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Original Article

Statistical optimization of lovastatin and confirmation of nonexistence of citrinin under solid-state fermentation by *Monascus sanguineus*

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ABSTRACT

Lovastatin is a well-known natural statin, which is used for lowering plasma cholesterol levels by inhibiting 3-hydroxy-3-methyl glutaryl coenzyme A reductase. Different strains of *Aspergillus* and *Monascus* sp. have been exploited for statin production but *Monascus sanguineus* is still unexplored. In this study, lovastatin production from *Monascus sanguineus* under solid state fermentation was optimized using response surface methodology. The optimized value of the lovastatin yield was 20.04 mg/gds with soybean concentration of 20 g/L, CaCl₂ concentration of 2.5 g/L, acetic acid concentration of 25 μ L and inoculum size of 3.4 mL. This study also documented spectrometric characterization and fragment pattern of lovastatin with the help of Fourier transfer infrared spectrometry and mass spectrometry. Citrinin was not detected in any of the samples used for this study.

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1. Introduction

Coronary heart disease and most heart attacks are due to hypercholesterolemia, which is the accumulation of cholesterol in blood plasma that results in atherosclerosis (blockage of arteries). Almost one-third of the total body cholesterol comes from food intake and the other two-thirds is synthesised in the body [1]. A potent hypocholesterolemic agent, known as lovastatin (mevinolin and monacolin K) can inhibit the rate-limiting enzyme 3-hydroxy-3-methyl glutaryl coenzyme A reductase. This enzyme has a regulatory and rate-

limiting function in cholesterol biosynthesis [2]. Lovastatin was the first natural statin; it is a fungal secondary metabolite and was approved by the US Food and Drug Administration in August 1987 [3,4]. The microorganisms used for statin production under solid-state fermentation (SSF) conditions mostly belong to *Aspergillus* and *Monascus* sp. There are also a few reports of statin production in rice fermented by *Penicillium* and *Monascus* sp., which is also called Angkak. It is known to contain several valuable secondary metabolites such as lovastatin, γ -aminobutyric acids, monascodilone, monascorubramine, monascin, ankaflavin, rubropunctatin and citrinin [5–7].

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Citrinin is a mycotoxin with nephrotoxic, hepatotoxic and carcinogenic activities. *Penicillium citrinum*, *Aspergillus* sp. and *Monascus* sp. along with other secondary metabolites are known to produce citrinin, which greatly limits the application of the *Monascus* fermented products. Due to a potential risk to livestock and human health, extensive research on citrinin biosynthesis is being conducted. Comprehensive details are not known about the biological background of citrinin biosynthesis [8].

Recent studies have mainly focused on the production of microbial secondary metabolites using SSF approaches. The development of good-quality fermenters along with temperature, humidity, aeration, and sterility control has accelerated research using SSF. However, research should also explore new strains for improved statin production [9]. Mass spectrometry (MS) with electrospray ionization is considered an expanding approach for the structural elucidation of organic species. It can provide molecular mass information along with fragment pattern. It is widely used for the identification of impurities and degrades in the pharmaceutical industry [10].

Response surface methodology (RSM) can be used to assess the relative significance of several affecting factors. It is an empirical statistical modeling technique used for multiple regression analysis using quantitative data. These data are obtained from appropriately designed experiments required to solve multivariable equations simultaneously. RSM is being increasingly used for optimization of the process in fermentation [11].

The purpose of present study was to determine suitable conditions for scale-up of lovastatin production, verification of the absence of citrinin, and spectrometric characterization of lovastatin. The experiments were carried out under SSF with *Monascus sanguineus* in order to achieve optimum SI yield. Characterization of lovastatin was done with UV spectrophotometry and Fourier transform infrared (FT-IR) techniques. Liquid chromatography (LC)-MS was used to study fragment ions and mass using an electrospray ionization approach.

2. Methods

2.1. Culture and inoculum preparation

Pomegranate (*Punica granatum*) was used to isolate the wild strain of *Monascus*, which was identified as *M. sanguineus*. The spores were scraped off under aseptic conditions to produce a spore suspension that was prepared in 0.9% saline water [12].

