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Comparative studies on the production of statins using different microbial strains

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Abstract

In this study, we aimed to increase the yield of mevastatin and lovastatin in solid state fermentation (SSF) using three different fungi, *Aspergillus terreus* MTCC 279, *Penicillium citrinum* MTCC 1751, and *Penicillium brevicompactum* MTCC 549. Initially, we screened various substrates for maximizing the production of mevastatin and lovastatin, and barley powder was found to be the most suitable substrate for *A. terreus.* We applied response surface methodology (RSM) to determine the optimal parameters for initial moisture content, temperature, and inoculum size. The use of RSM resulted in maximizing the production of mevastatin to 288.13 mg/gram dry substrate (gds) and lovastatin to 329.51 mg/gds. For further studies, we used the high yielding strain of *A. terreus* with barley as a substrate. In the validation experiment, the maximum amount of mevastatin (297.98 mg/gds) and lovastatin (340.71 mg/gds) was obtained using *A. terreus* with barley as a substrate. This is the first report on the simultaneous production of mevastatin and lovastatin using a high yielding strain in SSF.

Key words: solid state fermentation, mevastatin, *Aspergillus terreus*, *Penicillium citrinum*, *Penicillium brevicompactum*, response surface methodology

Introduction

Statins, a class of fungal secondary metabolites, have attracted considerable attention because of their ability to influence the *de novo* synthesis of endogenous cholesterol, which is ~66% of the cholesterol in human body (Alberts, 1988). All statins possess a common main polyketide portion, i.e., a hydroxy-hexahydro naphthalene ring, to which different side chains are linked at C6 and C8. Lovastatin contains a methyl butyric side chain and a β-hydroxylactone, and the latter is present as the corresponding β-hydroxy acid in the pharmaceutically active drug.

Both mevastatin and lovastatin are derived from fungal sources, while pravastatin and simvastatin are chemical modifications of mevastatin and lovastatin, respectively (Chakravarti and Sahai, 2004; Alberts et al., 1980a). Mevastatin was first isolated as an antifungal metabolite from the fermentation broth of *Penicillium brevicompactum* in 1976 (Endo et al., 1977). Mevastatin was then commercially producing using *Penicillium citrinum* (Endo et al., 1977; Endo et al., 1986), and its production by solid state fermentation (SSF) was reported by Biocon using a large-scale solid matrix bioreactor (Suryanarayan et al., 2001); however, there is not much readily available information on the procedure that was followed by Biocon. Reducing the cost of fermentation by finding inexpensive and efficient substrates and optimization of the mode of fermentation is of high importance. Solid state fermentation (SSF) offers the advantage of using inexpensive agro-industrial residues as substrates, which also act as a support matrix and provide nutrients that are required for the growth of fungi. SSF provides higher yields compared to conventional submerged fermentation (SmF) and provides a natural habitat for fungal organisms; moreover, it requires lower capital investment, generates a lower volume of polluting effluents, and requires minimal instrumentation (Perez-Guerra et al., 2003).

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Lovastatin is formed when extracting mevinolinic acid from the cultivation media (Kumar et al., 2000; Barrios-González and Mejía, 2007; Bizukojc and Ledakowicz, 2009). Lovastatin, monacolin J, monacolin L, and mevastatin can be produced from *Monascus ruber* (Endo, 1979), *P. brevicompactum*, and *A. terreus* (Alberts et al., 1980b). Moreover, fermentation-derived lovastatin acts as a precursor for simvastatin, which was obtained via the selective enzymatic deacylation of lovastatin (Daborah et al., 1992), and is a powerful semi-synthetic statin that is commercially available as Zocor™.

In this study, we evaluate the simultaneous production of mevastatin and lovastatin by three different microorganisms, i.e., *A. terreus*, *P. citrinum*, and *P. brevicompactum*, in SSF using various substrates. The simultaneous production of mevastatin and lovastatin, allows reduction in space, time, and the amount of medium used for fermentation. We further optimized it with the best microorganism (*A. terreus*) and substrate (barley powder) using response surface methodology (RSM), which is a powerful and an efficient mathematical approach that is extensively applied for optimizing the fermentation process (Syed et al., 2014). RSM provides information about the interaction between process parameters as well as information that is necessary for both design and process optimization. We optimized the factors that have significant effects on production (initial moisture content, incubation temperature, and inoculum size) using a central composite design (CCD) and response surface analysis. The effects of process parameters, such as the initial moisture content, temperature and inoculum volume, were investigated using RSM.

Materials and methods

Media components

We purchased potato dextrose agar (PDA), glucose, malt extract, magnesium sulfate, and manganese sulfate from Hi-Media Limited, India. All substrates for SSF were purchased from local markets located at Chidambaram, Tamil Nadu, India. HPLC-grade acetonitrile (ACN) and ethanol were purchased from Rankem, New Delhi, India. All chemicals (Hi-Media, Mumbai, India) were of analytical grade. Mevastatin was purchased from Sigma Chemicals, Bangalore, India, and pharmaceutical grade lovastatin (lactone form) tablets containing 40 mg of lovastatin per tablet were obtained from Merck Laboratories.

Culture maintenance

For this study, *A. terreus*, *P. citrinum*, and *P. brevicompactum* were obtained from the Institute of Microbial Technology (MTCC), Chandigarh, India. The cultures were maintained on potato dextrose agar slants at 25 °C for 12 days, and the slants were sub-cultured every 30 days. A spore suspension (10^6 spores/ml) prepared from such slants was used to inoculate 100 ml of the sterile seed medium in 250 ml flasks at 25 °C, 120 rpm for 2 days in an incubator shaker. We used the potato dextrose broth as a seed medium for all the three cultures/fungal strains that were tested.

