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RESPONSE SURFACE METHODOLOGY FOR OPTIMIZATION OF PRODUCTION OF MEVASTATIN BY SOLID STATE FERMENTATION USING SESAME OIL CAKE

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ABSTRACT

Mevastatin, also known as compactin or ML-236B is a member of the class of statins belonging to the polyketide group. Polyketides are rich sources of pharmaceuticals, including antibiotics, anticancer drugs, and cholesterol-lowering drugs, immunosuppressants and other therapeutics [1]. This study was performed for the production of mevastatin using sesame oil cake as substrate with *Pencillium citrinum* MTCC 1256 by Solid State FermentationThe present study was carriemevastatin by solid state fermentation with *Pencillium citrinum* MTCC 1256 using sesame oil cake. Different agro residues as substrates and different organisms were screened and various physico-chemical parameters were optimized. In this paper we explained in detail on the four significant factors i.e, fermentation time (X_1, days), temperature (X_2,^oC), pH (X_3), nitrogen source (X_4,%w/v), which significantly influence the production of mevastatin were selected by using Plackett-Burman statistical design. These variables were further optimized using a 2^4 full factorial CCD (Central Composite Design) and a second order polynomial model equation was obtained. In the present study the value of the regression coefficient R^2 = 0.9938 which indicates that 99.38% of the variability in the response could be explained by the model. The adjusted R^2 value is 0.9859 which is also very high to advocate the significance of the model.

KEYWORDS:Mevastatin, *Pencillium citrinum* MTCC 1256, Fermentation time, Temperature, pH, Nitrogen source, Carbon source.

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INTRODUCTION

Mevastatin, also known as compactin or ML-236B is a member of the class of statins belonging to the polyketide group. Polyketides are rich sources of pharmaceuticals, including antibiotics, anticancer drugs, and cholesterol-lowering drugs, immune suppressants and other therapeutics. [1] One of the major causes of death in developed countries is coronary heart disease. Approximately 10.8% of all deaths are caused due to this disease. Coronary heart disease actually is a wide assortment of diseases. The basic manifestation of many of them is atherosclerosis, caused when fatty deposit called plaque buildup on the inner walls of arteries. Cholesterol is a major component of the atherosclerotic plaque. Many scientists believe that a high level of cholesterol in the blood is a major contributor to the development of atherosclerosis. In humans, the greater part of the cholesterol in the body is synthesized, mostly in the liver, the search for drugs to inhibit cholesterol biosynthesis has long been pursued as a means to lower the level of plasma cholesterol and so it helps to prevent and treat atherosclerosis.[2] Mevastatin is a specific and potent inhibitor of cholesterol biosynthesis, and also acts as an antifungal agent.[3] Mevastatin competitively inhibits the regulatory enzyme, 3-hydroxy-3-methylglutaryl-coenzyme- A-reductase. Mevastatin is also a precursor of pravastatin, which is also an anti-hypercholesterolemic agent. Among the few commercially used microbial strains for the production of mevastatin are *Penicillium citrinum*, *P. cyclopium* and *Aspergillus terreus.* [3, 4, and 5]The application of statistical experimental design techniques in fermentation process development can result in improvement of product yield, reduce process variability, give closer confirmation of the output response to nominal and reduce overall costs. Conventional practice of single factor reduce optimization by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all the factors involved. This method is also time consuming and requires a number of experiments to determine optimum levels, which are unreliable. These limitations can be eliminated by optimizing all the affecting parameters collectively by RSM. RSM can be used to evaluate the relative significance of several factors even in the presence of complex interactions[6]. Statistical approach for the optimization of the media effectively tackles the problem, which involves the specific design of the experiment that effectively decreases the error in determining the effect of variables[7]. In this paper we used the Planket Burman design to find the significant variables that influence the production of mevastatin and these variables were further optimized using an RSM, incorporating a 2^4 full factorial CCD.

MATERIALS AND METHODS

MATERIALS

Microorganisms

Penicillium citrinum MTCC 1256, Procured from MTCC, Institute of Microbial Technology, Chandigarh, India.

Maintenance of culture

The culture was maintained on potato dextrose agar (PDA) at 4^{o}C and the subculture was done for every three weeks in the laboratory. Fresh slants were prepared for running experiments.