2.2. SSF

Ten grams of wheat bran as a substrate were taken and placed in a 250-mL conical flask to which 30 mL of basal medium was added. The basal medium composition included 100 g dextrose, 10 g peptone, 2 g KNO₃, 2 g NH₄ H₂ PO₄, 0.5 g MgSO₄·7H₂O, and 0.1 g in 1 L distilled water. The pH of the medium was adjusted to 6.0 [13].

2.3. Extraction of lovastatin

Acetonitrile was used as extraction solvent. One gram of fermented dry substrate was dissolved in 20 mL acetonitrile and kept in a shaker incubator for 2 hours at 180 rpm and 70°C. It was then filtered with Whatman filter paper (Sigma Aldrich, USA) and the filtrate was centrifuged at 3000 × g for 8 minutes [14].

2.4. Screening of citrinin

Fermented dry substrates were dissolved in ethyl acetate and acetified up to pH 5. The aqueous layer was removed and the organic layer was concentrated. Ten microliters of these samples was applied to Silica gel 60 F254 Aluminum sheets (Merck, Germany). Ethyl acetate: acetone: water (4: 4: 1, by volume) was used as the mobile phase. Subsequently, the drying plates were examined under UV light at 350 nm to observe fluorescent yellow bands [15].

2.5. Estimation of lovastatin by UV spectrophotometry

Lovastatin was purified from the sample with the help of thin layer chromatography (TLC). Purified spots from TLC were scraped and transferred into glass tubes and acetonitrile was added. The tubes were centrifuged, filtered, and the filtrate was estimated at 238 nm using UV-visible spectrophotometry. Lovastatin was estimated in its acid form (mevinolinic acid) [16].

2.6. Identification of lovastatin by FT-IR and LC-MS

For FT-IR analysis, the sample was kept in vacuum desiccators over solid KOH for 48 hours and IR analysis was carried out using Thermo-Nicolet 6700 Fourier Transfer Infrared spectrometer. LC-MS analysis was carried using a 250 mm × 4.6 mm internal diameter Lichrosper 100 C18 column of particle size 5 µm, loop injector of 20 µL, and Shimadzu CLASS-VP version 5.032 software (Shimadzu, Japan). The mobile phase used for this analysis was acetonitrile: water (65: 35 v/v and pH 3.5). The flow rate was set to 1.0 mL/min and the detection was carried out using a wavelength of 235 nm by UV detector SPD10A VP (Shimadzu Europe, Germany) [17].

2.7. Experimental design (RSM)

The experimental design was formulated according to the central composite design of RSM using MATLAB software for four selected parameters: soya bean meal, CaCl₂, acetic acid and inoculum size (Table 1). A set of 30 experiments was required with each variable being at five levels. All the flasks

Table 1 – Four variables in coded and natural units.

Variables with designate	Code	Actual factor level at coded factor levels of				
		–2	–1	0	1	2
Soybean meal (g/L)	X1	4	8	12	16	20
CaCl ₂ (g/L)	X2	0.5	1	1.5	2	2.5
Acetic acid %(v/v)	X3	0	0.025	0.05	0.075	0.1
Inoculum size % (v/v)	X4	5	8	11	14	17

were incubated for 16 days. The relation between the coded values and actual values, independent variable and the response were calculated according to the second-order quadratic model (Table 2). The relative effects of two variables on response were examined from the three-dimensional surface and contour plots. MATLAB version 7.5.0.342 (R2007b) from Math Works, USA was used for the regression and graphical analysis of the experimented data and for analyzing the response surface and contour plots. Analysis of variance was used to estimate the statistical parameters with a significance level of $p < 0.001$ [18].