Fermentation procedure

Fifteen different solid substrates such as besan flour, ragi flour, millet powder, rice flour, wheat bran, black gram powder, green gram powder, green peas powder, yellow peas powder, white bean powder, rice bran, long grain rice, barley powder, soya bean powder, and sago powder were individually screened using statin-producing microorganisms such as *A. terreus*, *P. citrinum*, and *P. brevicompactum*. The experiments were performed in 250 ml Erlenmeyer flasks containing 5 g of a substrate with the initial moisture content of 66% (w/w). The contents of the flasks were mixed and autoclaved at 121 °C at 15 psi for 20 min. The seed medium was inoculated with 10^6 spores/ml and incubated at 30 °C for 48 h. To inoculate the production medium, we used five percent of this preculture (glucose (5 g/100 ml), malt extract (5 g/100 ml), magnesium sulfate (0.2 g/100 ml), and manganese sulfate (0.1 g/100 ml)). Fermentation was performed at 30 °C for all the three microorganisms, i.e., *A. terreus*, *P. brevicompactum* (7 days), and *P. citrinum* (10 days), but at different incubation times (Syed et al., 2015).

Extraction of mevastatin and lovastatin

After fermentation, to obtain the intracellular product, the cultures were harvested and homogenized (using a motor pestle). For the analysis, a total of 2 g of dry substrate (dried in an oven) was used: 1 g from the fermentate was used to extract mevastatin and 1 g was used to extract lovastatin. We added equal volumes of ethanol to the substrates, and the suspensions were maintained in a rotary shaker that was incubated for 1 h at 200 rpm at 40 °C. The suspension was filtered using a Whatman filter paper, and then through a micro filter

(Millipore) having a pore diameter of 0.22 μm. Furthermore, using HPLC, we analyzed 20 μl of the filtrates for mevastatin and lovastatin.

HPLC analysis of mevastatin and lovastatin

Both mevastatin and lovastatin were analyzed in a Shimadzu HPLC (LC20 AT prominence) at 238 nm in the Luna C18 column having an ID of 250X 4.6 mm, UV detector (SPD 20 A) and a column oven (CTO-10 AS VP) at 45°C. The binary gradient system was used and the samples were manually injected using the Rheodyne injector of 20 μl. As the mobile phase, we used acetonitrile and 0.1% orthophosphoric acid in a 60:40 ratio. The eluent was pumped at a flow rate of 1.5 ml/min. For the standard curve, various concentrations of 20, 40, 80, 100, and 120 μg of mevastatin were dissolved in acetonitrile and analyzed using HPLC. The equation of the standard curve for the various concentrations of mevastatin (Y) versus the peak area (X) was $Y = 49870X$ with $R^2 = 0.9952$. Similarly, for the standard curve, various concentrations of lovastatin dissolved in acetonitrile were prepared and analyzed using HPLC. The equation of the standard curve for the various concentrations of lovastatin (Y) versus the peak area (X) was $Y = 44250X$ with $R^2 = 0.993$. During fermentation, lovastatin was produced as a mixture of lactone and free β-hydroxy acid form; therefore, the standards were prepared in both forms. Note that the retention time of lovastatin in its beta hydroxyacid form is 8 min.

Results

The screening of solid substrates in SSF using A. terreus, P. citrinum, and P. brevicompactum

The selection of a substrate for the SSF process depends primarily on its cost and availability and may involve screening of several starchy substrates that are locally available. Compared to SmF, the availability of water in substrates is an important factor in SSF. A lower moisture content within the substrate limits the growth and metabolism of the microorganisms. Note that barley powder was found to be the best substrate (Tables 1–3).

Comparison of A. terreus, P. citrinum, and P. brevicompactum efficiency in mevastatin and lovastatin production from different substrates

When barley flour was used as a substrate, *A. terreus* yielded 272.16 mg/gds (grams of dry substrate) of mevastatin and 206.8 mg/gds of lovastatin, followed by 110.92 mg/gds of mevastatin and 71.76 mg/gds of lovastatin for long grain rice (Table 1). Note that *A. terreus* produced mevastatin in almost all the substrates that were tested. As shown in Table 1, barley (288.13 mg/gds

Table 1. Results of various solid substrates for the production of mevastatin and lovastatin using *A. terreus*

Substrates	Lovastatin [mg/gds]	Mevastain [mg/gds]
Green peas	13.3	122.28
Long grain rice	71.76	110.92
Ragi	148.32	69.92
Barley powder	206.8	272.16
Beans	19.08	75.24
Sago powder	80.6	90
Green gram	4.48	74.36
Besan flour	9.6	70.36
White bean powder	2.32	67.48
Millet	0.48	71.24
Rice bran	2.04	68.16
Yellow peas	56.68	82.24
Rice flour	30.31	81.6
Wheat bran	5.36	72.64

Table 2. Results of various solid substrates for the production of mevastatin and lovastatin using *P. citrinum*

Substrates	Lovastatin [mg/gds]	Mevastain [mg/gds]
Green peas	0	0
Long grain rice	2.87	74.87
Black gram	11.96	68.44
Ragi	31.92	217.05
Barley powder	8.16	75.92
Beans	10.64	71.52
Sago powder	1.96	0
Green gram	0.4	66.76
Besan flour	2.52	0
White bean powder	0	0
Millet	0	72.6
Rice bran	0	0
Yellow peas	0.44	0
Rice flour	0	70.4
Wheat bran	4.92	66.96

Table 3. Results of various solid substrates for the production of mevastatin and lovastatin using *P. brevicompactum*

Substrates	Lovastatin [mg/gds]	Mevastain [mg/gds]
Green peas	0	0
Long grain rice	0	0
Black gram	0	0
Ragi	3.64	125.07
Barley powder	6.52	68.72
Beans	0	0
Sago powder	0	0
Green gram	0	0
Besan flour	0	0
White bean powder	0	0
Millet	0	67.12
Rice bran	3.64	68.6
Yellow peas	0	0
Rice flour	1.36	67.36
Wheat bran	0	0

of mevastatin and 329.51 mg/gds of lovastatin) and long grain rice (long grain rice is four to five times as long as normal rice) were found to be better than other substrates that were used for mevastatin and lovastatin production. Hence, we performed further parameter optimization for *A. terreus* using barley as a substrate.