Substrate

Agro industrial residues such as wheat bran, green gram husk, sesame oil cake, and coconut oil cake were brought from local market in Visakhapatnam, A.P, and grounded well and sieved to remove unwanted materials.Locally available wheat bran, green gram husk, sesame oil cake, coconut oil cake were grounded well and sieved to remove unwanted materials. Initially, all the substrates were screened to determine the potentiality of the above substrates for mevastatin production using SSF method.

METHODS

Inoculum preparation

Cultures of *Penicillium citrinum* and *Penicillium brevicompactum* was grown on potato dextrose agar (PDA) slants at 30°C and 25°C for 5 and 7 days respectively and maintained at 4°C. Distilled water was added to each slant and the spores were scrapped by using an inoculation loop.

Fermentation procedure for mevastatin production

Five grams of substrate in total was taken and supplement solution was added to it with initial moisture content being 60% (v/w). The pH of the supplement solution was maintained at 6 using 2N H_3PO_4. All media components were sterilized at 121°C for 15 min and inoculated with 3ml of seed culture. The fermentation was carried out at 26°C for 7 days

Extraction

At the end of SSF, fermented solid culture was adjusted to pH 6.5 with either diluted acid (aq.H_3PO_4) or alkali (aq.NaOH) and then 25 ml absolute ethyl alcohol was added to it for extraction by keeping in an orbital shaking incubator at 180 rpm for 1h.The residue was filtered with filter paper and then centrifuged at 6000 rpm for 15 min. Then the supernatant was collected and analyzed for quantitative determination of mevastatin.

EXPERIMENTAL DEISGNS

The Plackett-Burman experimental design (PBD, 7/8 runs)

Out of the variables studied, the variables which influenced the fermentation significantly were studied by PBD. PBD is a two level factorial design which allows the investigation of '*n*-1' variables with at least '*n*' experiments. The main effect was calculated as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (−1) of each factor .This model describes no interaction among factors and it is used to screen and evaluate the important factors that influence production of mevastatin. The factors that have a confidence level above 95% are considered the most significant factors that affect the mevastatin production. The main effect of the fermentation components, regression coefficient, F values and P values of the factors were investigated. The design matrix generated by 7/8 runs of PBD of was shown in Table 1.

Table 1
The design matrix for PBD.

Runs	A	B	C	D	E	F	G	Final response
1.	-	-	-	+	+	-	-	_
2.	+	-	-	-	-	-	+	_
3.	-	+	-	-	+	+	+	_
4.	+	+	-	+	-	+	-	_
5.	-	-	-	+	-	+	+	_
6.	+	-	-	-	+	+	-	_
7.	-	+	-	-	-	-	-	_
8.	+	+	+	+	+	-	+	_

Central composite design (CCD)

Once the variables having significant influence on the response were identified, the variables were further optimized using response surface methodology and a CCD. Twenty six experiments were conducted with 16 factorial points (2^4), 8 axial points (2×4) and 2 replications at the center points (n_o=2) according to CCD and the concentration of mevastatin was measured in each case. The design matrix generated by CCD was shown in Table 2.

Table 2
Central composite design consisting of 26 experiments for the study of four experimental factors in coded values.

Runs	A	B	C	D	Final response
1.	-1	-1	-1	-1	_
2.	-1	-1	-1	1	_
3.	-1	-1	1	-1	_
4.	-1	-1	1	1	_
5.	-1	1	-1	-1	_
6.	-1	1	-1	1	_
7.	-1	1	1	-1	_
8.	-1	1	1	1	_
9.	1	-1	-1	-1	_
10.	1	-1	-1	1	_
11.	1	-1	1	-1	_
12.	1	-1	1	1	_
13.	1	1	-1	-1	_
14.	1	1	-1	1	_
15.	1	1	1	-1	_
16.	1	1	1	1	_
17.	-2	0	0	0	_
18.	2	0	0	0	_
19.	0	-2	0	0	_
20.	0	2	0	0	_
21.	0	0	-2	0	_
22.	0	0	2	0	_
23.	0	0	0	-2	_
24.	0	0	0	2	_
25(c)	0	0	0	0	_
26(c)	0	0	0	0.	_

