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Enhanced Lipase Recovery through RSM Integrated Differential Evolutionary Approach from the Fermented Biomass

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ABSTRACT

The aim of this work was to apply a modeling integrated optimisation approach for a complex, highly nonlinear system for an extracellular lipase extraction process. The model was developed using mutation, crossover and selection variables of Differential Evolution (DE) based on central composite design of Response Surface Methodology. The experimentally validated model was optimized by DE, a robust evolutionary optimization tool. A maximum lipase activity of 134.13 U/gds (more than 36.28 U/gds compared to one variable at a time approach) was observed with the DE-stated optimum values of 25.01% dimethyl sulfoxide concentration, 40 mM buffer, 128.52 min soaking time and 35^{o}C with the DE control parameters, namely number of population, generations, crossover operator and scaling factor as 20, 50, 0.5 and 0.25, respectively. The use of DE approach improved the optimization capability and decision speed, resulting in an improved yield of 36.28 U/gds compared to the one variable at a time approach for the extracellular lipase activity under the non-optimized conditions. The developed mathematical model and optimization were generic in nature, which seemed to be useful for the scale-up studies of maximum recovery of lipase from the fermented biomass.

Key words: Modeling, Optimization, Lipase extraction, Response surface methodology, Differential evolution, Solid state fermentation

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases with the natural function of hydrolyzing the ester bonds at an oil-water interface (Reetz 2002). Among the hydrolases, lipases occupy a prominent place in industrial arena due to their use in different applications (Houde et al. 2004; Hasan et al. 2006) such as in resolution of chiral drugs (Barbosa et al. 2011), modification of fats, synthesis of cocoa butter substitutes, biofuels (Kumari et al. 2009a; Lee et al. 2011), personal care products and flavour enhancers (Mahapatra et al. 2009a; Kumari et al. 2009b; Dheeman et al. 2011). Fungal lipases represent the major commercial source because of their ability to utilise a wide spectrum of substrates, high stability towards extreme temperature, pH, organic solvents and its chemo-, regio- and enantioselectivity (Jaeger and Eggert 2002).

In contrast to submerged (liquid state) fermentation, solid-state fermentation (SSF) process occurs in absence or near absence of free water and thus the lipase strongly adsorbs on the insoluble biomass, which need to be extracted with

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efficient extraction process. Downstream processing involving enzyme extraction from the fermented biomass is a vital step to enhance lipase activity and recovery. The influence of extraction parameters and the interrelation among them during enzyme extraction from the fermented biomass is essential for optimised enzyme recovery with enhanced activity in SSF. The optimisation of different extraction parameters on enzyme recovery from the fermented biomass has been reported in the literature where Volken de Souza et al. (2008) optimised the transglutaminase extraction from the SSF biomass of *Bacillus circulans* BL32. In another instance, extraction conditions were optimised for the production of xylanase (Heck et al. 2005) and amylase (Palit and Banerjee 2001) from SSF biomass of *B. circulans*. The production (Sun et al. 2009; Rajendran and Thangavelu 2009), purification (Yasuda et al. 1999; Hiol et al. 2000), characterisation (Yu et al. 2009), sequencing (Sayari et al. 2005) and immobilisation (Karra- Châabouni et al. 2008; Kumari et al. 2008) of *Rhizopus* lipases as well as its utilisation in various fields (Adachi and Kobayashi 2005; Hama et al. 2006; Ben Salah et al. 2007; Pereira et al. 2009; Mahapatra et al. 2009b) have already been reported. However, the modeling and optimisation studies of lipase extraction from SSF biomass, a vital step in reducing the capital cost of lipase production, have not been explored till-date.