3. Results and discussion

3.1. Verification of absence of citrinin

TLC revealed that *M. sanguineus* strain did not produce citrinin in the present experimental conditions, although citrinin was detected in our earlier study using potato dextrose broth [12]. You et al [19] have also reported a lower amount of citrinin produced by *M. sanguineus* compared to other *Monascus* species. The reported concentration was 386 mg/L, 120 mg/L and 78 mg/L for *Monascus purpureus*, *Monascus ruber* and *M. sanguineus*, respectively. There is a possibility of citrinin amount

being diminutive enough to be detected by TLC. The concentration of optimized nutrients (acetic acid, CaCl_2 and soybean) would have provided a physiological balance between organism growth and lovastatin yield from *M. sanguineus* and inhibited citrinin production. Sani et al [20] have reported a reduction in citrinin concentration and enhanced yield of lovastatin by *M. purpureus* in SSF conditions. The optimized conditions were 2% (v/w) glycerol, 0.14% (w/w) methionine, and 0.01% (w/w) NaNO_3 at 25°C for 16 days. The fungus yielded monacolin K and citrinin of 5900 mg/kg substrate dry weight (sdw) and 0.26 mg/kg sdw, respectively.

3.2. Optimization of lovastatin

The quadratic polynomial explaining the relationship of the four variables for lovastatin yield is given below

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_1X_2 + b_6X_1X_3 + b_7X_1X_4 + b_8X_2X_3 + b_9X_2X_4 + b_{10}X_3X_4 + b_{11}X_1^2 + b_{12}X_2^2 + b_{13}X_3^2 + b_{14}X_4^2 \quad (1)$$

Where Y is the response of the quadratic model (lovastatin yield), b_i ($i = 0-14$) are the regression coefficients and X_1, X_2, X_3 and X_4 are the coded independent factors (Table 1). Applying the regression analysis, equation (1) can be rewritten as below:

Table 2 – Full factorial central composite design of four variables in coded and natural units along with the observed responses.

Run No.	Soybean g/L	CaCl_2 g/L	Acetic acid (μL)	Inoculum size (mL)	Lovastatin (mg/gds)	
					Experimental	Predicted
1.	8	1	10	1.6	6.75	6.72
2.	16	1	10	1.6	4.20	4.06
3.	8	2	10	1.6	3.45	3.56
4.	16	2	10	1.6	4.80	4.54
5.	8	1	20	1.6	7.65	7.75
6.	16	1	20	1.6	5.70	6.00
7.	8	2	20	1.6	5.10	5.48
8.	16	2	20	1.6	6.90	7.36
9.	8	1	10	2.8	5.73	5.14
10.	16	1	10	2.8	3.90	3.55
11.	8	2	10	2.8	5.10	4.83
12.	16	2	10	2.8	7.10	6.87
13.	8	1	20	2.8	6.60	6.89
14.	16	1	20	2.8	6.45	6.21
15.	8	2	20	2.8	7.26	7.46
16.	16	2	20	2.8	5.70	5.58
17.	4	1.5	15	2.2	5.40	5.59
18.	20	1.5	15	2.2	6.18	5.98
19.	12	0.5	15	2.2	4.20	4.48
20.	12	2.5	15	2.2	5.70	5.52
21.	12	1.5	0	2.2	5.10	5.54
22.	12	1.5	25	2.2	10.50	9.60
23.	12	1.5	15	1	6.24	5.73
24.	12	1.5	15	3.4	6.60	7.21
25.	12	1.5	15	2.2	5.88	5.98
26.	12	1.5	15	2.2	5.76	5.98
27.	12	1.5	15	2.2	5.82	5.98
28.	12	1.5	15	2.2	5.64	5.98
29.	12	1.5	15	2.2	5.49	5.98
30.	12	1.5	15	2.2	6.60	5.98

All experiments were carried in duplicate; the above-mentioned values are mean values.

$$\begin{aligned} \text{lovastatin yield(mg/gds)} = & 26.3668 - 0.8767X_1 - 8.5303X_2 \\ & - 0.5716X_3 - 6.6755X_4 \\ & + 0.4544X_1X_2 + 0.0114X_1X_3 \\ & + 0.0114X_1X_4 + 2.3688X_2X_4 \\ & - 0.9783X_2^2 + 0.0133X_3^2 \end{aligned} \quad (2)$$

The influence of the individual independent variable, keeping other variables at their central level, is shown in the subplots of Fig. 1. The trend for the lovastatin yield was similar for acetic acid, inoculum size and soybean meal, which initially showed a reduction in the yield. The yield increased subsequently with the increase in the values of the above-mentioned parameters. The relationship between the lovastatin yield and CaCl_2 concentration was almost linear with the yield increasing steadily.