Among the fungal stains that were tested, *P. citrinum* resulted in the maximum mevastatin production of 217.05 mg/gds and lovastatin production of 31.92 mg/gds when ragi flour was used as a substrate (Table 2). *P. citrinum* was tested with 15 different substrates, but only few showed a positive result for mevastatin and lovastatin production, whereas other substrates afforded poor yields. *P. citrinum* showed better yield for mevastatin compared to *P. brevicompactum* for ragi flour. The second highest mevastatin and lovastatin production levels were obtained for barley powder, i.e., 75.92 mg/gds and 8.16 mg/gds, respectively. Note that the maximum production levels were obtained after 10 days of incubation.

P. brevicompactum produced mevastatin and lovastatin in only five of the tested substrates: ragi flour, barley powder, millet, rice bran, and rice flour. Note that the highest amounts of mevastatin and lovastatin were produced when *P. brevicompactum* was grown in a medium where ragi flour was used as a substrate (Table 3). Furthermore, compared to *A. terreus* and *P. citrinum*, *P. brevicompactum* was the weakest producer of mevastatin and lovastatin. The above results demonstrate that the three microorganisms that were tested have different incubation periods, and the maximum yield of mevastatin and lovastatin production was achieved in 7 days for *A. terreus*, 10 days for *P. citrinum*, and 7 days for *P. brevicompactum*.

A. terreus was superior in mevastatin and lovastatin production compared to the other two microorganisms that were tested. *P. citrinum* produced more mevastatin and lovastatin compared to *P. brevicompactum*. All the three microorganisms produced higher titers of mevastatin and lovastatin in starch-rich substrates. *P. citrinum* and *P. brevicompactum* produced a maximum yield in the same substrate (ragi), whereas *A. terreus* produced the maximum yield in barley powder. Table 3 shows *P. brevicompactum* grown on all the substrates, but the production is negligible. In almost all the substrates, *P. citrinum* growth was observed; however, poor production of mevastatin and lovastatin was observed in only a few of them such as green peas, sago powder, besan flour, white bean powder, rice bran, and yellow peas (Table 1). Note that *A. terreus* is a potential producer of lovastatin and the same *A. terreus* was used for producing mevastatin; thus, it has shown dominance over the other two microorganisms (Syed et al., 2015).

Optimization of mevastatin and lovastatin production using Response surface methodology

To optimize the production of mevastatin and lovastatin using RSM, we studied the effect of various process parameters such as initial moisture content, incubation temperature, and inoculum size. Based on initial experimental results (3 fungal strains and 15 substrates), further optimization was performed using the high yielding stain of *A. terreus* and barley powder as a substrate. Note that these process parameters have considerable influence on the fungal growth and secondary metabolite production. To determine the optimum for each significant variable, we applied a full factorial central composite design (2^3) and RSM. To identify the optimum levels for different process parameters influencing mevastatin and lovastatin production, SSF was performed in conical flasks containing previously optimized nutrients. We studied the individual and inter-

Table 4. Experimental range and levels of the independent variables for *A.terreus*

Variables	Media [g/l]	−1.682	−1	0	+1	+1.682
X_1	temperature	21.6	25	30	35	38.4
X_2	moisture content	1	3	6	9	11
X_3	inoculum size	1	3	6	9	11

Table 5. Full factorial central composite design matrix of three variables in coded and natural units along with the observed responses

Runs	Temperature	Initial moisture	Innoculum size	Mevastatin [mg/gds]		Lovastatin [mg/gds]	
				experimental	predicted	experimental	predicted
1	−1	−1	−1	227.71	228.019	312.92	286.783
2	1	−1	−1	80.84	131.336	86.24	134.503
3	−1	1	−1	83.05	123.784	8.93	84.004
4	1	1	−1	119.31	134.111	176.94	125.768
5	−1	−1	1	98.41	127.021	8.28	95.352
6	1	−1	1	87.47	90.148	145.95	106.777
7	−1	1	1	68.32	61.236	2.52	-9.842
8	1	1	1	88.27	131.373	133.59	195.627
9	−1.682	0	0	67.4	51.124	17.83	-38.383
10	1.682	0	0	73.92	28.802	0.9	6.343
11	0	−1.682	0	265.63	237.746	291.73	267.401
12	0	1.682	0	218.27	184.761	198.04	171.598
13	0	0	−1.682	246.02	203.719	270.59	260.529
14	0	0	1.682	135.58	116.487	199.01	158.300
15	0	0	0	281.8	280.991	309.75	306.767
16	0	0	0	267.22	280.991	289.27	306.767
17	0	0	0	278.24	280.991	293.62	306.767
18	0	0	0	272.08	280.991	297.26	306.767
19	0	0	0	287.94	280.991	312.48	306.767
20	0	0	0	288.13	280.991	329.51	306.767

