed seed cultures of *M. purpureus* and *M. ruber*. Box–Behnken response surface design (Sayyad et al. 2007) was followed to design fermentation process conditions such as temperature, fermentation time, inoculum volume, and pH of the solid medium for different experimental runs at different levels (Table 1).

Extraction of Lovastatin

Fermented rice (1 g) was suspended in 5 ml ethyl acetate and kept in a shaker incubator at 180 rpm and 70 °C for 1.5 h. The mixtures were centrifuged at $3{,}000\times g$ for 8 min, supernatant (1 ml) was collected, and 1% trifluoroacetic acid (10 ml) was added for lactonization of the lovastatin. The resultant was concentrated at 80 °C (without applying vacuum), diluted to 1 ml with acetonitrile and filtered through a 0.45-μm filter for high-performance liquid chromatography (HPLC) analysis (Su et al. 2003).

Quantitative Analysis of Lovastatin

Procedure given by Samiee et al. for HPLC analysis was slightly modified. Lovastatin was estimated by HPLC (SHIMADZU, Japan) using 250×4.6 mm ID Lichrosper® 100 C_{18} column of 5 μm particle size, 20 μl loop injector, and Shimadzu CLASS-VP version 5.032 software. Acetonitrile/water (65:35 *v*/*v*), acidified with *ortho*-phosphoric acid to the concentration 0.1%, was used as mobile phase with a flow rate of 1.5 ml/min, and detection was carried out by UV detector (SPD10A VP) at 235 nm (Samiee et al. 2003; Sayyad et al. 2007).

Results and Discussion

Fermentation process parameters such as temperature, fermentation time periods, inoculum volume, and pH of the solid medium are selected for lovastatin production under coculture of *M. purpureus* MTCC 369 and *M. ruber* MTCC1880 during solid-state fermentation, and RSM for process optimization was followed.

To identify the optimum levels of different process parameters influencing lovastatin production, solid-state fermentation was carried out in conical flasks containing optimized nutrients. Four process parameters (temperature, fermentation time, inoculum volume, and pH of the solid medium) were chosen for study by borrowing methodology

Table 1 Box–Behnken design for process parameters with lovastatin concentration (actual and predicted) under the coculture system

Run	Temperature (°C)	Fermentation time (days)	Inoculum volume (ml)	pH of the solid medium	Lovastatin (mg/g)	
					Actual	Predicted
1	25 (−1)	10 (−1)	5 (0)	6 (0)	1.88	1.47
2	35 (+1)	10 (−1)	5 (0)	6 (0)	1.67	1.19
3	25 (−1)	18 (+1)	5 (0)	6 (0)	1.79	1.66
4	35 (+1)	18 (+1)	5 (0)	6 (0)	1.1	0.9
5	30 (0)	14 (0)	3 (−1)	5 (−1)	2.46	2.02
6	30 (0)	14 (0)	7 (+1)	5 (−1)	2.15	1.65
7	30 (0)	14 (0)	3 (−1)	7 (+1)	2.09	1.98
8	30 (0)	14 (0)	7 (+1)	7 (+1)	2.01	1.84
9	25 (−1)	14 (0)	5 (0)	5 (−1)	1.05	1.48
10	35 (+1)	14 (0)	5 (0)	5 (−1)	0.56	0.73
11	25 (−1)	14 (0)	5 (0)	7 (+1)	1.01	1.32
12	35 (+1)	14 (0)	5 (0)	7 (+1)	0.99	1.03
13	30 (0)	10 (−1)	3 (−1)	6 (0)	1.67	1.95
14	30 (0)	18 (+1)	3 (−1)	6 (0)	1.95	2.38
15	30 (0)	10 (−1)	7 (+1)	6 (0)	2.13	2.17
16	30 (0)	18 (+1)	7 (+1)	6 (0)	1.46	1.65
17	25 (−1)	14 (0)	3 (−1)	6 (0)	1.98	1.73
18	35 (+1)	14 (0)	3 (−1)	6 (0)	1.46	1.54
19	25 (−1)	14 (0)	7 (+1)	6 (0)	1.76	1.81
20	35 (+1)	14 (0)	7 (+1)	6 (0)	0.56	0.95
21	30 (0)	10 (−1)	5 (0)	5 (−1)	1.37	1.75
22	30 (0)	18 (+1)	5 (0)	5 (−1)	1.57	1.52
23	30 (0)	10 (−1)	5 (0)	7 (+1)	1.46	1.64
24	30 (0)	18 (+1)	5 (0)	7 (+1)	2.03	1.78
25	30 (0)	14 (0)	5 (0)	6 (0)	2.81	2.81
26	30 (0)	14 (0)	5 (0)	6 (0)	2.82	2.81
27	30 (0)	14 (0)	5 (0)	6 (0)	2.81	2.81
28	30 (0)	14 (0)	5 (0)	6 (0)	2.8	2.81
29	30 (0)	14 (0)	5 (0)	6 (0)	2.82	2.81