The interactive effects of CaCl_2 and soybean concentration on lovastatin yield showed an interesting trend (first subplot of Fig. 2). Keeping one variable at a lower level and increasing the others resulted in a decrease in lovastatin yield. Hence, the maximum yield was obtained with both the variables at their maximum (lovastatin yield of 7.83 mg/gds with CaCl_2 concentration of 2.5 g/L and soybean concentration of 20 g/L) or minimum (lovastatin yield of 7.38 mg/gds with CaCl_2 concentration of 0.5 g/L and soybean concentration of 4 g/L) levels. This predominant behavior can be correlated with the high t value obtained for the interaction of CaCl_2 and soybean (Table 3). A similar trend, though smaller, was noticed for the interactive effects of inoculum size and CaCl_2 concentration (data not shown).

The effect of the concentration of acetic acid and soybean meal showed a valley-type trend, with almost no variation with the increase in the concentration of soybean. The lovastatin yield increased in a parabolic way with the increase in concentration of acetic acid. The maximum value of the lovastatin yield was ~10.12 mg/gds with acetic acid concentration of 25 μL and soybean concentration of 20 g/L (second subplot of Fig. 2).

Inoculum size and soybean concentration showed slight variation with the change in the values of the variables. The peaks were obtained with either the maximum values of the variables or with their minimum values. The above observation is also endorsed by the insignificant p value obtained for the interaction effect of these two variables (Table 3). The maximum value of lovastatin yield was ~6.99 mg/gds with inoculum size of 3.4 mL and soybean concentration of 20 g/L (third subplot of Fig. 2).

The lovastatin yield dependency on the concentration of acetic acid and CaCl_2 also showed a valley-type trend, with almost no variation with the increase in concentration of CaCl_2 . The lovastatin yield increased in a parabolic way with the increase in concentration of acetic acid. The maximum value of the lovastatin yield was ~10.1 mg/gds with acetic acid concentration of 25 μL and CaCl_2 concentration of 2.19 g/L (fourth subplot of Fig. 2).

From the analysis of variance it can be seen that the model represented by equation (1) was highly significant with a p value of 0.00001. Also the R^2 (determination coefficient) value for the lovastatin yield was 0.9214, indicating that 92.14% of the total variation in the observed response value could be explained by the model, or by experimental parameters and their interactions (Table 4). The coefficient estimated in the

