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Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*

Rekha Kaushik, Saurabh Saran, Jasmine Isar, R.K. Saxena *

Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

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Abstract

A response surface approach has been used to study the production of an extracellular lipase from *Aspergillus carneus*, which has the property of immense industrial importance. Interactions were studied for five different variables (sunflower oil, glucose, peptone, agitation rate and incubation period), which were found influential for lipase production by one-at a time method. We report a 1.8-fold increase in production, with the final yield of 12.7 IU/ml in comparison to 7.2 U/ml obtained by one-at-a-time method. Using the statistical approach (response surface methodology (RSM)) the optimum values of these most influential parameters were as follows: sunflower oil (1%), glucose (0.8%), peptone (0.8%), agitation rate (200 rpm) and incubation period (96 h) at 37 °C. The subsequent verification experiment confirmed the validity of the model.

Keywords: Lipase; *Aspergillus carneus*; Response surface methodology; FCCCD

1. Introduction

Lipases also known as triacylglycerol ester hydrolase (EC 3.1.1.3) belong to the class of serine hydrolases that catalyse the breakdown of triacylglycerol to diacylglycerol, monoglycerol, free fatty acids and glycerol [1]. However, in the absence of water or micro-aqueous conditions, they catalyse the reverse reaction of synthesis [2]. Today, lipases are the choice of biocatalysts as they show unique chemo-, regio-, enantioselectivites, which enable the production of novel drugs, agrochemicals and fine products [3–7]. Many lipases due to their ability to perform both hydrolytic and synthetic reactions find immense applications in industries like foods, detergents, pharmaceuticals, leather, textile and dairy [7–9].

We have earlier reported that *Aspergillus carneus* lipase has several properties of immense industrial importance, in particular, pH and temperature tolerance and stability, 1,3-regiospecificity, excellent chemo- and regiospecificity in aqueous and non-aqueous media, high enatioselectivity and remarkable esterification and transesterification abilities [4]. Such a unique and extraordinary combination of properties has not been found in any other lipase.

Realizing the immense potential of this *A. carneus* lipase, it was worthwhile to process optimize the production of this lipase for the maximum yields. Therefore, attempts were made to enhance its production by developing processes that are economically viable. Conventional methods for optimization of medium and fermentation conditions involves varying one parameter at a time and keeping the others constant, is time consuming and expensive, when a large number of variables are to be evaluated. To overcome this difficulty and to evaluate and understand the interactions between different physiological and nutritional parameters, response surface methodology (RSM) has been widely used [10–12]. This methodology brings about the effect of interaction of various parameters, generally resulting in higher production yields and simultaneously limits the number of experiments. It is currently used for optimization studies in several biotechnological and industrial processes [9,13–17].

We report the use of a statistical approach called face-centered central composite design (FCCCD) falling under response surface methodology to optimize the physiological and nutritional parameters, which were found optimum by classical 'one-at-a-time' method. The aim was to study the effect of interactions

Abbreviations: RSM, response surface methodology; FCCCD, face-centered central composite design

* Corresponding author. Tel.: +91 11 24116559; fax: +91 11 24115270.
E-mail addresses: rksmicro@yahoo.co.in, rksmicro@hotmail.com (R.K. Saxena).

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Table 1
Experimental range and levels of the five independent variables used in RSM in terms of actual and coded factors

Variables	Range of levels					
	Actual	Coded	Actual	Coded	Actual	Coded
Sunflower oil (%)	1	−1	1.5	0	2	+1
Peptone (%)	0.2	−1	0.5	0	0.8	+1
Glucose (%)	0.8	−1	1.0	0	1.2	+1
Agitation rate (rpm)	200	−1	250	0	300	+1
Inoculum period (h)	72	−1	96	0	120	+1

among these most influential parameters on lipase yield of *A. carneus*.

2. Experimental

2.1. Chemicals

p-Nitrophenyl palmitate (*p*-NPP) for lipase assay was purchased from Sigma Chemicals (St. Louis, USA). All other media components and chemicals used were of analytical grade available commercially from Hi-Media, Qualigens and Sisco Research Laboratories Ltd., India .