RESULTS AND DISCUSSION

Response surface methodology (RSM)
The Plackett-Burman experimental design (PBD, 7/8 runs):

To evaluate the significant fermentation variables Plackett- Burman statistical design was used. This is a two level factorial design which allows the investigation of '*n*-1' variables with at least '*n*' experiments. The main effect is calculated as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (−1) of each factor .This model describes no interaction among factors and it is used to screen and evaluate the important factors that influence the production of mevastatin. Variables to be monitored in Plackett-Burman statistical design for the production of mevastatin are fermentation time, temeperature, pH ,moisture content, inoculums volume, carbon source, nitrogen source. The factors that have confidence level above 95% are considered the most significant factors that affect the mevastatin production. The effect of the fermentation factors were investigated (Fig 1). Shows the pareto chart for the determination of the significant factors which influenced the production of mevastatin.

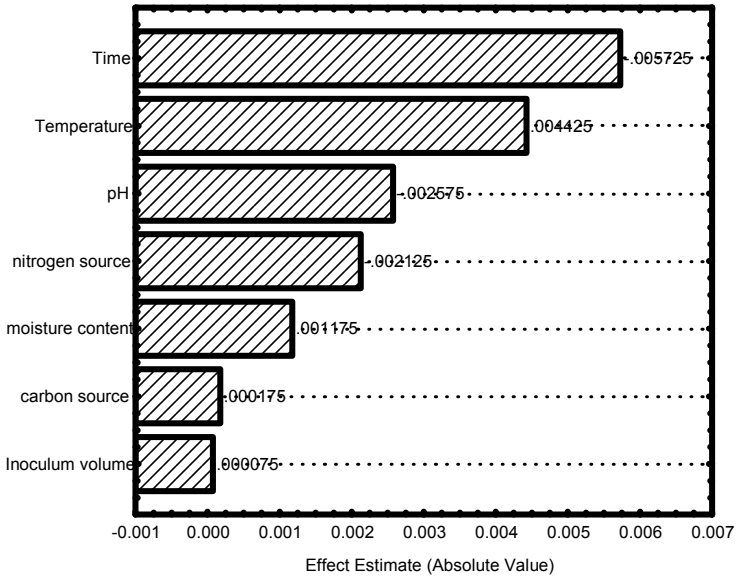

Figure 1
Pareto chart for the determination of the significant factors Which influenced the production of mevastatin.

From the pareto chart of PBD the variables that were selected in the screening were fermentation time (days), temperature (^{o}C), pH, and nitrogen source (%w/v).

Central composite design (CCD)

With the individual optimum values obtained from the previous experiments, which were obtained on one parameter at a time basis, an RSM with2^4 full factorial, central composite design was used to get the accurate optimized values which were independent and which gave maximum production of mevastatin. The central values (zero level) chosen for experimental design were fermentation time (X_1), temperature (X_2), pH (X_3), and nitrogen source (X_4). Table 3 shows the range and coded levels of these variables.

Table 3
Experimental range and levels of independent variables for CCD

Variable	Name	Range and levels				
		-2	-1	0	1	2
X_1	Fermentation time (days)	3	4	5	6	7
X_2	Temperature (^{O}C)	24	26	28	30	32
X_3	pH	4	5	6	7	8
X_4	Nitrogen source (%w/v)	0.6	0.8	1.0	1.2	1.4

With these variables, twenty six experiments were conducted with 16 factorial points (2^4), 8 axial points (2×4) and 2 replications at the center points (n_o=2) according to CCD and the concentration of mevastatin was measured in each case and tabulated in Table 4.

Table 4

Central composite design consisting of 26 experiments for the study of four experimental factors in coded and real values and comparison of experimental and predicted values of concentration of mevastatin.