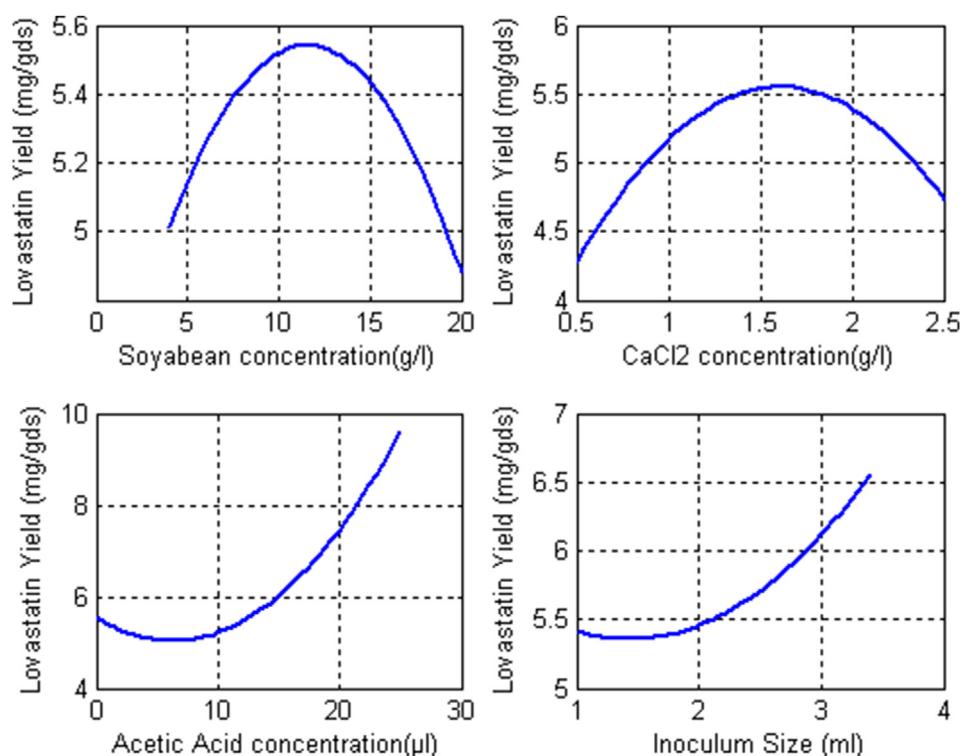


Fig. 1 – Response of the model depicting influence of individual variables.

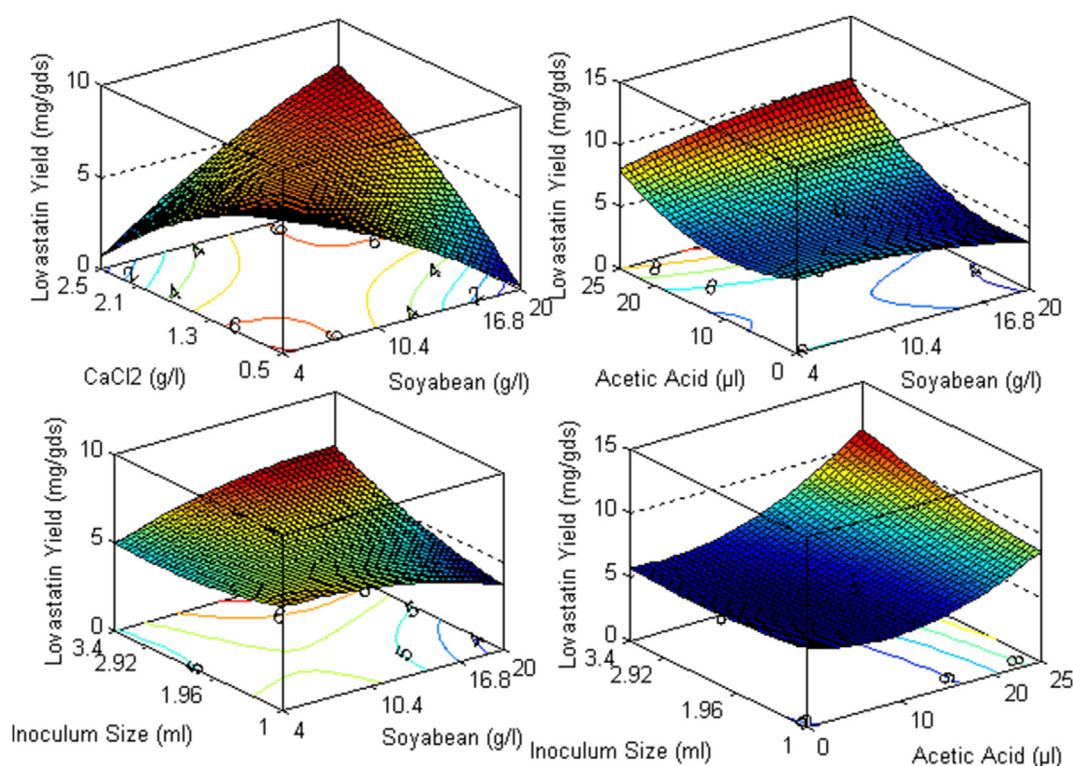


Fig. 2 – 3D Response surface and contour subplots showing the relative effect of tested variables.

Table 3 – Regression coefficient results from the data of central composite designed experiments for lovastatin yield.

