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Fermentative production and optimization of mevastatin in submerged fermentation using *Aspergillus terreus*☆

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ABSTRACT

The main objective of the study is to enhance the mevastatin production using Plackett–Burman (PB) and central composite design (CCD) by *Aspergillus terreus* in submerged fermentation (SmF). Eight nutrients were chosen for a PB design with 12 experimental runs. A maximum mevastatin production of 170.4 mg L^{-1} was obtained in PB design. Response surface methodology (RSM) is a sequential procedure with an initial objective to lead the experimenter rapidly and efficiently along a path of improvement toward the general vicinity of the optimum. The individual and interactive effects of these variables were studied by conducting the fermentation run at randomly selected and different levels of all five factors. Experiments were conducted to optimize the medium constituents like glycerol, $CuCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$. At the optimum condition, a maximum mevastatin production of 701 mg L^{-1} was obtained.

1. Introduction

Mevastatin is the competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (3-HMG-CoA) reductase, the enzyme which is responsible for the conversion of 3-HMG-CoA into mevalonate. Mevastatin is capable of decreasing the level of the endogenous cholesterol in the animals and it is used against hypercholesterolemia. Coronary artery disease represents the most important causes of death which is caused by fatty depositions called plaque build-up on the inner walls of arteries and progression of atherosclerotic lesions, related to the primary risk factor of hypercholesterolemia. Statins which are produced directly from the fermentations are called as natural statins (lovastatin, mevastatin and pravastatin). Natural statins can be obtained from different genera and species of filamentous fungi. Generally statins are synthesized mainly by strains of *A. terreus* [4]. Statins interfere with events involved in bone formation and impede tumor cell growth. Recently, there are emerging interests in their use as anti-cancer agents based on preclinic evidence of their anti proliferative, pro-apoptotic, anti-invasive and radio sensitizing properties. Mevastatin production is affected by various nutritional and environmental factors either in submerged or solid state fermentation. Another active compound related to lovastatin (named monacolin K) was isolated from the fungus *Monascus ruber*. In addition to these products, several related metabolites were isolated from cultures of these fungi, which include dihydromevastatin from *Penicillium citrinum*, dihydromevinolin from *A. terreus*, monacolin J and L from *M. ruber* and dihydromonacolin L and monacolin X from a mutant strain of *M. ruber*, which are structurally related to each other [7–9].

The present study was aimed at screening of nutrients and optimization of the selected nutrients in SmF using Planckett–Burmann method and central composite design (CCD) to enhance the mevastatin production.

2. Materials and methods

2.1. Microorganisms and culture conditions

A. terreus was obtained from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained on potato dextrose agar slants at 4 °C and the slants were sub-cultured every month.

2.2. Media components

Potato dextrose agar (PDA), dextrose, galactose, mannose, sucrose, lactose, maltose, fructose, xylose, glycerol, peptone, soybean meal, yeast extract, malt extract, urea, ammonium chloride, ammonium sulphate, KH_2PO_4, $CaCl_3{\cdot}H_2O$, $CuCl_2{\cdot}2H_2O$,

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$FeSO_4 \cdot 7H_2O$ and $MgSO_4 \cdot 7H_2O$ were purchased from Hi-Media Limited, India. HPLC grade acetonitrile (ACN) and ethanol were purchased from Rankem, New Delhi, India. All the chemicals used were of analytical grade. Mevastatin standard was purchased from Sigma chemicals, Bangalore, India.

2.3. Inoculum preparation

Actively growing slants were used to prepare the spore suspension of *A. terreus* in sterile water. A spore suspension 10^6 spores mL^{-1} prepared from such slants was used to inoculated into conical flasks containing the seed medium: 100 g dextrose, 10 g peptone, 2 g KNO_3, 2 g $NH_4H_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.1 g $CaCl_2$ in 1000 mL of distilled water. The pH is adjusted to 6. These cultures were incubated at 30 °C for 48 h in a shaking incubator at 120 rpm. 5 percent of this pre-culture was used to inoculate into the production medium. Fermentation experiments were carried out at 30 °C for 7 days using *A. terreus* in 250 mL Erlenmeyer flasks containing 100 mL of production media, as per the experimental design.