Run No.	Fermentation time (days) (X_1)	Temperature (^oC) (X_2)	pH (X_3)	Nitrogen source conc. (%w/v) (X_4)	Concentration of mevastatin (mg/ml)		
					Experimen-tal	Predicted	Residuals
1	4(-1)	26(-1)	5(-1)	0.8(-1)	0.040700	0.040481	0.000219
2	4(-1)	26(-1)	5(-1)	1.2 (1)	0.041000	0.041111	-0.000111
3	4(-1)	26(-1)	7 (1)	0.8(-1)	0.037000	0.037441	-0.000441
4	4(-1)	26(-1)	7 (1)	1.2 (1)	0.042990	0.042343	0.000647
5	4(-1)	30 (1)	5(-1)	0.8(-1)	0.037990	0.038359	-0.000369
6	4(-1)	30 (1)	5(-1)	1.2 (1)	0.043290	0.042747	0.000543
7	4(-1)	30 (1)	7 (1)	0.8 (-1)	0.033729	0.032851	0.000878
8	4 (-1)	30 (1)	7 (1)	1.2 (1)	0.041089	0.041511	-0.000422
9	6 (1)	26(-1)	5(-1)	0.8(-1)	0.035666	0.035385	0.000281
10	6 (1)	26(-1)	5(-1)	1.2 (1)	0.036590	0.037258	-0.000668
11	6 (1)	26(-1)	7 (1)	0.8(-1)	0.038440	0.038772	-0.000332
12	6 (1)	26(-1)	7 (1)	1.2 (1)	0.045144	0.044917	0.000227
13	6 (1)	30 (1)	5(-1)	0.8(-1)	0.036844	0.037280	-0.000436
14	6 (1)	30 (1)	5(-1)	1.2 (1)	0.043210	0.042911	0.000300
15	6 (1)	30 (1)	7 (1)	0.8(-1)	0.038167	0.038198	-0.000031
16	6 (1)	30 (1)	7 (1)	1.2 (1)	0.048093	0.048101	-0.000009
17	3(-2)	28 (0)	6 (0)	1.0 (0)	0.038221	0.038659	-0.000438
18	7(2)	28 (0)	6 (0)	1.0 (0)	0.040521	0.040153	0.000368
19	5 (0)	24(-2)	6 (0)	1.0 (0)	0.039763	0.039640	0.000123
20	5 (0)	32 (2)	6 (0)	1.0 (0)	0.040511	0.040703	-0.000192
21	5 (0)	28 (0)	4(-2)	1.0 (0)	0.042337	0.042182	0.000155
22	5 (0)	28 (0)	8 (2)	1.0 (0)	0.044109	0.044333	-0.000224
23	5 (0)	28 (0)	6 (0)	0.6 (-2)	0.031964	0.031814	0.000150
24	5 (0)	28 (0)	6 (0)	1.4 (2)	0.042129	0.042348	-0.000219
25	5 (0)	28 (0)	6 (0)	1.0 (0)	0.051700	0.051700	-0.000000
26	5 (0)	28 (0)	6 (0)	1.0 (0)	0.051700	0.051700	-0.000000

By applying multiple regression analysis on the experimental data using STATISTICA 6.0, the following second order polynomial equation was found to represent the concentration of mevastatin.

$$Y= -0.6071- 0.0031X_1 -0.0007X_2 -0.0021X_3 – 36.5473X_4 + 0.0059 X_1^2 + 0.0375 X_2^2 + 0.0211 X_3^2 +1.8064 X_4^2+ 0.0005X_1X_2+0.0016 X_1X_3 +0.0311X_1X_4 – 0.0003 X_2X_3 + 0.0470 X_2X_4 + 0.1068 X_3X_4 --------1$$

where, Y is the response which is the concentration of mevastatin and X_1, X_2, X_3, X_4 are the real values of test variables, i.e., fermentation time (days), temperature (^oC), pH, nitrogen source (%w/v) respectively. The coefficients of the regression model calculated were listed in Table 5.

Table 5
Regression data of the model

	Coefficient	Regression	Std .Error	t-value	p-value
Constant	b_0	-0.6071	0.038071	-15.9470	0.000000
X_1	b_1	-0.0031	0.000136	-22.6204	0.000000
X_2	b_2	-0.0007	0.000034	-21.2110	0.000000
X_3	b_3	-0.0021	0.000136	-15.5334	0.000000
X_4	b_4	-36.5473	1.358760	-26.8975	0.000000
X_1^2	b_{11}	0.0059	0.002653	2.2086	0.049335
X_2^2	b_{22}	0.0375	0.002014	18.6093	0.000000
X_3^2	b_{33}	0.0211	0.002762	7.6518	0.000010
X_4^2	b_{44}	1.8064	0.265251	6.8103	0.000029
X_1X_2	b_{12}	0.0005	0.000071	7.0754	0.000021
X_1X_3	b_{13}	0.0016	0.000142	11.3209	0.000000
X_1X_4	b_{14}	0.0311	0.014192	2.1889	0.051067[a]
X_2X_3	b_{23}	-0.0003	0.000071	-4.3484	0.001159
X_2X_4	b_{24}	0.0470	0.007096	6.6210	0.000038
X_3X_4	b_{34}	0.1068	0.014192	7.5257	0.000012