Values	Coefficients	Standard error	t value	p value
Constant	26.3668	4.8142	5.48	0.00006
Soybean meal (g/L)	−0.8767	0.2943	−2.98	0.00936
CaCl ₂ (g/L)	−8.5303	2.1415	−3.98	0.00120
Acetic acid % (v/v)	−0.5716	0.1904	−3.00	0.00894
Inoculum size % (v/v)	−6.6755	1.8441	−3.62	0.00252
Soybean meal × CaCl ₂	0.4544	0.0662	6.87	0.00001
Soybean meal × acetic acid	0.0114	0.0066	1.72	0.10620
Soybean meal × inoculum size	0.1114	0.0551	2.02	0.06149
CaCl ₂ × acetic acid	0.0883	0.0551	1.60	0.13029
CaCl ₂ × inoculum size	2.3688	0.4595	5.16	0.00012
Acetic acid × inoculum size	0.0598	0.0459	1.30	0.21274
Soybean meal × soybean meal	−0.0084	0.0067	−1.26	0.22813
CaCl ₂ × CaCl ₂	−0.9783	0.3962	−2.47	0.02604
Acetic acid × acetic acid	0.0133	0.0025	5.32	0.00009
Inoculum size × inoculum size	0.3415	0.2751	1.24	0.23361

regression model for lovastatin production is presented in Table 3. The table shows that the linear effect of the acetic acid concentration affected the yield most. This implies that the acetic acid concentration played an important role in lovastatin yield, because it can act as a carbon source and as a pH regulator. The quadratic effects of acetic acid and soybean meal were more significant than those of the other factors. The interactive effects of CaCl₂ with soybean meal and inoculum size were found to be more significant than the other interactive effects.

Several attempts have been made for statin production under SSF conditions. Complex and simple carbon sources and several agro-industrial residues such as wheat bran, bagasse, barley, soybean meal, gram bran and fruit waste, and supplementation with organic and inorganic nitrogen and mineral salts have been used to synthesize and optimize the lovastatin yield. Among several types of agro-industrial waste tested, wheat bran has emerged as the most suitable substrate for lovastatin production by different microorganisms. Valera et al [1] have reported lovastatin yield of 16.65 mg/gds with *Aspergillus flavipes* BICC 5174 and wheat bran as substrates. With the same substrate, Patil et al [21] have reported a lovastatin yield of 12.5 mg/gds with *Aspergillus terreus* PM3. As far as *Monascus* strains are concerned, few attempts have been made to screen and optimize the lovastatin yield. *M. purpureus* MTCC 369 along with rice supplemented with ammonium

Table 4 – Analysis of variance for response surface quadratic model.

Sum of squares	Degree of Freedom	f value	p value	Mean square	R ²	Adjusted R ²
3.99	14	12.56	0.00001	0.27	0.9214	0.8480

chloride yielded 3.422 mg/gds [14]. Panda et al [22] have also attempted to optimize the lovastatin yield (2.80 mg/gds) with combination of *M. purpureus* MTCC 369 + *M. ruber* MTCC 1880 and rice supplemented with malt extract and dextrose as substrate. Xu et al [23] have reported a maximum yield of lovastatin (4–6 mg/gds) from *M. ruber* when rice was supplemented with soybean powder, sucrose, yeast extract, glycerol, sodium nitrate and acetic acid. They used one variable at a time to optimize the yield.

The present study successfully established the optimum conditions for lovastatin yield with wheat bran as substrate and absence of citrinin. The cumulative effects of variables such as soybean meal, inoculum size, and acetic acid and CaCl_2 concentrations were also explored and encouraging results were obtained. Such a high yield of lovastatin with a wild strain of *M. sanguineus* opens the door for further research in this direction. The efficiency of this strain can meet the requirements for the current demand for production of natural statins.

