Optimization of Lipase Production from *Aspergillus terreus* by Response Surface Methodology and Its Potential for Synthesis of Partial Glycerides Under Solvent Free Conditions

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Received: 19 June 2009 / Accepted: 11 May 2010 / Published online: 25 January 2011
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Abstract *Aspergillus terreus* produces lipase 7.01 IU/ml in 96 h after optimization by one variable at a time method. Using the significant factors i.e. corn oil (A), sodium nitrate (B), casein (C), agitation rate (D) and incubation period (E) RSM was carried out resulting in 19.65 IU/ml from the combination +1(A), −1(B), −1(C), +1(D) and 0(E). The interactions between sodium nitrate, casein and agitation with corn oil were most significant. Scale up of production from 250 ml shake flask to 30 l bioreactor resulted in increased productivity of 0.52 IU/ml/h as against 0.2 IU/ml/h obtained in shake flasks. This lipase could carryout solvent free synthesis of partial glycerides of oleic acid with 96% efficiency in 12 h.

Keywords Lipase · *Aspergillus terreus* · Production · Response surface methodology · Glyceride synthesis

Abbreviations

RSM Response surface methodology
FCCCD Face centered central composite design
OVAT One variable at a time

Introduction

Lipases (triacylglycerol acylhydrolase, E.C.3.1.1.3) are important biocatalysts used in a variety of industrial applications [1]. The applicability of lipases range from food & dairy, pharmaceuticals, detergents, oleochemicals and many others [1]. In the last few decades microbial lipases from *Candida* sp., *Thermomyces* sp. have gained significant applications in various industries [2–5].

Among various fungi evaluated as lipase producers, aspergilli are considered to be a potential source. Lipase production from *Aspergillus carneus*, *Aspergillus terreus*, *Aspergillus niger* have been reported by several investigators [3, 6, 7]. Previous investigations in our laboratory has shown *Aspergillus carneus* and *Aspergillus terreus* as potent lipase producers. We have reported certain results on production of these lipases [3, 7, 8]. In continuation of this, here we present some further studies carried out on process optimization of lipase from *Aspergillus terreus* by a statistical design method and its use in glyceride synthesis.

Materials and Methods

Chemicals

p-Nitrophenyl palmitate (*p*-NPP) was purchased from Sigma (St. Louis, USA). Other chemicals used were of analytical grade from Hi-Media (India).

Culture Conditions

Lipase production by *A. terreus* was carried out in modified Czapek-Dox medium with medium composition (g/l): Corn

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oil, 20; Sodium nitrate, 2; casein, 1; KCl, 0.52; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; KH_2PO_4 , 1.52; $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (traces) pH 8.0. Flasks containing production medium (50 ml in 250 ml flask) were inoculated with 5×10^7 spores per 50 ml (5-day-old slants used) and incubated at 37°C at 250 rpm for 96 h.

Enzyme Activity

Lipase activity in the culture filtrate was determined spectrophotometrically using *p*-nitrophenyl palmitate as the substrate using the procedure of Winkler and Stuckman [9].

One International Unit (IU) of lipase activity is defined as the amount of enzyme required to release 1 μM of *p*-nitro phenol per ml per minute under the standard assay conditions.

Experimental Design for Lipase Production Using RSM

The levels of five variables viz. corn oil (A), sodium nitrate (B), casein (C), agitation (D) and incubation period (E), obtained as the most influential parameters after optimization of enzyme production by one variable at a time methods were selected for RSM studies (Table 1). Face centered central composite design (FCCCD) was employed to study the interaction of these parameters as it incorporates replication of the medial point (000). The ranges of these variables were decided according to the ranges determined by OVAT method with other variables at optimum level. Other variables were set at their optimum levels as obtained by OVAT method. Experiments were performed in triplicates to estimate the experimental error.

The statistical software package 'Design-Expert® 6.0 Stat-Ease, Inc.' (Minneapolis, USA) was used to analyze the experimental design. A set of 32 experiments were generated (Table 2). The design matrix with different variables was set at three levels (−1, 0, +1). All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were investigated.

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
Corn oil (%)	1.5	−1	2.0	0	2.5	+1
Sodium nitrate (%)	0.1	−1	0.2	0	0.3	+1
Casein (%)	0.05	−1	0.1	0	0.15	+1
Agitation rate (rpm)	200	−1	250	0	300	+1
Incubation period (h)	72	−1	96	0	120	+1

Statistical Analysis and Modeling

In each case, the data on lipase production was subjected to Analysis of Variance (ANOVA) appropriate to the design of the experiments. The mathematical relationship of the independent variable and the response (lipase production) was calculated by the second order polynomial equation. i.e.