Insignificant (P ≥ 0.05)

$$Y= -0.6071- 0.0031X_1 -0.0007X_2 -0.0021X_3 – 36.5473X_4 + 0.0059 X_1^2 + 0.0375 X_2^2 + 0.0211 X_3^2 +1.8064 X_4^2+ 0.0005X_1 X_2+0.0016 X_1X_3– 0.0003 X_2X_3 + 0.0470 X_2X_4 + 0.1068 X_3X_4 \quad \text{..... 2}$$

Table 6
ANOVA for the model

Source of variation	SS	df	Mean square(MS)	F-value	*P*> F
Model	0.000568	14	0.0000406	0.00886	0.000001
Error	0.000004	11	0.00000036		
Total	0.000572	25			

df, degree of freedom; SS, sum of squares; F, factor F; P, probability. R^2=0.9938; Adj R^2:0.9859

The correlation coefficient (R^2) provides a measure of the model's variability in the observed response values. The closer the R^2value to 1, the stronger the model is and it predicts the response better. In the present study the value of the regression coefficient R^2 = 0.9938 which indicates that 99.38% of the variability in the response could be explained by the model. The adjusted R^2 value is 0.9859 which is also very high to advocate the significance of the model.

Interaction effects of fermentation variables

The concentration of mevastatin over different combinations of independent variables was visualized through three-dimensional view of response surface plots in Fig 2 to 7. All the plots were represented as a function of two factors at a time, holding the other factors fixed at zero level. All the response surface plots revealed that at low and high levels of the variables the concentration of mevastatin is maximal, however, it was noted that there existed a region where neither an increasing nor a decreasing trend in the concentration of mevastatin. This phenomenon conformed that there was an existence of optimum for the fermentation variables in order to maximize the concentration of mevastatin.

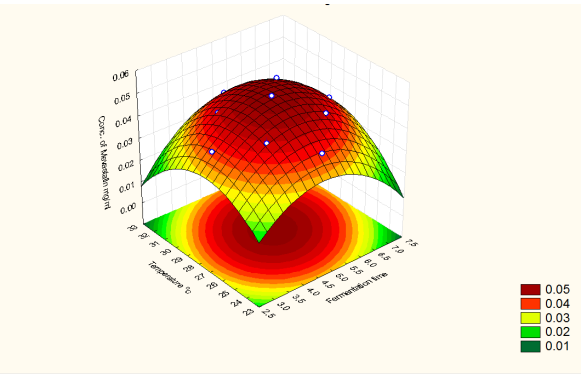

Figure 2
Effect of temperature and fermentation time on the concentration of mevastatin keeping nitrogen source and pH as constant.

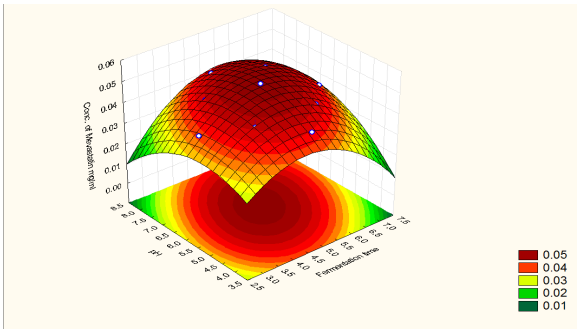

Figure 3
Effect of pH and fermentation time on the concentration of mevastatin keeping nitrogen source and temperature as constant.