active effects of process parameter (temperature, moisture content, and inoculums size) variables by conducting the fermentation run at randomly selected and different levels of all three factors (Table 4). The response was measured as mevastatin and lovastatin production levels. Moreover, to optimize the process parameters, 20 experiments with six replicates were performed. These conditions were tested at five coded levels, namely, −1.682, −1, 0, +1 and +1.682, which are listed in Table 4. The optimal levels of the selected variables were obtained by solving the regression equation using MATLAB and by analyzing the response surface and contour plots. Table 4 lists the coded values and the levels of the variables temperature, moisture content, and inoculum size. Table 5 lists the experimental and predicted values along with the CCD experimental design. A multiple regression analysis of the CCD experimental design yields the following quadratic polynomial equation for the biosynthesis for mevastatin and lovastatin:

$$Y = 280.991 - 6.63657x_1 - 15.7525x_2 - 25.9340x_3 + - 85.2161x_{12} - 24.6559x_{22} - 42.7402x_{32} + 26.7525x_1x_2 + + 14.9525x_1x_3 + 9.61250x_2x_3 \quad (1)$$

$$Y = 306.767 + 13.2972x_1 - 28.4822x_2 - 30.3930x_3 + - 114.123x_{12} - 30.8536x_{22} - 34.4192x_{32} + 48.5113x_1x_2 + + 40.9262\ x_1x_3 + 24.3963x_2x_3 \quad (2)$$

Tables 6A and 6B list the results of the regression analysis from the data of central composite design experiments. The analysis of variance of the quadratic regression model demonstrated that in Eq. (1 and 2) was a highly significant. It is evident from the Fisher's *F*-test with a very low probability value [(*P* model > *F*) = 0.0001]. To check the significance of each coefficient, Student's *t*-test and *P* values were used as tools, which also indicated the interaction strength between each independent variable. The larger the magnitude of the *t*-value and the smaller the *P* value, the more significant was the corresponding coefficient. Tables 7A and 7B list the analysis of variance for mevastatin and lovastatin production by *A. terreus*. The linear effect of x_3 and the squared effect of x_{12}, x_{22}, and x_{32} were found to be significant as the *P* value was less than 0.05 for mevastatin (Table 7A). Note that the squared effect of x_{12} and x_{32} and the interactive effect of x_1x_2 were found to be significant as the *P* value was less than 0.05 for lovastatin (Table 7B). The correctness of fit of the model based on RSM can be verified by the coefficient of determination (R^2), which provides a measure of how much the variation in the observed response values can be explained via experimental factors and their interactions. The closer the R^2 value is to 1, the stronger the model, and the better it can predict the response. In this case, the value of the determination coefficient (R^2 = 91.29%) indicated that only 8.71% of the total variations were not explained by the model for mevastatin. Moreover, the value of the determination coefficient (R^2 = 89.23%) indicated that only 10.77% of the total variations were not explained by the model for lovastatin. This model resulted in six response surface plots (3D) with their corresponding contour plots (2D). The response surface plots with the contours of the calculated model for mevastatin and lovastatin production were generated by the Design-Expert software and are shown in Figure 1 (A, B, and C) and Figure 2 (A, B, and C). Three-dimensional graphs were generated from the pairwise combination of the three factors, and elliptical contours were obtained when there was a perfect interaction between the substrates. Figure 1 (A, B and C) shows the contour and response surface plot of the temperature, moisture content, and inoculum size for the mevastatin production at a fixed substrate concentration. The elliptical contour indicated greater interaction among the independent variables. The response surface plot and the contour plot shown in Figure 2 (A, B, and C) show the effects of temperature, inoculum size, and moisture content. The elliptical contour indicated an interaction among the variables x_1, x_2, and x_3 for lovastatin production at a fixed substrate concentration.

Effect of temperature on mevastatin and lovastatin production levels

We performed experiments with five different temperature ranges (21.6, 25, 30, 35, and 38.4 °C) using RSM. The results of the study suggested that the temperature had influenced the mevastatin and lovastatin production by *A. terreus*. When the experimentation was carried out at 30 °C, any further increase in temperature lead to a decrease in mevastatin and lovastatin production (Tables 1 and 2).

Effect of initial moisture on mevastatin and lovastatin production levels

We used an initial moisture content of 66% for mevastatin and lovastatin production. The moisture content influenced the production of mevastatin and lovastatin. An increase in the moisture content to above optimal levels (moisture content 4.52 ml) caused aeration problems. A decrease in the moisture content below optimal levels led to poor growth in the fungal strain and lower productivity. Note that the optimum moisture content depends on the nature of microorganisms and the substrate that was used.

Effect of inoculum size on mevastatin and lovastatin production levels

The inoculum volume influenced the production levels of mevastatin and lovastatin. First, the inoculum volume (5 ml) was kept constant for all three microorganisms. The inoculum volume (48-h old) used in RSM was as follows: 1, 3, 6, 9, and 11 ml. The optimal inoculum volume was found to be 4.82 ml for *A. terreus*, and the 48 h seed growth culture supported the maximum production with mevastatin and lovastatin yield for *A. terreus*.