Modeling and optimisation play an important role in a complex, highly nonlinear, dynamic system such as fermentation process. The dynamic mathematical model coupled with the optimisation facilitates a better understanding of the different phenomena occurring in the system under investigation. Moreover, it simultaneously helps in the maximisation of desirable properties of a system by overcoming the limitations associated with the conventional "change one factor at a time" approach. This multi-faceted task can be easily accomplished by using the statistical integrated biologically inspired the evolutionary algorithms. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimise the response (Montgomery 1997). Differential evolution (DE), a biologically inspired evolutionary algorithm proposed by Storn and Price (1997), is one of the alternatives to the classical optimisation techniques (Storn and Price 1997). These techniques are associated with the various computational and convergence-based drawbacks in solving different non-linear tasks by handling discrete search spaces (Price et al. 2005; Baskar and Renganathan 2010). The advantages of DE over other evolutionary algorithms (EAs) such as genetic algorithm include simple structure, ease of use, efficient memory utilisation, lower computational complexity, lower computational effort, faster convergence and greater freedom in designing a mutation distribution (Price et al. 2005). In case of alkaline exo-polygalacturonase, an enhanced enzyme recovery from SSF biomass has been reported where the effect of extraction variables such as fermented bran to solvent ratio and shaking conditions on the recovery of exo-polygalacturonase was studied and optimised through statistical approach (Gupta et al. 2008). Factorial design approach was used in tranglutaminase extraction study from the SSF biomass of *B. circulans* BL32, where the optimal enzyme recovery was obtained using water-bran ratio of 1:6 at 7^{o}C, with an extraction time of 5 min and agitation speed of 250 rpm (Volken de Souza et al. 2008).

Therefore, after studying the effect of different extraction parameters on lipase activity (U/gds) (Units per gram of dry fermented substrate) by one-variable at a time approach, modeling and optimisation using RSM integrated DE approach was attempted. The proposed approach could facilitate an easy understanding of biophysical and chemical effects of biomass processing on the lipase extraction process with enhanced lipase recovery from the fermented biomass.

MATERIALS AND METHODS

Materials

p-nitrophenyl palmitate (*p*-NPP) was purchased from Sigma Chemical Co., USA and the culture media components from Hi Media Laboratories (Mumbai, India). Coconut oil and wheat bran were purchased from the local market. Dimethyl sulfoxide (DMSO), solvents and reagents were of AR grade and were obtained from Merck (India).

Microorganism and inoculum preparation

Rhizopus oryzae NRRL 3562 was isolated from local soil at IIT Kharagpur, India and maintained on PDA medium. For inoculation, spore

suspension of *R. oryzae* NRRL 3562 (6 x 10^5 spores.mL^{-1}) was used, which was initially prepared in sterile distilled water using 3-day old slant.

Substrate, medium and inoculation

Medium-sized sieved wheat bran (4 g) was used as a solid substrate for the cultures. Czapek-dox medium (6.0 mL) supplemented with glucose (5%) and coconut oil (10%) was used for moistening of wheat bran taken in 100 mL Erlenmeyer flask. The autoclaved substrate was inoculated with 6 × 10^5 spores. mL^{-1} of 3-d old slant and incubated for 5 d at 35°C and 90% relative humidity.

Selection of extraction parameters for lipase recovery

The fermented biomass incubated for 5-d was soaked in different solvents (water, methanol, ethanol, glycerol, Tris-HCl buffer (pH 8.0) and mixtures of solvents in water and Tris-HCl buffer (pH 8.0)) of different volume ratios (1:1-1:5 v/w) for 1-5-h incubation periods at 10-40°C with different leaching (stationary, agitation and recirculation) and washing (1^{st}-5^{th} wash) conditions. The soaked fermented biomass was squeezed using a cheese cloth. The supernatants obtained after the centrifugation at 6987 g at 4°C for 10 min were assayed for lipase activity.

Lipase assay and biomass yield

Lipase activity was spectrophotometrically determined using *p*-NPP as substrate (Mahapatra et al. 2009b). The substrate solution was prepared by adding a solution of *p*-NPP in propane-2-ol to a solution of gum Arabic and Triton X-100 by drop wise with intense stirring. The assay mixture consisted of substrate solution, Tris-HCl buffer (50 mM, pH 8.0) and suitably diluted enzyme. Reaction mixture was incubated at room temperature for 10 min and the released *p*-nitrophenol was measured at 410 nm using a spectrophotometer. One unit (U) of enzyme was defined as the amount of enzyme that liberated one micromole of *p*-nitrophenol per minute under the assay conditions. Enzyme activity is expressed in U/gds.

Modeling and optimisation approach

Modeling of lipase extraction using RSM

The experiments were carried out following a four variable central composite design at three levels using MINITAB 14 software. DMSO concentration (%), buffer molarity, soaking time (h) and temperature (°C) were considered as input variables whereas lipase activity (E.A (U/gds)) was taken as the response. The parameters along with their coded and real values are presented in Table 1.

The actual three level-four variable central composite design (CCD), used as an experimental design, in the current study is presented in Table 2.

Table 1 - Extraction parameters and levels for central composite design.