2.2. Reconfirmation of lipase production

Earlier it has been reported that 7.2 U/ml of lipase was produced by *A. carneus* in modified Asiaka & Terda medium with composition as follows: peptone 0.5%, glucose 1%, sunflower oil 1.5%, KH_2PO_4 0.25%, KCl 0.05% and $MgSO_4$ 0.05%, by inoculating 5×10^7 conidia per 50 ml of the medium, pH 8.0 and incubating the flasks at 37 °C, 250 rpm in an incubator shaker (Multitron, Switzerland) for 96 h [7]. Broth was filtered through four to five layers of muslin cloth. After the filtration with the muslin cloth, it is the growth supernatant (culture filtrate devoid of organism) which was the source of crude enzyme was cen-

Table 2
Design matrix of face-centered central composite design for lipase production

Serial no.	Sunflower oil (%)	Peptone (%)	Glucose (%)	Incubation period (h)	Agitation rate (rpm)	Mean observed (IU/ml)	Mean predicted (IU/ml)
1	0	−1	0	0	0	6.77	6.79
2	−1	+1	+1	−1	+1	9.00	9.00
3	0	0	0	0	0	7.01	6.97
4	−1	−1	−1	0	+1	9.42	9.40
5	−1	+1	−1	−1	0	12.7	12.67
6	−1	−1	+1	0	−1	8.82	8.83
7	0	+1	0	−1	0	7.95	8.03
8	0	0	−1	0	0	8.45	8.61
9	0	0	0	0	0	6.97	6.97
10	0	0	0	−1	0	6.1	6.08
11	+1	−1	−1	+1	+1	5.34	5.30
12	+1	+1	+1	−1	−1	5.45	5.47
13	+1	+1	−1	+1	−1	10.65	10.61
14	−1	0	0	0	0	9.6	9.61
15	0	0	0	0	0	7.12	6.97
16	+1	−1	−1	−1	−1	8.21	8.21
17	+1	+1	+1	+1	+1	5.05	5.03
18	−1	−1	+1	+1	+1	8.45	8.47
19	+1	+1	−1	0	+1	6.05	6.04
20	+1	−1	+1	+1	−1	4.87	4.86
21	0	0	0	0	0	7.09	6.97
22	0	0	0	0	+1	6.0	6.07
23	−1	+1	+1	+1	−1	10.6	10.62
24	0	0	0	0	0	6.98	6.97
25	0	0	+1	0	0	6.48	6.42
26	+1	0	0	0	0	5.65	5.73
27	0	0	0	0	0	7.0	6.97
28	0	0	0	0	−1	7.7	7.72
29	+1	−1	+1	−1	+1	3.27	3.30
30	−1	−1	−1	+1	−1	11.02	11.02
31	0	0	0	+1	0	6.7	6.78
32	−1	+1	−1	+1	+1	11.2	11.19

trifuged at 8000 × *g* (Sorvall RC 5C Plus) at 4 °C for 15 min. The concentrated culture filtrate was analyzed for lipase activity.

2.3. Lipase determination

Lipase activity in the culture filtrate was determined spectrophotometrically using the procedure of Winkler and Stuckman [18]. Experimentally, *p*-nitrophenyl palmitate (30 mg) was dissolved in 10 ml of isopropanol and subsequently mixed with 90 ml of 0.5 M glycine–NaOH buffer (pH 9.0). To 2.4 ml of freshly prepared *p*-nitrophenyl palmitate solution, 0.1 ml of the enzyme sample (concentrated culture filtrate) was added. This reaction mixture was incubated at 37 °C for 5 min. The reaction was terminated by adding 50 μl of 0.3 M $CaCl_2$ solutions. The sample was immediately centrifuged at 8000 × *g* and absorbance of the supernatant was read at 410 nm. The control was treated with heat-inactivated enzyme.

One International Unit (IU) of lipase activity is defined as the amount of enzyme required to release 1 μM of fatty acid per milliliter per minute under the standard assay conditions.