as these process parameters mostly influence the growth of different fungal strains and secondary metabolite production during solid-state fermentation. An experimental design of 29 runs containing five central points was made according to Box–Behnken's response surface design for four selected parameters. The individual and interactive effects of these (process parameters) variables were studied by conducting the fermentation run at different levels of all factors. The response was measured in milligram of lovastatin per gram of fermented rice. The results of experimental and simulated values are listed in Table 1. Lovastatin production in each experimental run was analyzed using the software Design Expert 7.1 (Statease, USA) and fitted into a multiple nonlinear regression model. The model proposes the following equation.

Multiple Nonlinear Regression Model:

$$\text{Lovastatin (mg/g)} = 2.81 - 0.26 \times \text{temperature} - 0.023 \times \text{fermentation time} - 0.13 \times \text{inoculum volume} + 0.035 \times \text{pH of the solid medium} - 0.12 \times \text{temperature} \times \text{fermentation time} - 0.17 \times \text{temperature} \times \text{inoculum volume} +$$

Table 2 The analysis of variance of the calculated model of process parameters for lovastatin production in the coculture system

Parameters	Values
Regression analysis of model	
Sum of squares	10.03
df	14
Mean squares	0.72
F value	4.97
p value	0.0025
Residual	
Sum of squares	2.02
df	14
Mean squares	0.14
Correlation coefficient (R^2)	0.8325
Coefficient of variation (CV%)	21.08
Test for lack of fit	
Sum of squares	2.02
df	10
F value	2,887.56
Adequate precision	7.633 (>4)