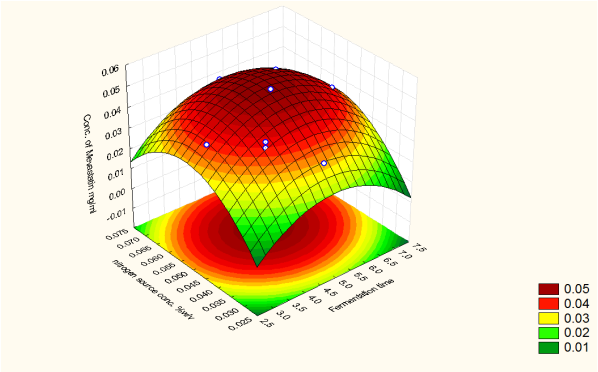

Figure 4
Effect of nitrogen source and fermentation time on the concentration of mevastatin keeping temperature and pH as constant.

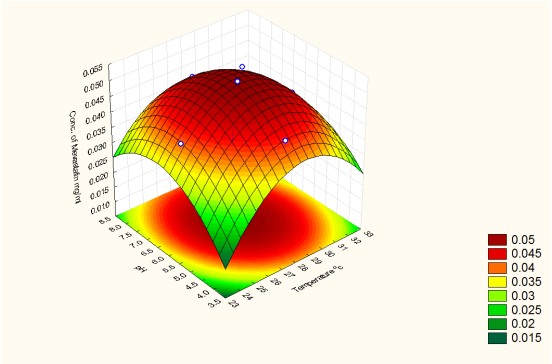

Figure 5
Effect of pH and temperature on the concentration of mevastatin Keeping nitrogen source and fermentation time as constant.

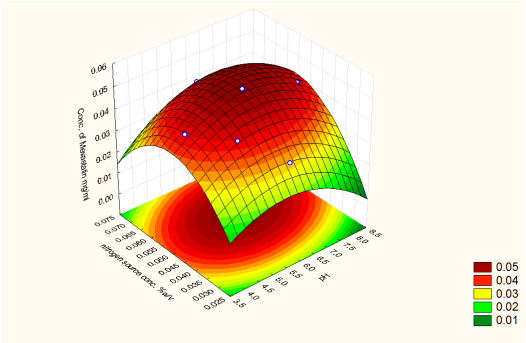

Figure 6
Effect of nitrogen source and pH on the concentration of mevastatin Keeping temperature and fermentation time as constant.

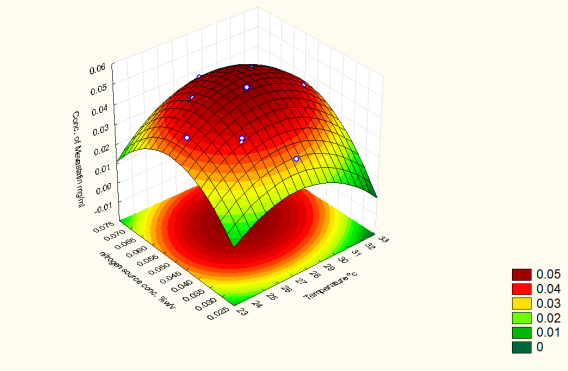

Figure 7
Effect of nitrogen source and temperature on the concentration of Mevastatin keeping temperature and fermentation time as constant.

CONCLUSION

The optimum values of the test variables obtained from RSM and CCD were fermentation time (5.17543 days), temperature (28.23067 ^{o}C), pH (6.28461) and nitrogen source (1.0848

%w/v). The maximum production of mevastatin that was predicted using the above values of the variables was 0.052383 mg/ml. The goodness of the fitness of the model was checked by the value of the correlation coefficient R^2 value. The correlation coefficient (R^2) = 0.9938 which indicates that 99.38% of the variability in the response could be explained by the model. The adjusted R^2 value is 0.9859 which is also very high to advocate the significance of the model.The parity plot shows a satisfactory correlation between experimental and predicted values of concentration of mevastatin produced wherein the points cluster around the diagonal line which indicated the good fit of the model.The optimum values of the test variables obtained from RSM and CCD were fermentation time: 5.17543 days , temperature: 28.23067 ^oC , pH: 6.28461, Nitrogen source :1.0848 %w/v.The model predicts that the maximum production of mevastatin that could be obtained using the above values of the variables was 0.052383 mg/ml.Thus the present study enables to obtain the maximum production of mevastatin by solid state fermentation with *Pencillium citrinum* MTCC 1256 from sesame oil cake.

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