2.4. Extraction of mevastatin

After fermentation, the harvested samples were homogenized to recover the product from broth. An equal volume of ethanol was added to fermentation broth and the suspension was kept in an incubated rotary shaker for 1 h at 200 rpm and 40 °C. The suspension was filtered through a Whatman filter paper and then through a micro filter (Millipore) of 0.22 mm pore diameter. 20 μL of the filtrate was analyzed for mevastatin using HPLC.

2.5. Analysis of mevastatin

Analysis of mevastatin was carried out in Shimadzu HPLC (LC20 AT prominence) at 238 nm in Luna C18 column of particle size 5m and (250 × 4.6) mm I.D, UV detector (SPD 20A) and the column oven (CTO-10 AS vp) at 45 °C. Binary gradient system with isocratic conditions was used and the samples were injected manually using Rheodyne injector of 20 μL. The mobile phase used was acetonitrile and 0.1% orthophosphoric acid in the ratio of 60:40 respectively. The eluent was pumped at a flow rate of 1.5 mL min^{-1}. Mevastatin standard was obtained from Sigma–Aldrich and various concentrations of mevastatin were prepared by dissolving in acetonitrile. The equation of the standard curve for the various concentrations of mevastatin (Y) versus peak area (X) is $Y = 49{,}870X$ with $R^2 = 0.9952$. The retention time of mevastatin elutes at 9.4 min of a fermented sample.

2.6. Plackett–Burman design

The PB design was proved to be a powerful tool to rapidly determine the effects of medium constituents on mevastatin production. In this part, the PB design was used to evaluate the relative importance of various nutrients for mevastatin production in batch fermentation. This design does not consider the interaction effects among the variables and is used to screen the important variables affecting the mevastatin production. Each variable was set at two levels, that is, high level and low level. The highest level of each variable was set far enough from the low level to identify which ingredients of the media have significant influence on the mevastatin production.

2.7. Central composite design and response surface methodology

Statistical methods provide an efficient alternative methodology for traditional one factor at a time approach to optimize a particular process by considering the mutual interactions among the variables and to give an estimate of the combined effects of these variables [5]. The total run number for CCD with respect to the concentration of the components is determined by full factorial points 2^k, where k is the number of variables, at centre points and two axial points for each variable ($a = 2^k/4$, which is =2 for $k = 3$). For statistical calculation, the test factors were coded by the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i}, i = 1, 2, 3, \ldots, k \quad (1)$$

where x_i in Eq. (1) is the dimensionless value of an independent variable, X_i is the real value of an independent variable; X_0 is the real value of the independent variable at the centre point; ΔX_i is the step change value. The experimental data obtained was fitted to the following quadratic polynomial equation:

$$Y = X_0 + \sum_i x_i + \sum_{ii} x_i^2 + \sum_{ij} x_i x_j \quad (2)$$

where yield (Y) is the predicted response variable in Eq. (2),i and j are the linear and quadratic coefficients respectively, β is the regression coefficient of the model and x_i, x_j ($i = 1, 3$; $j = 1, 3$, $i = j$) represent the independent variables (media components) in the form of coded values. The accuracy and general ability of the above polynomial model could be evaluated by the coefficient of determination R^2. Design expert software (version 6.0.5; Stat-Ease. Inc., MN, USA) was used for the regression and graphical analysis of the experimental data. The optimum levels of the selected variables were obtained by solving the regression equation using MATLAB software and by analyzing the response surface and contour plots.