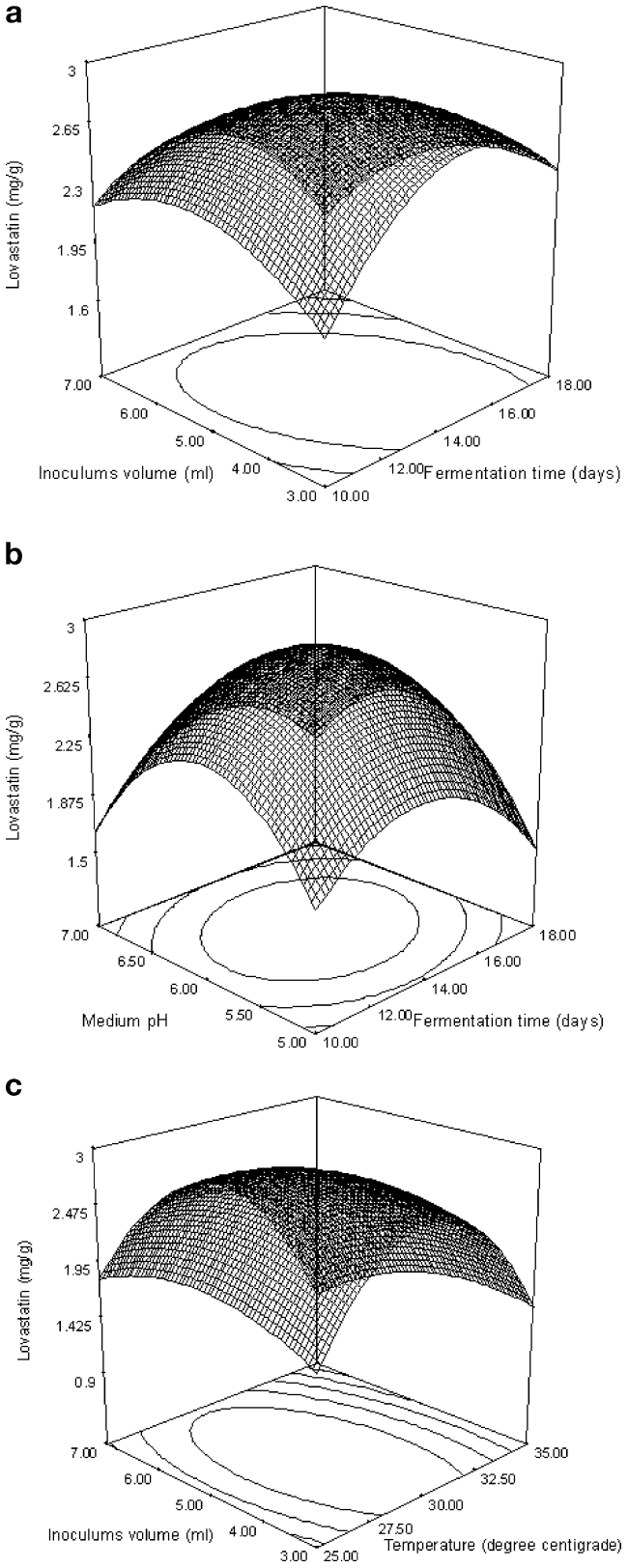

Fig. 1 Response surface plots showing relative effects of different process parameters on lovastatin production during solid-state fermentation

$0.12 \times \text{temperature} \times \text{pH of the solid medium} - 0.24 \times \text{fermentation time} \times \text{inoculum volume} + 0.092 \times \text{fermentation time} \times \text{pH of the solid medium} + 0.058 \times \text{inoculum volume} \times \text{pH of the solid medium} - 1.02 \times \text{temperature}^2 - 0.49 \times \text{fermentation time}^2 - 0.29 \times \text{inoculum volume}^2 - 0.65 \times \text{pH of the solid medium}^2$

This multiple nonlinear quadratic model resulted in six response surface graphs. A few representative response surface plots of the calculated model for lovastatin production are shown in Fig. 1a,b, and c. The analysis of variance of the model for lovastatin production is represented in Table 2.

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 temperature at 29.46 °C, fermentation time for 13.89 days, inoculum volume of 4.95 ml, and at a medium pH of 6.03 were determined. These values predict 2.83 mg/g of lovastatin production by coculture of *M. purpureus* and *M. ruber* under solid-state fermentation. These optimized values of process parameters were validated by solid-state fermentation of rice containing previously optimized medium parameters (malt extract 9.68 g/l, dextrose 38.90 g/l, $MnSO_4.H_2O$ 1.96 g/l, and $MgSO_4.7H_2O$ 0.730 g/l), and an average 2.80 mg/g of lovastatin production in solid substrate was obtained. This shows 98.93% validity of the predicted model.