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_5E + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{44}D^2 + \beta_{55}E^2 + \beta_{12}AB + \beta_{13}AC + \beta_{15}AE + \beta_{23}BC + \beta_{24}BD + \beta_{25}BE + \beta_{34}CD + \beta_{35}CE + \beta_{45}DE.$$

where *Y* is the predicted response; β_0 the intercept; $\beta_1, \beta_2, \beta_3, \beta_4, \beta_5$ the linear coefficient; $\beta_{11}, \beta_{22}, \beta_{44}, \beta_{55}$ the squared coefficients; and $\beta_{12}, \beta_{13}, \beta_{15}, \beta_{23}, \beta_{24}, \beta_{25}, \beta_{34}, \beta_{35}, \beta_{45}$ are the interaction coefficients. This equation is used to evaluate the linear, quadratic and interactive effect of independent variables on the selected response.

Verification of Model in a Bioreactor

The lipase production was validated in a 30 l bioreactor (Scigenics, India) with a working volume of 22 L. Medium was inoculated (5×10^7 spores/ml) and fermentation was carried out at 37°C, 300 rpm for 72 h with controlled pH. DO and air flow rate were maintained above 20% saturation and 1.0 vvm respectively. Samples were analyzed after every 6 h.

Glyceride Synthesis

The reaction mixture for esterification reaction consisted of freeze dried lipase (50 mg), oleic acid (1,770 μM) and glycerol (5,910 μM) in stoichiometric amounts. The reaction was carried out under solvent free conditions at 37°C and an initial a_w of 0.33 (achieved with using $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). The effect of ratio of oleic acid to glycerol was studied in the range from 1:1 to 1:10 molar ratio of the reactants, the effect of the temperature, initial water activity and effect of water removal through molecular sieves were studied. The reaction products were analyzed by HPLC using C18 column (column temp., 40°C; mobile phase, methanol; flow rate, 1.0 ml/min; UV detector detecting at 215 nm).

Results and Discussion

A five factor RSM using corn oil, sodium nitrate, casein, agitation rate and incubation period was carried out. FCCCD model was used using the optimal values of these variables. Lipase production (response) of the experiment for each individual run along with the predicted responses are presented in Table 2 where maximum lipase titers of

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

S.No.	Corn oil (%)	Sodium nitrate (%)	Casein (%)	Agitation rate (rpm)	Incubation period (h)	Mean	
						Observed (IU/ml)	Predicted (IU/ml)
1	−1	−1	−1	−1	1	9.5	9.45
2	0	0	1	0	0	8.5	8.5
3	−1	1	−1	1	1	12.5	12.5
4	0	0	0	0	0	7	7.1
5	0	0	0	1	0	8.5	8.5
6	0	0	0	0	0	7.5	7.51
7	1	1	1	1	1	15.12	15.1
8	1	−1	1	−1	1	12.5	12.49
9	0	0	−1	0	0	9.5	9.61
10	−1	−1	1	−1	−1	8.5	8.5
11	−1	−1	1	1	1	7	7.15
12	−1	1	1	−1	1	13	13.02
13	1	−1	1	1	−1	11	11.12
14	0	0	0	0	0	7.5	7.61
15	0	−1	0	0	0	6.5	6.51
16	0	0	0	0	1	7.5	7.52
17	−1	1	1	1	−1	11.5	11.78
18	0	0	0	0	0	7.5	7.57
19	1	1	1	−1	−1	17	17.12
20	1	1	−1	1	−1	16.5	16.35
21	−1	1	−1	−1	−1	14	14.02
22	0	1	0	0	0	11	11.12
23	−1	0	0	0	0	5.23	5.25
24	0	1	−1	−1	0	19.65	19.56
25	1	0	0	0	0	9	8.95
26	0	0	0	−1	0	10	9.98
27	1	−1	−1	−1	−1	13.5	13.44
28	0	0	0	0	0	7.5	7.96
29	1	−1	−1	1	1	11.95	11.81
30	0	0	0	0	0	7.5	7.01
31	0	0	0	0	0	7.5	7.67
32	−1	−1	−1	1	−1	8	8.12