3. Results and discussion

3.1. Screening of carbon and nitrogen sources for A. terreus in SmF

Various carbon and nitrogen sources were screened for the production of mevastatin. Initially various carbon sources have been tested for suitable growth of *A. terreus* and for maximum production of mevastatin. Mevastatin is the secondary metabolites and the maximum growth of the organism is required and which in turn depends on the type of carbon source. The various carbon sources used in our experiments are glucose, fructose, galactose, mannose, sucrose, lactose, maltose and xylose. Among the above carbon sources only few had influenced the growth of the organism and on production of mevastatin, no nitrogen sources were added. Glucose produced maximum mevastatin of 86.6 mg L^{-1}. Second highest production was obtained from lactose 68.74 mg L^{-1} of mevastatin as shown in Table 1. The glucose is selected as the sole carbon source for further optimization experiments. High productivity is only possible in the presence

Table 1
Production of mevastatin by various carbon sources using *A. terreus*.

S. no	Carbon sources (50 g L^{-1})	Mevastatin production (mg L^{-1})
1	Glucose	86.6
2	Galactose	45.2
3	Fructose	64.1
4	Sucrose	32.4
5	Lactose	68.74
6	Maltose	26.4
7	Mannose	58.3
8	Xylose	56.7
9	Glycerol	67.2

Table 2
Production of mevastatin by various nitrogen sources using *A. terreus*.

S. no	Nitrogen sources (50 g L^{-1})	Mevastatin production (mg L^{-1})
1	Peptone	22.1
2	Soybean meal	110.78
3	Yeast extract	67.39
4	Urea	69.78
5	Ammonium chloride	80.64
6	Ammonium sulphate	23.3
7	Malt extract	12.9

of sufficient amounts of carbon source and additional precursors in the medium.

Nitrogen sources influence the production of mevastatin; hence screening of various nitrogen sources was carried out keeping glucose and other medium constituents constant. Various nitrogen sources used in this study are peptone, soybean meal, yeast extract, urea, ammonium chloride, ammonium sulphate and malt extract. Among the above nitrogen sources soybean meal had a high influence of mevastatin production. When a nitrogen source was added there is a sharp increase in the production of mevastatin. The soybean meal and glucose combination produced maximum mevastatin of 110.78 mg L^{-1} as shown in Table 2.

3.2. Production of mevastatin by Plackett–Burman method using A. terreus

Initially the carbon and nitrogen sources were screened and among them the best carbon and nitrogen sources were selected for further optimization using PB design. PB design was adopted to optimize various medium components for the production of mevastatin fermentation by *A. terreus*. Various media components were investigated for their effect in the process of mevastatin production. Table 3 shows the medium components for the independent variables and their respective high and low concentrations used in PB optimization study with respect to mevastatin production. Eight nutrients such as (glucose, glycerol, soybean meal, KH_2PO_4, $CuCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, $CaCl_3{\cdot}H_2O$ and $MgSO_4{\cdot}7H_2O$) were chosen for PB design with a 12 experimental runs was shown in Table 4. PB design was used to study the effect among the eight constituents of the medium [6]. The effects of the variables and their significance in the production were found using their *P* values ($P < 0.05$). The effect of each variable was determined by the following equation:

$$E_{xi} = \frac{2(\Sigma H_{xi} - \Sigma L_{xi})}{N} \quad (3)$$

where E_{xi} is the concentration effect of the tested variable, H_{xi} and L_{xi} are the concentration of mevastatin at high level and low level of the same variable, among the variables tested, the variables which were found to be dominant on the production of mevastatin in their order are: glycerol, $CuCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, glucose, $CaCl_3{\cdot}H_2O$, soybean meal. In defined medium the carbon and nitrogen sources play an important role as a source of precursors for biomass and mineral salts acts as cofactors for the enzymatic reactions in mevastatin production. If the main effect of the components is negative, it indicates that the concentration required for enhancing mevastatin production is lower than the concentration used in the PB design. Similarly if the effects are positive, the amount of required for the production of mevastatin was higher than the concentration used in the design.