Solid-state fermentation runs were designed according to Box–Behnken design of RSM at randomly selected different levels. The process parameters temperature, fermentation time periods, and inoculum volume was negatively significant factors, and the pH of the fermenta-

Table 3 Analysis of variance of model parameters

Model parameters	*df*	*F* value	*p* value
Temperature	1	5.65	0.0322
Fermentation time	1	0.045	0.8346
Inoculum volume	1	1.37	0.2616
pH of the solid medium	1	0.11	0.7488
Temperature × Fermentation time	1	0.40	0.5378
Temperature × Inoculum volume	1	0.80	0.3860
Temperature × pH of the solid medium	1	0.38	0.5462
Fermentation time × Inoculum volume	1	1.56	0.2318
Fermentation time × pH of the solid medium	1	0.24	0.6339
Inoculum volume × pH of the solid medium	1	0.092	0.7666
Temperature^2	1	46.71	< 0.0001
Fermentation time^2	1	10.61	0.0057
Inoculum volume^2	1	3.67	0.0759
pH of the solid medium^2	1	19.11	0.0006

tion medium was a positively significant factor. From the quadratic model, it was conformed that the pH of the fermentation medium interacts positively to all the process parameters. Fermentation temperature interacts negatively with fermentation time and inoculum volume, but fermentation time and inoculum volume interact negatively with each other. Out of the total model parameters, 30% of the parameters significantly influenced the lovastatin production (Table 3). The "lack-of-fit *F* value" of 2,887.56 was obtained. A high lack-of-fit value could occur due to noise. However, adequate precision (measures the signal-to-noise ratio) of the model was found to be at 7.633, and the value is larger then the desirable value of 4 (Table 2). A high adequate precision ratio indicates an adequate signal in the quadratic model. Therfore, the model can be used to navigate the design space.

The optimum values of temperature at 29.46 °C, fermentation time for 13.89 days, inoculum volume of 4.95 ml, and at a medium pH of 6.03 were determined by the point prediction tool of the software with 98.93% validity. Fermentation under optimized process yields 2.80 mg/g of lovastatin in the solid substrate, which is much higher than the lovastatin or monacolin K concentration obtained under monoculture of *M. ruber* M 82121,1005 (Chang et al. 2002), *M. pilosus* M12-69 (Chen and Hu 2005), *M. purpureus* NTU 601, 301, and *M. purpureus* BCRC 31499, 31504, 31530, 31540, 32966, 32807, 32808, 32809 on rice (Lee et al. 2006b).

Different *Monascus* species such as *M. ruber*, *M. purpureus*, *M. anka*, and *M. pilosus* are used in China for production of red yeast rice (red mold rice, angkak), a functional food, and are considered to be nonpathogenic (Kohama et al. 1987; Chiu et al. 2006). This functional food has a wide variety of therapeutic applications including serum lipid management due to the presence of slovastatin (monacolin K) in fermented rice (Lee et al. 2006a). The present research shows that lovastatin concentration can be increased in rice (red mold rice) so as to increase its hypocholestrolemic effects, when fermented by mixed cultures of *Monascus* rather then monocultures of *Monascus* under optimized medium and process parameters.

Moreover, downstreaming of pure lovastatin from the fermented medium is not required as solid fermented materials can be consumed directly after sterilization, and this can produce multiple therapeutic benefits, i.e., blood pressure-lowering effects due to presence of γ-aminobutyric acid (Kohama et al. 1987), antiinflammatory effects due to the presence of monascin (Lee et al. 2006b), anticancer effects due to the presence of ankaflavin (Su et al. 2005), and antioxidant effects due to the presence of a free radical scavenger dimerumic acid (Taira et al. 2002), including lowering of serum lipids levels.

Conclusion

Lovastatin concentration in red mold rice (Chinese functional food) can be increased by mixed-culture fermentation of rice with two different *Monascus* species (*M. purpureus* and *M. ruber*). Solid-state fermentation of rice with pH 6.03 at 29.46 °C for 13.89 d, predict 2.83 mg/g and yielded 2.80 mg of lovastatin/gram of fermented rice with 98.93% validity. Moreover, it can be eaten directly to gain better therapeutic benefits.

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