Bold values are the highest yield obtained

19.5 IU/ml was achieved in 96 h of incubation at 1.5%, corn oil; 0.3%, sodium nitrate; 0.05%, casein and agitation rate of 200 rpm. There was approximately 2.8 fold increase in the lipase titers as against the yield obtained by OVAT. The result obtained after FCCCD was then analyzed by standard Analysis of Variance (ANOVA), which gave the following regression equation:

$$\begin{aligned}
 Y = & +7.34 + 2.06 * A - 2.36 * B - 0.62 * C - 0.87 * D \\
 & + 0.071 * E - 0.11 * A^2 + 1.25 * B^2 + 1.78 * C^2 \\
 & + 2.03D^2 + 0.29 * E^2 + 0.091 * A * B - 0.13 * A * C \\
 & - 0.14 * A * D + 0.079A * E * -0.14 * B * C - 0.13 \\
 & * B * D + 0.085 * B * E + 0.091 * C * D - 0.13 * C \\
 & * E - 0.13 * C * E
 \end{aligned}$$

where Y is the lipase activity; A the corn oil; B the sodium nitrate; C the casein; D the agitation rate; and E is the incubation period and the interactions AB, AC, AD were significant. The ANOVA for the responses (Table 3) indicated that the model was significant. R^2 value when expressed as percentage, is interpreted as the percent variability in the response explained by the statistical model. This implies that the sample variation of 99.85% for lipase production, here was attributed to the independent variables and only 0.15% of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. Also the closer this value is to 1 the stronger the model is Haaland [10]. An adequate precision of 77.09 for

lipase production, indicated an adequate signal (Table 3). The “Pred R^2 ” of 0.9303 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. 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 373.67 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’ 1.79 for lipase activity implied the lack of fit is insignificant and the model is adequate.

The plotting of response surface curves showed the interaction between the significant factors corn oil (A), Sodium nitrate (B) and casein (C). Maximum lipase titer of 19.69 IU/ml was obtained at the oil concentration of 1.5% and sodium nitrate concentration of 0.3% (Fig. 1) while 19.69 IU/ml of lipase was obtained at oil concentration of 1.5% and casein concentration of 0.05% (Fig. 2) as its concentration significantly affected lipase yield. Corn oil (A) was found to be the most influential carbon and lipid

source for lipase production. Corn oil was also found to be most influential factor in lipase production by *Rhizopus arrhizus* [11] using 2^2 full-factorial central composite design. However, soybean oil, corn steep liquor and ammonium nitrate influenced lipase production by *Geotrichum* sp. using 2^4 central composite design [12].

Table 4 presents the data for validation and the lipase production and results show that the model holds true as the results obtained are close to the predicted values.

Similar RSM using both nutritional and physiological factors have been reported by Kaushik et al. [7] and Wang et al. [13]. However, Sharma et al. [14] used only three physical parameters i.e. pH, temperature and incubation period to optimize lipase production from *Arthrobacter* sp.

Further, the model was scalable to 30 l bioreactor with a working volume of 22 l. Lipase production started as early as 24 h and reached a maximum of 28.2 IU/ml in only 54 h at 200 rpm (Fig. 3). This increase in the production could be due to increased/controlled oxygen transfer rates in the bioreactor, which supported both higher growth as well as enzyme yields.

Synthesis of Partial Glycerides of Oleic Acid by *A. terreus* Lipase Under Solvent Free Conditions

Partial glycerides of fatty acids i.e. mono and diacylglycerols are an important class of compounds extensively used as emulsifiers [15]. These are normally produced by alcoholysis of triacylglycerols with glycerol in presence of a metal catalyst at high temperatures of 210–240°C [16]. The yields are often low and result in odiferous and coloured by products. *A. terreus* lipase mediated esterification between different fatty acids of varying carbon chain

Table 3 ANOVA for response surface quadratic model

Model terms	Values
R^2	0.9985
Adj R^2	0.9959
Pred R^2	0.9303
Adeq precision	77.090
Model F -value	373.67
Lack of fit-value	1.79

Fig. 1 Response surface curve of lipase production from *Aspergillus terreus* showing interaction between sodium nitrate and corn oil

Lipase unit
X = A: Corn oil
Y = B: Sod. nitrate
Actual Factors
C: Casein
D: Agitation
E: Incubation period

Fig. 2 Response surface curve of lipase production from *Aspergillus terreus* showing interaction between casein and corn oil

Lipase unit

X = A: Corn oil

Y = C: Casein

Actual Factors

B: Sod. nitrate

D: Agitation

E: Incubation period

Fig. 3 Fermentation profile of lipase produced from *A. terreus*

Table 4 Validation of FCCCD using different levels of corn oil, sodium nitrate and casein at a constant rate of 200 rpm and incubation period of 96 h