Table 3
Plackett–Burman design and media components for mevastatin production by *A. terreus*.

Variables	Medium components	Lower level (−1) (g 100 mL^{-1})	Higher level (+1) (g 100 mL^{-1})
A	Glucose	5	7
B	Soybean meal	4	6
C	Glycerol	0.5	0.7
D	KH_2PO_4	0.3	0.5
E	$CuCl_2{\cdot}2H_2O$	0.01	0.1
F	$CaCl_3{\cdot}H_2O$	0.02	0.1
G	$FeSO_4{\cdot}7H_2O$	0.02	0.2
H	$MgSO_4{\cdot}7H_2O$	0.01	0.2

Table 4
Plackett–Burman experimental design with 12 runs with corresponding mevastatin production.

S. no	A	B	C	D	E	F	G	H	Mevastatin (mg L^{-1})
1	+	+	–	+	+	+	–	–	100
2	+	–	+	+	+	–	–	–	95.6
3	–	+	+	+	–	–	–	+	170.4
4	+	+	+	–	–	–	+	–	148
5	+	+	–	–	–	+	–	+	139.2
6	+	–	–	–	+	–	+	+	95.6
7	–	–	–	+	–	+	+	–	146
8	–	–	+	–	+	+	–	+	136.5
9	–	+	–	+	+	–	+	+	112.4
10	+	–	+	+	–	+	+	+	108.4
11	–	+	+	–	+	+	+	–	0
12	–	–	–	–	–	–	–	–	0

The Pareto plots offer a convenient view of the results obtained by PB design. The main effects plot is very useful in determining the mevastatin production at intermediate levels of different combination of the independent variable. The pre-optimized medium was determined based on the main effects. The component having positive main effect were kept the concentration at higher levels and the component which is having negative main effect were kept the concentration at lower levels. The variables which are having positive main effects, means the concentration of glucose, soybean meal, KH_2PO_4, $CuCl_2{\cdot}2H_2O$ and $CaCl_3{\cdot}H_2O$ can be increased. The variables which are having negative effects mean that concentration of glycerol, $FeSO_4{\cdot}7H_2O$ and $MgSO_4{\cdot}7H_2O$ can be decreased. The maximum mevastatin production was 170.4 mg L^{-1} was obtained in PB optimization, hence it is proven that PB design is to evaluate the dominant factors present in the medium. Further optimization can be done using response surface methodology (RSM) using above significant factors evaluated from PB experimental design.

3.3. Optimization of process parameters using CCD and RSM for mevastatin production using A. terreus

The effect of various medium constituents was studied using RSM. Optimization of medium constituents using *A. terreus* was done keeping the other nutrients concentration as constant level. These medium constituents mostly influence the fungal growth and secondary metabolite production. RSM is a sequential procedure with an initial objective to lead the experimenter rapidly and efficiently along a path of improvement toward the general vicinity of the optimum. Response surface methodology

Table 5
Experimental ranges and the levels of the independent variables for *A. terreus*.

S. no	Medium components (g 100 mL^{-1})	Coded values				
		−2	−1	0	+1	+2
1	Glycerol (x_1)	1	2	3	4	5
2	$CuCl_2{\cdot}2H_2O$ (x_2)	0.02	0.06	0.1	0.14	0.18
3	$FeSO_4{\cdot}7H_2O$ (x_3)	0.01	0.025	0.04	0.055	0.07
4	K_2HPO_4 (x_4)	0.3	0.4	0.5	0.6	0.7
5	$MgSO_4{\cdot}7H_2O$ (x_5)	0.01	0.03	0.05	0.07	0.09

Table 6
CCD matrix of three variables in coded units along with the observed responses for *A. terreus*.