Table 6A. Estimated regression coefficients for mevastatin production by *A. terreus*

Term	Coefficient	Standard error of coefficient	*t*-value	*P*-value
Constant	280.991	15.202	18.484	0.000
A	−6.637	10.086	−0.658	0.525
B	−15.753	10.086	−1.562	0.149
C	−25.934	10.086	−2.571	0.028
A*A	−85.216	9.819	−8.679	0.000
B*B	−24.656	9.819	−2.511	0.031
C*C	−42.740	9.819	−4.353	0.001
A*B	26.752	13.178	2.030	0.070
A*C	14.953	13.178	1.135	0.283
B*C	9.613	13.178	0.729	0.482

R^2 = 91.29%

Table 6B. Estimated regression coefficients for lovastatin production by *A. terreus*

Term	Coefficient	Standard error of coefficient	*t*-value	*P*-value
Constant	306.77	22.98	13.348	0.000
A	13.30	15.25	0.872	0.404
B	−28.48	15.25	−1.868	0.091
C	−30.39	15.25	−1.993	0.074
A*A	−114.12	14.84	−7.688	0.000
B*B	−30.85	14.84	−2.079	0.064
C*C	−34.42	14.84	−2.319	0.043
A*B	48.51	19.92	2.435	0.035
A*C	40.93	19.92	2.054	0.067
B*C	24.40	19.92	1.225	0.249

R^2 = 89.23%

Validation of the models

The validation experiment was carried out in a 250 ml Erlenmeyer flask under the optimum combination of the process parameters predicted by the polynomial model. The optimum values predicted by the model for mevastatin were as follows: temperature 29.23°C, moisture content 4.52 ml, and inoculum size 4.82 ml. The maximum mevastatin production of 290.156 mg/gds was predicted by the model. In fact, we obtained mevastatin production of 297.98 mg/gds, which is even higher than the predicted production level, thereby validating the proposed model. The optimum values predicted by the model for lovastatin were as follows: temperature 28.04°C, moisture content 2.38 ml, and inoculum size 2.68 ml. The maximum response of 338.22 mg/gds of lovastatin production was predicted using this model. Moreover, using the predicted model, the lovastatin production of 340.71 mg/gds was obtained, which is also higher than the predicted values.

Discussion

A substrate that provides all the required nutrients to the microorganism for an enhanced yield of the product could be considered as an ideal substrate (Pandey et al., 2001). Glucose, a form of starch present in the tested

Table 7A. Analysis of variance for mevastatin production by *A. terreus*

Source	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*-value	Source
Regression	9	145700	145700	16189	11.65	0.000
Linear	3	13176	13176	4392	3.16	0.073
A	1	602	602	602	0.43	0.525
B	1	3389	3389	3389	2.44	0.149
C	1	9185	9185	9185	6.61	0.028
Square	3	124271	124271	41424	29.82	0.000
A*A	1	91882	104652	104652	75.32	0.000
B*B	1	6064	8761	8761	6.31	0.031
C*C	1	26325	26325	26325	18.95	0.001
Interaction	3	8253	8253	2751	1.98	0.181
A*B	1	5726	5726	5726	4.12	0.070
A*C	1	1789	1789	1789	1.29	0.283
B*C	1	739	739	739	0.53	0.482
Residual error	10	13893	13893	1389		
Lack-of-fit	5	13535	13535	2707	37.81	0.001
Pure error	5	358	358	72		
Total	19	159594				

Table 7B. Analysis of Variance for lovastatin production by *A. terreus*

Source	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*-value	Source
Regression	9	263008	263008	29223	9.20	0.001
Linear	3	26109	26109	8703	2.74	0.099
A	1	2415	2415	2415	0.76	0.404
B	1	11079	11079	11079	3.49	0.091
C	1	12615	12615	12615	3.97	0.074
Square	3	199912	199912	66637	20.99	0.000
A*A	1	171883	187692	187692	59.11	0.000
B*B	1	10956	13719	13719	4.32	0.064
C*C	1	17073	17073	17073	5.38	0.043
Interaction	3	36988	36988	12329	3.88	0.045
A*B	1	18827	18827	18827	5.93	0.035
A*C	1	13400	13400	13400	4.22	0.067
B*C	1	4761	4761	4761	1.50	0.249
Residual error	10	31752	31752	3175		
Lack-of-fit	5	30636	30636	6127	27.46	0.001
Pure error	5	1116	1116	223		
Total	19	294760				