3.3. Spectrometric analysis of lovastatin

Purified lovastatin from *Monascus*-fermented substrate had maximum UV absorption near to 238 nm (Fig. 3 inset). Such an absorption band corresponded to a π – π^* transition owing to the conjugated double bonds. This compound was analyzed with FT-IR and LC-MS. The FT-IR spectra of the purified compound (Fig. 3) showed characteristic peaks at 3470.25 cm^{-1} (non-hydrogen-bonded free O–H stretching vibration also appeared to the left of this peak, indicating the alcohol/phenolic group); 2260.69 cm^{-1} (methyl and methylene C–H asymmetric stretching); 2071.67 cm^{-1} (weak

overtone for the aromatic ring); 1645.23 cm^{-1} (lactone and ester carbonyl stretch without any *cis* or *trans* conformations); 1491.53 cm^{-1} (band due to methylene scissoring and methyl and methylene bending vibration); 1342.55 cm^{-1} (weak peak for C–O–H bending vibrations); 850 cm^{-1} (alcohol C–OH stretch); 804.67 cm^{-1} (strong peak for tri-substituted olefinic C–H); 758.32 cm^{-1} (meta di-substituted benzene rings); and 686.53 cm^{-1} (C–H out of plane bending vibrations). All these FT-IR spectra confirmed the presence of lovastatin in the sample. A lovastatin-containing lactone ring gave a characteristic peak at 1645.23 cm^{-1} [24].

LC-MS data of the *Monascus*-fermented substrate (Fig. 4) gave major fragment ions at m/z 360.47, 388.47, 344.73 and 104.8, along with a base peak at m/z 242.4. Minor fragment ions including m/z 427.27, 460.33, 285, 254.6, 238.27 and 91.13 were also detected. These results suggested that an identical ion at m/z 285 was formed after the loss of the ester side-chain and thereby a dehydration process. A base peak at m/z 242.4 appeared to be due to the neutral gain of 44 Da from the ion at m/z 199, as reported in most of the literature. In particular, the ion at m/z 243 was observed only from lovastatin, as it has an OH group. An equivalent ion at m/z 229 was also detected for a compound that also had a hydroxylactone moiety. The ion at m/z 242.4 generated an abundant product ion at m/z 143 and minor ions at m/z 157 and 128. This indicated that the OH group was involved in the formation of the ion at m/z 243 via intramolecular proton transfer, and facilitated the neutral loss of 60 Da (CH_2COOH or a combination of CH_2 , CO and H_2O) from the ion at m/z 303. The m/z of 460.33 corresponded to the carboxylic acid form of lovastatin, whereas m/z of 427.27 contained the Na salt of lovastatin. After removal of the OH group from parent M^+ 405, 388.47 fragment ions were