2.4. Experimental design for lipase production (response surface methodology)

Earlier one-at-a-time approach had been followed to identify the parameters (variables) having significant effect on lipase production from *A. carneus* [19]. Subsequently, a statistical approach, response surface method was employed to study the interaction of these parameters. Here, face-centered central composite design was used to optimize the levels of these parameters. FCCCD was performed as it incorporates replication of medial point as (0 0 0). The levels of five parameters, viz. sunflower oil (A), peptone (B), glucose (C), incubation period (D), and agitation rate (E) were selected for evaluation. These were the most influential parameters for lipase production as obtained by the classical one-at-a-time method [19].

Each parameter was studied at three different levels (−1, 0, +1). All parameters were taken at a central coded value considered as zero (Table 1). A matrix of 32 experiments with five factors was generated using the software package, Design Expert 6.0 (Stat Ease Inc. Minneapolis, USA). The minimum and maximum ranges of parameters were investigated and the full experimental plan with respect to their values in actual and coded form are listed in Table 2. The average maximum lipase activity was taken as the dependent variable or response (*Y*). A second order polynomial equation was then fitted to the data by multiple regression procedure. This resulted in an empirical model that related the response measured in the independent parameters. All tests were performed in triplicates and the data represented is a mean of the three. For a five-factor system, the model equation was:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2 + \beta_{12} AB + \beta_{13} AC + + \beta_{15} AE + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} DE$$

where, *Y* is the predicted response, β_0 is the intercept, β_1, β_2, β_3, β_4, β_5, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, β_{55}, squared coefficients; β_{12}, β_{13}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35}, β_{45} interaction coefficients.

2.5. Validation of the model

In order to determine the accuracy of the model, the concentrations of three factors (sunflower oil, glucose, and agitation rate), which have a major influence on lipase production as obtained by response surface methodology were randomly selected within the design space. The remaining factors in this experiment were at fixed levels. Six sets of experiment were generated and carried out.

3. Results and discussion

The variables found as optimum by one-at-a-time method were the following: sunflower oil (1.5%), glucose (1%), peptone (0.5%), incubation period (96 h) and agitation rate (250 rpm). These values which resulted in the production of 7.2 U/ml of lipase were fitted in the model FCCCD falling under RSM. Lipase production (i.e. the response) of the experiment (FCCCD) for each individual run along with the predicted responses are presented in Table 2. The maximum lipase activity, 12.7 U/ml was achieved in 96 h of incubation at sunflower oil concentration of 1%, glucose 0.8%, peptone 0.8%, and agitation rate of 200 rpm. There is approximately 1.8-fold increase in the lipase activity. The result obtained after FCCCD were then analyzed by standard analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) -

$$Y = +28.22 + 5.49A - 7.35B + 34.3C + 0.19D + 0.41E - 4.76A^2 - 0.93B^2 - 3.16C^2 + 0.59D^2 + 0.022E^2 + 0.026AB - 0.029AC + 0.40AD + 0.18AE + 0.25BC + 0.028BD + 0.011BE + 0.17CE + 0.039DE$$

where Y: lipase activity, A: sunflower oil, B: peptone, C: glucose, D: incubation period and E: agitation rate.

Analysis of variance (ANOVA) showed that the factor B (peptone), and D (incubation period) were insignificant factors and AC, CE, AE were significant model terms. The R^2 value 0.9993 for lipase production, point to the accuracy of the model. The ANOVA for the responses (Table 3) indicated that the model was significant. The R^2 value provides a measure of how much variability in the observed response values can be explained by

Table 3
ANOVA for response surface quadratic model

Model terms	Value
R^2	0.9993
Adj R^2	0.9981
Pred R^2	0.9908
Adeq precision	129.21
Model *F*-value	874.64
Lack-of-fit-value	3.06

the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1.00, the stronger the model is and the better it predicts the response [20]. When expressed as a percentage, R^2 is interpreted as the percent variability in the response explained by the statistical model. This implies that the sample variation of 99.93% for lipase production, was attributed to the independent variables and only 0.01 of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to the noise. Adequate precision measures signal-to-noise ratio, a ratio greater than 4 is desirable [21]. An adequate precision of 129.29 for lipase activity, indicated an adequate signal (Table 3). The 'Pred R^2' of 0.9908 indicated a good agreement between the experimental and predicted values for lipase production (Table 3). The adjusted R^2 corrects the R^2 values for the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2 [20]. In this case, the adjusted R^2 value is very close to the R^2 value. The coefficients of regression equation were calculated using design expert. The model F-value of 874.64 for lipase activity implied that the model is significant. Values of 'Pred > F' less than 0.05 indicated that the model terms are significant. The 'Lack of fit F-value' 3.06 for lipase activity implied the lack of fit is insignificant and the model is adequate.