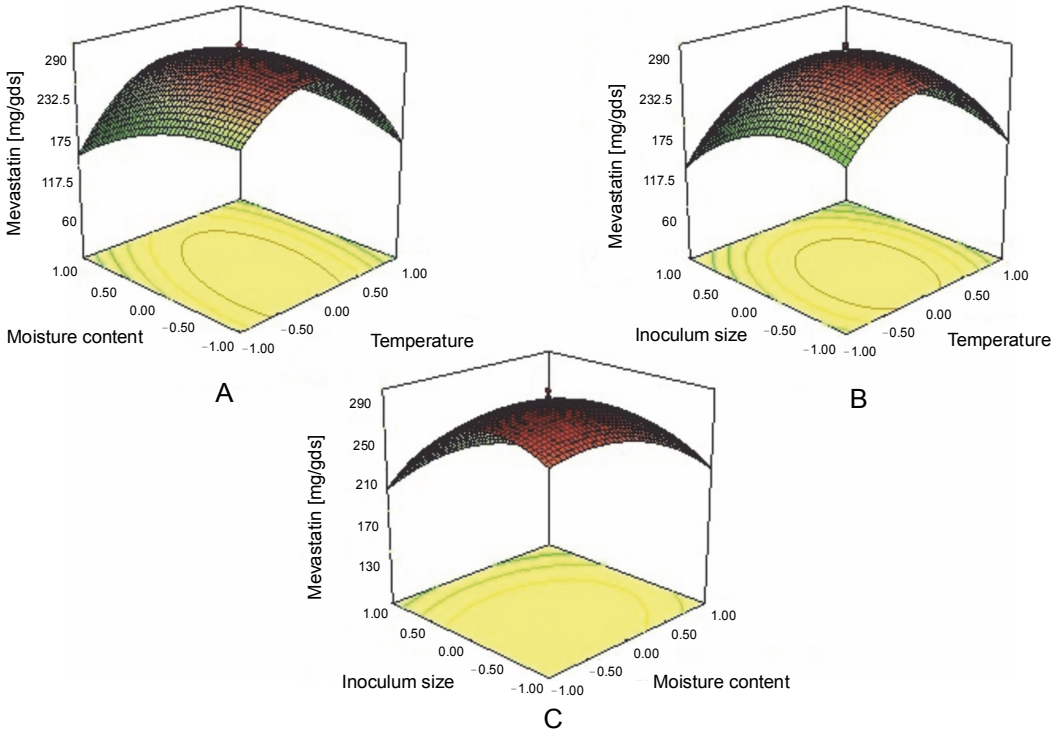

Fig. 1. A, B, C) response and contour plot showing the effects of interaction between the substrates temperature, moisture content and inoculum size for mevastatin

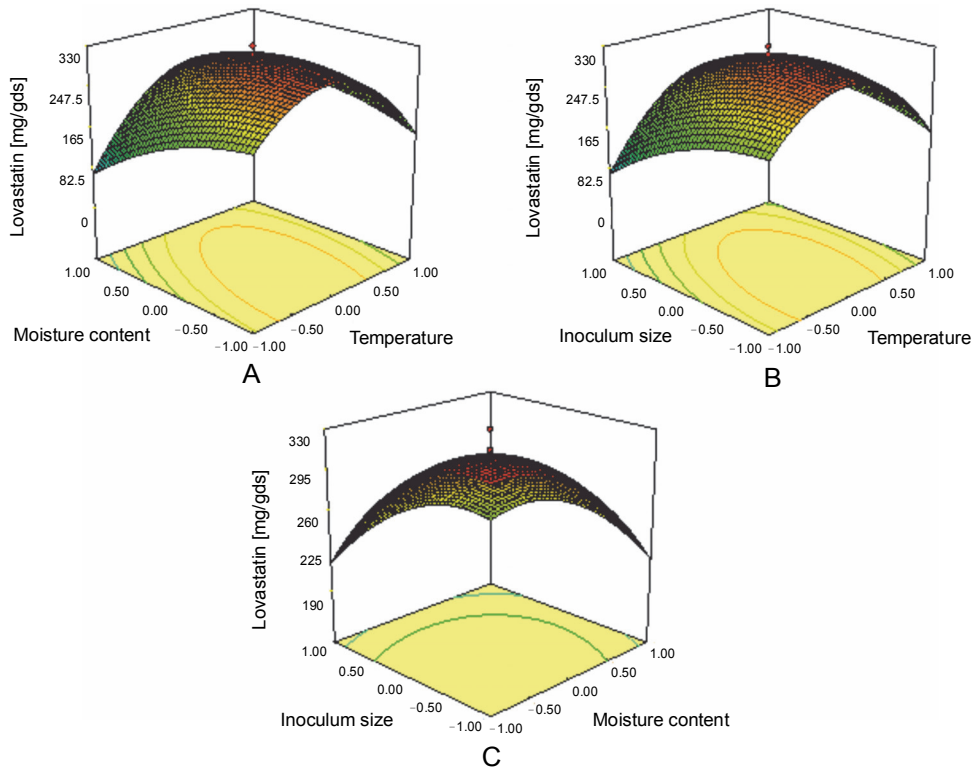

Fig. 2. A, B, C) response and contour plot showing the effects of the interaction between the substrates temperature, moisture content and inoculum size for lovastatin

substrates, was found to be an excellent supplement for growth, possibly because of its rapid utilization by the fungal culture. However, the production of secondary metabolites has been found to be independent of growth (Drew and Wallis, 1983). Glucose, which is essential for growth, helps microorganisms to adapt to new environments. Once it is exhausted from the medium, the solid substrate (barley powder in this case) acted as a carbon source. SSF on inert supports is very convenient for microorganisms to grow and may help increase the production rate (Oojikaas et al., 2000). As described above, glucose was used as a nutrient supplement for the fungi to adapt to a new environment. Moreover, a solid substrate such as barley powder provided support for the microorganism to grow. Biocon produced lovastatin using *A. terreus* on wheat bran SSF (Suryanarayan, 2003). Note that a novel SSF process, using a high-density polyurethane foam (PUF) as an inert support, was developed for producing lovastatin nearly 10 years ago (Baños et al., 2009). A comparison of lovastatin yield of 19.95 mg/gdc, obtained using SSF with PUF, resulted in 30 times higher levels than those obtained using liquid submerged fermentation (SmF; 0.57 mg/ml) (Baños et al., 2009). In this study, SSF resulted in a higher production of statins than SmF. Previously, RSM was used to optimize the culture medium for producing lovastatin by *M. ruber* and the maximum lovastatin yield obtained was 131 mg/l (Chang et al., 2002). The principal nutrients on the isolated *Monascus pilosus* mutant produced the highest level of lovastatin, i.e., 725 mg/l in the peptone medium containing glucose and glycerol (Miyake et al., 2006).