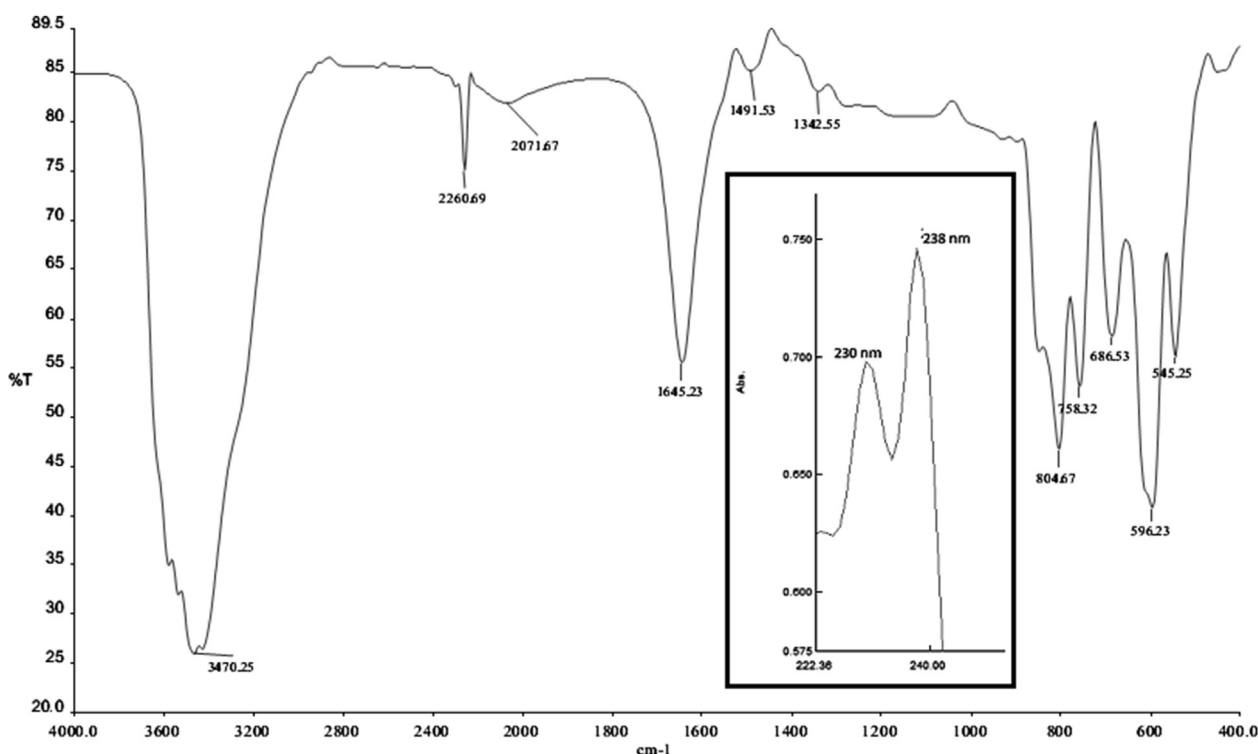


Fig. 3 – Fourier transform infrared spectra and UV absorption spectra of lovastatin (inset).

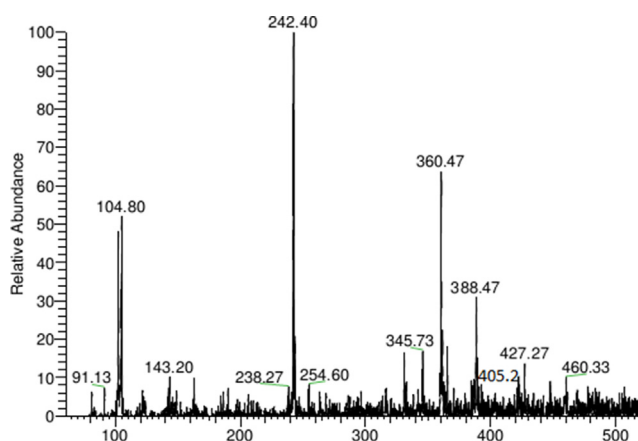


Fig. 4 – Mass spectra of lovastatin.

observed. 360.47 mass fragments were observed after removal of the propyl group. 345.75 mass fragments were obtained after removal of the $\text{CH}_3\text{CH}_2\text{CHO}$ group, and similarly, a 330 mass fragment was observed after elimination of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CHO}$ group. 104 corresponded to aromatic ketone compounds. All obtained fragments had a resemblance to the results of Wang et al [10].

3.4. Validation of the model

The optimized value of the lovastatin yield from the second order quadratic model defined by equation (2) was 20.04 mg/gds with a soybean concentration of 20 g/L, CaCl_2 concentration of 2.5 g/L, acetic acid concentration of 25 μL , and inoculum size of 3.4 mL. The experiment was carried out under the above-predicted optimum conditions in order to check the validity of the model and a yield of 19.05 mg/gds was obtained. This demonstrated a good match between the experimental and predicted values, thus substantiating the proposed model.

Monascus sp. has primarily been exploited for pigment production and there are only few reports available for the production of lovastatin. Mostly, *Aspergillus* strains have been exploited for lovastatin production. To the best of our knowledge, this is the first study of optimization of lovastatin production from *M. sanguineus*. This study also established the non-existence of citrinin in the samples under experimental conditions. This work can be considered as a model approach for enhanced yield of secondary metabolites from *M. sanguineus* strain for industrial usage.

Conflict of interest

The authors declare that they have no conflict of interest.

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