Run	Glycerol	$CuCl_2 \cdot 2H_2O$	$FeSO_4 \cdot 7H_2O$	K_2HPO_4	$MgSO_4 \cdot 7H_2O$	Mevastatin ($mg\,L^{-1}$) Experimental	Mevastatin ($mg\,L^{-1}$) Predicted
1	−1	−1	−1	−1	1	505.63	491.637
2	1	−1	−1	−1	−1	248.29	189.088
3	−1	1	−1	−1	−1	200.02	191.611
4	1	1	−1	−1	1	314.42	304.732
5	−1	−1	1	−1	−1	201.20	152.727
6	1	−1	1	−1	1	220.84	171.088
7	−1	1	1	−1	1	208.10	209.141
8	1	1	1	−1	−1	201.22	157.052
9	−1	−1	−1	1	−1	209.02	213.406
10	1	−1	−1	1	1	236.55	239.657
11	−1	1	−1	1	1	90.00	143.9
12	1	1	−1	1	−1	163.78	172.471
13	−1	−1	1	1	1	72.56	86.396
14	1	−1	1	1	−1	563.21	531.837
15	−1	1	1	1	−1	201.49	220.91
16	1	1	1	1	1	211.37	229.511
17	−2	0	0	0	0	227.61	198.939
18	2	0	0	0	0	206.06	270.366
19	0	−2	0	0	0	202.59	275.506
20	0	2	0	0	0	201.16	163.879
21	0	0	−2	0	0	334.56	327.347
22	0	0	2	0	0	237.54	280.387
23	0	0	0	−2	0	120.00	218.506
24	0	0	0	2	0	274.13	211.259
25	0	0	0	0	−2	437.41	499.157
26	0	0	0	0	2	537.01	510.897
27	0	0	0	0	0	549.21	551.831
28	0	0	0	0	0	540.34	551.831
29	0	0	0	0	0	594.63	551.831
30	0	0	0	0	0	571.56	551.831
31	0	0	0	0	0	567.45	551.831
32	0	0	0	0	0	523.43	551.831

(RSM) was used to optimize the fermentation medium for enhancing mevastatin production [10–12,1–3]. 2^5 full factorial central composite design and RSM were applied to determine the optimal for each significant variable. To identify the optimum levels for different medium constituents influencing mevastatin production, submerged fermentation was carried out in conical flasks containing optimized nutrients. The individual and interactive effects of these variables were studied by conducting the fermentation run at randomly selected and different levels of all five factors.

The response was measured in terms of mevastatin production. The total of 32 experiments was used to optimize the medium constituents glycerol, $CuCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$. These nutrients were tested at five coded levels namely −2, −1, 0, +1 and +2. The optimum levels of the selected variables were obtained by solving the regression equation using MATLAB software and by analyzing the response surface and contour plots. Table 5 gives the coded values and the levels of the variables. The experimental and the predicted values were presented along with the CCD experimental design in Table 6. Multiple regression analysis of the CCD experimental design gives the following quadratic polynomial equation for the biosynthesis for mevastatin shown in Eq. (4).

$$\begin{aligned} Y = {} & 551.831 + 17.8567x_1 - 27.9067x_2 - 11.7400x_3 \\ & - 1.81167x_4 + 2.93500x_5 - 79.2947x_1^2 - 83.0347x_2^2 \\ & - 61.9909x_3^2 - 84.2372x_4^2 - 11.7009x_5^2 - 5.58125x_1x_2 \\ & + 34.6825x_1x_3 + 45.7512x_1x_4 - 16.1175x_1x_5 \\ & + 12.2275x_2x_3 - 10.1562x_2x_4 + 15.2200x_2x_5 \\ & + 49.1425x_3x_4 - 48.7337x_3x_5 - 57.8300x_4x_5 \end{aligned} \quad (4)$$

The analysis of variance of the quadratic regression model demonstrated was a highly significant model, as it is evident from the Fisher's *F*-test with a very low probability value [(*P* model > *F*) = 0.0001]. The student's *t*-test and *P*-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength between each independent variable. The larger the magnitude of the *t*-value and smaller the *P* value, the more significant is the corresponding coefficient. Here the squared effect of x_1^2, x_2^2, x_3^2 and x_4^2 were found to be significant and the interactive effect x_1x_4, x_1x_4, x_3x_5 and x_4x_5 were significant as the *P*-value is less than 0.05 for mevastatin as shown in Table 7.