S.No.	Corn oil (%)	Sodium nitrate (%)	Casein (%)	Agitation (rpm)	Incubation period (h)	Lipase activity	
						Predicted	Observed
1	1.5	0.15	0.07	250	96	4.77	4.6
2	1.5	0.25	0.1	200	96	9.37	9.5
3	1.5	0.25	0.05	225	96	10.22	10.1
4	1.5	0.13	0.08	275	96	4.64	4.2
5	1.5	0.23	0.10	250	96	5.34	5.6
6	1.5	0.10	0.08	233	96	5.2	5.1
7	1.5	0.20	0.13	225	96	5.83	5.5
8	1.5	0.25	0.09	225	96	7.63	7.8

length and glycerol under solvent free conditions was attempted. The results showed that the lyophilized lipase (2.1 IU/mg) was able to synthesize mono-(MG), di-(DG) and triglycerides(TG) of different fatty acids. However, maximum conversions were obtained in case of oleic (75%) and palmitic acid (72%). In case of butyric acid, only MG's were formed and in case of caproic and caprylic acid only MG and DG were formed. Higher monoester formation with short chain fatty acids has also been reported by Janssen et al. [17]. They reported that the chain length was major controlling factor in the synthesis of different type of glyceride.

Molar ratio of glycerol to oleic acid had a profound effect on synthesis of different types of glycerides. It was observed that at high glycerol and oleic acid ratio of 1:7.5 and 1:10, all three types of glycerides that is MG, DG and TG were synthesized (Table 5). However, at molar ratio of 1:5 and less mainly MG and DG's were synthesized with 75.5% yields. Similar results were obtained by Kwon et al. [16] in synthesis of mono- and di-palmitoyl glycerol in *n*-hexane with 60% yields using *Mucor miehei* lipase. However, at high glycerol to oleic acid ratio, mainly triolein was synthesized by the same lipase as reported by Ergan et al. [18]. Xia et al. [19] reported an equimolar ratio of glycerol and capric acid favoured maximum production of partial glycerides of caprate and the rates accelerated on increasing glycerol concentration. However, molar ratio of caprylic acid to glycerol lower than one favoured production of only monoglycerides under microbial condition using *Candida antarctica* immobilized lipase [20].

Temperature increase to 45°C accelerated oleic acid partial glyceride synthesis partial glyceride with maximum yields of 86% in 12 h (Fig. 4). Initial a_w of 0.33 favoured high conversions (Fig. 5). Control of a_w by addition of molecular sieves increased both yields and rate of the reaction by shifting the equilibrium towards ester synthesis by absorbing excess water in the reaction system. 96% yields of partial glycerides of oleic were obtained in just 8 h. The effect of molecular sieves on accelerating the

Fig. 4 Effect of temperature on synthesis of partial glycerides of oleic acid by *A. terreus* lipase

Fig. 5 Effect of initial water activity on synthesis of partial glycerides of oleic acid

Table 5 Effect of glycerol to oleic acid on glyceride synthesis by *A. terreus* lipase at 37°C, 150 rpm and a_w of 0.33

Molar ratio of glycerol to oleic acid	MG	DG	TG	% Oleic acid esterified
1:1	+	+	–	45.0
1:2	+	+	–	60.0
1:5	+	+	–	75.5
1:7.5	+	+	+	76.7
1:10	+	+	+	75.5

MG Monoglyceride, DG diglyceride, TG triglyceride, + Presence of glyceride, – absence of glyceride

reaction rates has also been reported, especially for lipase mediated synthesis of sugar esters [21]. Similar results were obtained by Arcos et al. [22] for synthesis of esters of conjugated linoleic acid in a solvent free condition using *C. antarctica* lipase. In our study since conditions for synthesis of partial glycerides of oleic acid were optimized in such a way that TG's were not synthesized, the problem of their separation from the reaction mixture was thus overcome. The HPLC also proved that only mono and di glycerides of oleic acid were produced with major peaks of DG and MG at retention times of 5.6 and 3.4 min respectively and only a small peak of residual oleic acid at retention time of 3.1 min.

Conclusion

On summarizing Response Surface Methodology was useful tool for further process optimization of lipase production from *Aspergillus terreus*. A net 2.8 fold for lipase

production was noted with 19.65 IU/ml obtained after RSM as against 7.0 IU/ml obtained by One variable at a time. The model was also scalable to larger volumes and 28.02 IU/ml was achieved in a 30 l bioreactor in 54 h. The productivity was also significant with 0.52 IU/ml/h against 0.2 IU/ml/h initially. Corn oil was most significant for lipase while incubation period has minimum impact. This lipase could successfully be used for synthesis of partial glycerides with 96% efficiency at 0.33 a_w and 45°C.

Acknowledgment Authors acknowledge with thanks the help of Ms. Pinki Anand for critically evaluating the manuscript.

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