Higher yields of lovastatin, monacolin J, pravastatin, and mevastatin were produced by *A. terreus* strains compared to strains belonging to *Monascus* species (Manzoni and Rollini, 2002). *A. terreus* UV 1718 grown in a medium optimized by RSM and supplemented with mycological peptone produced a maximum of 3723.4 ± 49 μg/g DFM. The yield of lovastatin increased 2.6-fold compared to production in un-optimized media (Pansuriya and Singhal, 2010). The maximum yield of lovastatin (2.9 mg/g dry substrate) using rice as a substrate was reported by Wei et al. (2007) and was achieved by incubating *A. terreus* ATCC 20542 for 11 days at the following optimized SSF parameters: 50–60% initial moisture content, pH 5.5, and incubation temperature 28°C. A concentration of lovastatin of 6 mg/g was reported in the SSF of *M. ruber* as a result of adding soybean powder, glycerol, sodium nitrate, and acetic acid into the production medium (Xu et al., 2005). The optimal values for lovastatin were as follows: temperature 28.04°C, moisture content 2.38 ml, and inoculum size 2.68 ml. In this study, we obtained a lovastatin production of 340.71 mg/gds, which is higher than that reported previously (Syed et al., 2015).

To optimize the production of mevastatin by *P. citrinum*, the Plackett–Burman and central composite rotatable design have been used (Chakravarti and Sahai, 2002a). The optimization resulted in the production of 456 mg of mevastatin. In another study, a mutant strain of *P. citrinum* grown in a chemically defined medium yielded 145 mg/l of mevastatin. Note that the addition of KH_2PO_4 into the production medium did not increase the mevastatin production, while the addition of a surfactant, Tween 80, increased the mevastatin level to 175 mg/l (Chakravarti and Sahai, 2002b). The optimum values for mevastatin production obtained in this study were as follows: temperature 29.23°C, moisture content 4.52 ml, and inoculum size 4.82 ml. The amount of mevastatin produced was 297.98 mg/gds, which was higher than that reported in the literature.

The production of mevastatin by *P. brevicompactum* WA 2315 has been previously optimized using SSF (Shaligram et al., 2009). The feeding of glycerol (20% v/v) into the growth medium on day 3 resulted in further improvement of mevastatin yield to 1406 μg/gds. A twofold higher mevastatin concentration (1200 mg/l) than the control (without the addition of Triton X 100) has been reported by Choi et al. (2004) at the 10^{th} day of fermentation. An increase in mevastatin (ML-236B) production was also achieved by introducing a whole mevastatin biosynthetic gene cluster or the regulatory gene mlcR into the *P. citrinum* high-production mevastatin strain (Baba et al., 2009). In this case, glycerol was the most significant contributor to the mevastatin production rate. In another study, the addition of supplements at specific concentrations (glycerol 3.86 mg/100 ml, $CuCl_2 \cdot 2H_2O$ 0.102 mg/100 ml, $FeSO_4 \cdot 7H_2O$ 0.036 mg/100 ml, K_2HPO_4 0.003 mg/100 ml and $MgSO_4 \cdot 7H_2O$ 0.09 mg/100 ml) resulted in a mevastatin production of 771 μg/gds (Shaligram et al., 2008).

The moisture content has an important role in SSF, although fermentation with relatively no moisture to very high initial moisture levels has been reported (Prior

et al., 1992). However, it has been observed that high moisture content leads to the aggregation of substrate particles, poor aeration, and an occurrence of anaerobic conditions, while very low moisture content restricts fungal growth (Gervais and Molin, 2003). The optimal values of initial moisture content were determined at 4.52 ml for mevastatin and 2.38 ml for lovastatin, which resulted in the maximum levels of lovastatin and mevastatin production.

Conclusions

In this study, we focused on the simultaneous production of mevastatin and lovastatin, which act as excellent platforms for an industrial scale production. The most significant result of this study was the adoption of easily available starchy substrates for producing mevastatin and lovastatin using fungal strains of *P. citrinum*, *P. brevicompactum*, and *A. terreus*. In the fermentative production of mevastatin and lovastatin, ragi flour was found to be ideal for *P. citrinum*, and *P. brevicompactum* and barley powder proved to be the best substrate for *A. terreus*. Among the three microorganisms that were tested, the higher yielding microorganism *A. terreus* was selected for further parameter optimization using RSM. Under optimal conditions, the maximum mevastatin (288.13 mg/gds) and lovastatin (329.51 mg/gds) yields were achieved for *A. terreus*. In the validation experiment, the amount of mevastatin produced was 297.98 mg/gds and lovastatin of 340.71 mg/gds for *A. terreus*.

References

Alberts A.W., Chen J., Kuron G., Hunt V., Huff J., Hoffman C., Rothrock J., Lopez M., Joshua H., Harris E., et al. (1980a) *Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol lowering agent*. Proc. Natl. Acad. Sci. USA. 77: 3957–3961.

Alberts A.W., Chen J., Kuron G., Hunt V. (1980b) *Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent*. Proc. Natl Acad. Sci. USA: 77: 3957–3961.

Alberts A.W. (1988) *Discovery, Biochemistry and biology of lovastatin.* Am. J. Cardiol. 62: 10–15.

Baba S., Abe Y., Suzuki T., Ono C., Iwamoto K., Nihira T., Hosobuchi M. (2009) *Improvement of compactin (ML-236B) production by genetic engineering in compactin high-producing Penicillium citrinum.* Appl. Microbiol. Biotechnol. 83: 697–704.

Baños J.G., Tomasini A., Szakács G., Barrios-González J. (2009) *High lovastatin production by Aspergillus terreus in solid-state fermentation on polyurethane foam: an artificial inert support*. J. Biosci.Bioeng. 108 (2): 105–110.

Barrios-González J., and Mejía A. (2007) *Production of antibiotics and other commercially valuable secondary metabolites*. [in:] *Current developments in solid-state fermentation*. Eds. Pandey A., Larroche C., Soccol C.R., Rodríguez-León J.A. Chapter 14, Springer, London, Heidelberg: 262–296.