The goodness of fit of the model based on RSM can be checked by the coefficient of determination (R^2), which provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response. In this case, the value of the determination coefficient (R^2 = 94.02%) indicated that only 5.98% of the total variations were not explained by the model for mevastatin.

3.4. Validation of the model

The validation experiment was carried out in 250 mL Erlenmeyer flask under the optimum combination of the medium components predicted by the polynomial model. The optimum values for glycerol – 3.86 mg 100 mL^{-1}, $CuCl_2.2H_2O$ – 0.102 mg 100 mL^{-1}, $FeSO_4 \cdot 7H_2O$ – 0.036 mg 100 mL^{-1}, K_2HPO_4 – 0.003 mg 100 mL^{-1} and $MgSO_4 \cdot 7H_2O$ – 0.09 mg 100 mL^{-1}. The model predicted a maximum response of 693.212 $mg\,L^{-1}$ of mevastatin production. At these optimized conditions, a maximum mevastatin production (experimental) of 701 $mg\,L^{-1}$ was obtained, which is

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

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*T*	*P*
Regression	20	796354	796354	39818	8.65	20.389	0
Linear	5	29937	29937	5987	1.3		0.332
A	1	7653	7653	7653	1.66	1.289	0.224
B	1	18691	18691	18691	4.06	−2.015	0.069
C	1	3308	3308	3308	0.72	−0.848	0.415
D	1	79	79	79	0.02	−0.131	0.898
E	1	207	207	207	0.04	0.212	0.836
Square	5	571128	571128	114226	24.81		0
$A \times A$	1	119943	184438	184438	40.06	−6.329	0
$B \times B$	1	153773	202246	202246	43.93	−6.628	0
$C \times C$	1	88619	112724	112724	24.48	−4.948	0
$D \times D$	1	204778	208146	208146	45.21	−6.724	0
$E \times E$	1	4016	4016	4016	0.87	−0.934	0.37
Interaction	10	195289	195289	19529	4.24		0.013
$A \times B$	1	498	498	498	0.11	−0.329	0.748
$A \times C$	1	19246	19246	19246	4.18	2.045	0.066
$A \times D$	1	33491	33491	33491	7.27	2.697	0.021
$A \times E$	1	4156	4156	4156	0.9	−0.95	0.362
$B \times C$	1	2392	2392	2392	0.52	0.721	0.486
$B \times D$	1	1650	1650	1650	0.36	−0.599	0.561
$B \times E$	1	3706	3706	3706	0.8	0.897	0.389
$C \times D$	1	38640	38640	38640	8.39	2.897	0.015
$C \times E$	1	38000	38000	38000	8.25	−2.873	0.015
$D \times E$	1	53509	53509	53509	11.62	−3.409	0.006
Residual error	11	50647	50647	4604			
Lack-of-fit	6	47448	47448	7908	12.36		0.07
Pure error	5	3199	3199	640			
Total	31	847000					

F – Degree of freedom, SS – Sum of squares, MS – Mean square, *F* – *F*-value, *P* – *P*-value.

higher than the predicted mevastatin production, thereby validating the proposed model.

4. Conclusion

In the present study, various carbon and nitrogen sources have been screened to choose the best carbon and nitrogen for the maximum mevastatin production. The PB experimental design is the preliminary technique for rapid screening of the effects of various medium constituents. PB experimental design was used to evaluate the significance of various medium components and to enhance the mevastatin production in SmF. A maximum mevastatin production of 170.4 mg L^{-1} was obtained in PB screening study. In CCD, a maximum mevastatin production of 701 mg L^{-1} was obtained by *A. terreus*.

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