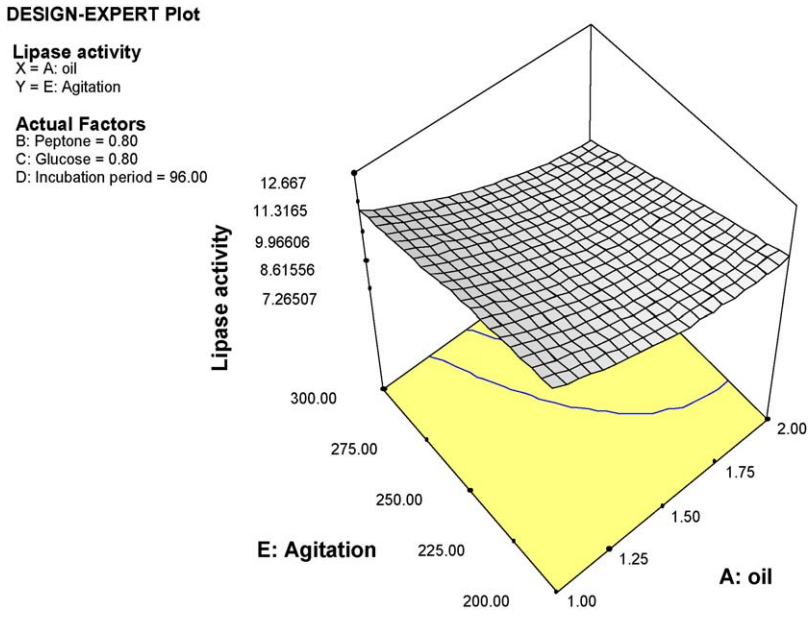

Fig. 1. Response surface curves of lipase production from *Aspergillus carneus* showing interaction between oil and agitation.

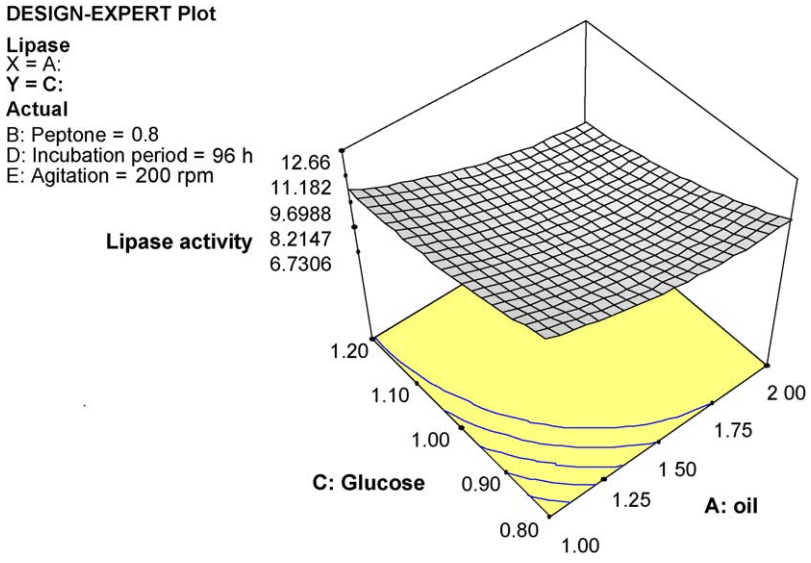

Fig. 2. Response surface curves of lipase production from *Aspergillus carneus* showing interaction between oil and glucose.