Bizukojc M., Ledakowicz S. (2009) *Physiological, morphological and kinetic aspects of lovastatin biosynthesis by Aspergillus terreus*. Biotechnol. J. 4: 647–664.

Chakravarti R., Sahai V. (2002a) *Optimization of compactin production in chemically defined production medium by Penicillium citrinum using statistical methods*. Process Biochem. 38: 481–486.

Chakravarti R., Sahai V. (2002b) *A chemically-defined medium for production of compactin by Penicillium citrinum*. Biotechnol. Lett. 24: 527–530.

Chakravarti R., Sahai V. (2004) *Compactin – a review*. Appl. Microbiol. Biotechnol. 64: 618–624.

Chang Y.N., Huang J.C., Lee C.C., Shih I.L., Tzeng Y.M. (2002) *Use of response surface methodology to optimize culture medium for production of lovastatin by Monascus ruber.* Enzyme Microbial Technol. 30: 889–894.

Choi D., Cho K., Cha W.S., Ryu S.R. (2004) *Effect of Triton X-100 on compactin production from Penicillium citrinum*. Biotechnol. Bioprocess Eng. 9: 171–178.

Daborah R.A., Lein J., Conder M.J., Tewalt G.L. (1992) *Enzymatic deacylation of simvastatin*. British Patent GB2255974.

Drew S.W., Wallis D.A. (1983) *Regulation of secondary metabolism and keys to its manipulation*. [in:] *Secondary metabolism and differentiation in fungi*. Marcel Dekker, NewYork: 35.

Endo A., Kuroda M., Terahara A., Yoshio T., Chhiro T. (1977) *Physiologically active substances and fermentative process for producing the same*. US Patent 4,049,495.

Endo A., Hasumi K., Yamada A., Shimoda R., Takeshima H. (1986) *The synthesis of compactin (ML-236B) and monacolin K in fungi*. J. Antibiot. 39: 1609–1610.

Endo A. (1979) *Monacolin K, a new hypocholesterolemic agent produced by a Monascus species*. J. Antibiot. 32: 852–854.

Gervais P., Molin P. (2003) *The role of water in solid-state fermentation*. Biochem. Eng. J. 13: 85–101.

Kumar M.S. Jana S.K., Senthil V., Shashanka S., Kumar S.V., Sadhukhan A.K. (2000) *Repeated fed-batch process for improving lovastatin production*. Process Biochem. 36: 363–368.

Manzoni M., Rollini M. (2002) *Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs*. Appl. Microbiol. Biotechnol. 58(5): 555–564.

Miyake T., Uchitomi K., Zhang M.Y., Kono I., Nozaki N., Sammoto H., Inagaki K. (2006) *Effects of the principal nu-*

trients on lovastatin production by Monascus pilosus. Biosci. Biotechnol. Biochem. 70(5): 1154–1159.

Oojikaas L.P., Weber F.J., Buitelaar R.M., Tramper J., Rinzema A. (2000) *Defined media and inert supports: their potential as solid-state fermentation production systems*. Trends Biotechnol. 18: 356–360.

Pandey A., Szakacs G., Soccol C.R., Rodriguez-Leon .J.A., Soccol V.T. (2001) *Production, purification and properties of microbial phytases*. Bioresource Technol. 77: 203–214.

Pansuriya R.C., Singhal R.S. (2010) *Response surface methodology for optimization of production of lovastatin by solid state fermentation*. Braz. J. Microbiol. 41: 164–172.

Perez-Guerra N., Torrado-Agrasar A., Lopez-Macias C., Pastrana L. (2003) *Main Characteristics and applications of solid substrate fermentation*. EJEAFChem. 2: 1–8.

Prior B.A., Preez J.C.D., Rein P.W. (1992) *Environmental parameters*. [in:] *Solid substrate cultivation*. Elsevier, London. pp. 65.

Shaligram N.S., Singh S.K., Singhal R.S., Szakacs G., Pandey A. (2008) *Compactin production in solid-state fermentation using orthogonal array method by P. brevicompactum*. Biochem. Eng. J. 41: 295–300.

Shaligram N.S., Singh S.K., Singhal R.S., Pandey A., Szakacs G. (2009) *Compactin production studies using Penicillium brevicompactum under solid-state fermentation conditions*. Appl. Biochem. Biotechnol. 159(2): 505–520.

Suryanarayan S., Sircar A., Khedkar A.P., Subramaniyam P., Tambe S.P., Anand K.N.S., Ramkrishna M., Ramavana G. (2001) *Novel process for the manufacture and purification of compactin*. WO, 081611.

Suryanarayan S. (2003) *Current industrial practice in solid state fermentations for secondary metabolite production; the Biocon India experience*. Biochem. Eng. J. 13: 189–195.

Syed M.B., Seraman S., Rajendran A., Thangavelu V. (2014) *Valorization of agricultural residues for compactin production by Aspergillus terreus MTCC 279 in mixed substrate solid state fermentation*. Waste Biomass Valorization 5: 715–724.

Syed M.B., Rajasimman M. (2015) *Fermentative production and optimization of mevastatin in submerged fermentation using Aspergillus terreus*. Biotechnol. Rep. 6: 124–128.

Wei P., Xu Z., Cen P. (2007) *Lovastatin production by Aspergillus terreus in solid-state fermentation*. J. Zhejiang. Univ. Sci. A. 8(9): 1521–1526.

Xu J., Wang Q.J., Jia X.Q., Sung C.K. (2005) *Enhanced Lovastatin production by solid state fermentation of Monascus rubber*. Biotechnol. Bioprocess Eng. 10: 78–84.