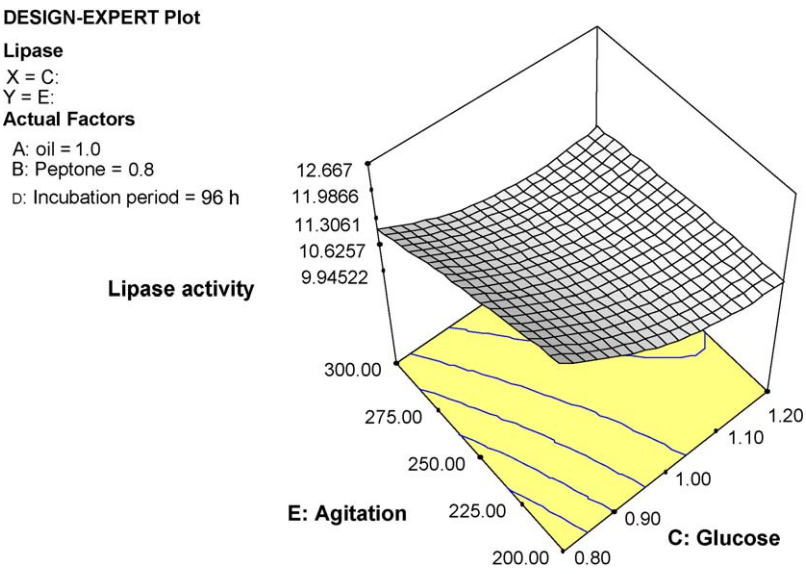

Fig. 3. Response surface curves of lipase production from *Aspergillus carneus* showing interaction between agitation and glucose.

Table 4
Validation of FCCCD using different levels of sunflower oil, glucose and agitation rate at a constant rate of 0.8% of peptone and 96 h

Serial no.	Sunflower oil (%)	Glucose (%)	Agitation rate (rpm)	Lipase activity (U/ml)	
				Predicted	Observed
1	1.25	0.8	275	9.38	9.01
2	1.5	1.2	200	6.66	6.11
3	1.0	2.0	300	6.17	5.99
4	1.0	0.90	250	7.78	7.45
5	1.0	0.8	275	7.93	7.24
6	1.0	0.8	200	12.67	12.72

The three-dimensional response surface curve was plotted by a statistically significant model to understand the interaction of the medium components and the optimum concentration of each component required for optimum lipase production. The interaction of two variables, viz. oil and agitation rate at constant glucose and peptone after four days of incubation is presented in Fig. 1. Maximum lipase titer of 12.7 U/ml was obtained at the peptone and glucose concentrations of 0.8%. Further increase or decrease in the concentration of either of these lead to the decrease in the enzyme production. Fig. 2 presents the interaction between oil and glucose. A maximum of 12.7 U/ml of lipase was obtained at an agitation rate of 200 rpm and oil concentration at 1% while Fig. 3 presents the interaction between agitation and glucose where a maximum of 12.7 U/ml of lipase was obtained at oil concentration at 1%.

3.1. Validation of the model equation in batch fermentation

In order to determine the accuracy of the model, six sets of experiments were performed by selecting oil, agitation rate and glucose as three factors, which have a major influence on lipase production. The remaining factors were set at their optimum levels. Table 4 presents the lipase yield of each individual experiment along with the predicted response. The results verify the previous model that oil at 1%, glucose at 0.8% and agitation rate of 200 rpm as the best combination for obtaining the maximum lipase production. The maximum yield 12.7 U/ml was obtained experimentally and this was closer to the predicted value 12.67 U/ml (Table 4).

4. Conclusions

Having established that *A. carneus* lipase is of immense importance in various industrial sectors, it is of utmost relevance to increase the enzyme yield during production. The results of the present investigations clearly showed that the influential parameters for lipase production are glucose (0.8%) as carbon, peptone (0.8%) as nitrogen source, sunflower oil (1%) as substrate, agitation rate (200 rpm) as a indicator the D.O. level and incubation period (96 h). However, the interaction among these parameters revealed that there in approximately 1.8-fold increase in the enzyme activity resulting in 12.7 U/ml. This itself is an important finding as very high titers of lipase production from wild type strain of fungi is not reported. This gives an idea that besides process engineering for the maximum production of *A. carneus* lipase it will be of equal importance to carry out investigations for the genes responsible for lipase production, their expression in a suitable organism and subsequent optimization using metabolic engineering.

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