Sl. No	Variable description	Notation		Levels		
		Coded	Uncoded	Low (-1)	Middle (0)	High (+1)
1	DMSO (%)	X_1	A	20	25	30
2	Buffer concentration (mM)	X_2	B	35	40	45
3	Soaking time (min)	X_3	C	60	100	140
4	Temperature (°C)	X_4	D	30	35	40

Statistical analysis

To determine the relationship between the factors and the response variable investigated, the collected data was analysed statistically using regression analysis. Regression analysis was performed on the collected data whereby an observed, empirical response was approximated based on a functional relationship between the output variable, *Y* and one or more input variables $x_1, x_2, \ldots, x_k$.

The non-linear relationship between a response and input variables has been represented by a polynomial quadratic equation (Eqn. 1) to describe the functional relationship between the response, *Y* and the input variables $x_1, x_{2\text{--------}} x_k$

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum \sum_{i<j}^{k} \beta_{ij} x_i x_j$$

---- **Eq. (1)**

The response include the linear terms $x_1, x_2, \ldots, x_k$, square terms $x_1^2, x_2^2, \ldots, x_k^2$, and interaction terms $x_1x_2, x_1x_3, \ldots, x_{k-1}x_k$. The least square technique was used to fit a model equation containing the input

variables by minimising the residual error measured by the sum of square deviations between the actual and the estimated responses. This involved the calculation of estimates for the regression coefficients, that is, the coefficients of the model variables including the intercept or constant term (Bethea et al. 1985). Response surfaces plots were drawn to illustrate the main and interactive effects of the parameters on the response and to determine the optimum conditions of lipase extraction.

DE-based optimisation approach

In the current study, "*DE/rand/1*" mutation scheme was employed to generate the trial vector where "*DE*" stood for Differential evolution. "*rand*" meant that base vectors were randomly chosen and "*1*" meant vector difference used for the contribution of differential vector. The dimensionality (*Di*) of the problem was equal to four, as there were four process variables (DMSO concentration, buffer molarity, soaking time and temperature) considered here.

RESULTS AND DISCUSSION

Selection of extraction parameters for lipase recovery

Selection of suitable extraction conditions is necessary for the recovery of the enzyme from the fermented biomass. The optimal lipase activity of 97±0.85 U/gds was obtained from the first wash of fermented biomass with 30% dimethyl sulfoxide (DMSO) in 30 mM Tris-HCl buffer (pH 8.0) as a solvent with 1:4 solid to liquid ratio, in a stationary leaching condition at 30°C and soaking time of 2 h. Organic solvents are the solvents of choice in most enzyme extraction processes compared to inorganic solvents due to their lower dielectric constants, which facilitated the higher interaction (Castilho et al. 1999). The maximum leaching in case of 30% DMSO was attributed to its low dielectric constant compared to the water, methanol, ethanol, glycerol and its excellent penetrating property and solvating ability. The lower dielectric constant facilitates the increased interaction between lipase and solvent based on the Debye-Huckel theory (Maron and Prutton 1965) (Eq. 2). The theory may be applied to SSF with the assumption that lipase present in the solid fermented mass may have some binding force (Palit and Banerjee 2001). The interaction between lipase and DMSO is considered to be a function of the solvent concentration, that is, charge of the attracting molecules and extraction power is considered to be a function of different conditions such as soaking time and temperature, which ultimately decide the distance of lipase and DMSO.

$$F_a(or)F_r = 1/\in_r D/(Q_1Q_2)/r^2 \qquad \text{--------Eq. (2)}$$

Where F_a or F_r = force of attraction or repulsion between lipase and DMSO in buffer; $\in_r$=dielectric constant of the DMSO (A) in buffer (B) (of Eq. 3); Q_1, Q_2 = charge of the attracting molecules, is a function of two variables (Concentration of DMSO (A) and Buffer (B) of Eq. 3; r = distance of two attracting molecules, is a function of process conditions such as soaking time (C) and temperature (D) of Eq. 3.

The presence of a 'hard' oxygen atom and a 'soft' sulphur atom facilitates the excellent penetration property and solvating ability of DMSO. The inhibitory effect of DMSO concentrations above 30% might be due to the denaturation of enzyme, and enzyme activity inhibition by secreted toxic products upon disruption of mycelia and due to distortion of enzyme structure at higher concentration of DMSO. Solid to liquid ratio plays an important role in getting maximum lipase recovery from the SSF biomass. The maximal lipase activity with 1:4 solid to liquid ratio in 2 h soaking time is attributed to the maximal penetration capability of the solvent through the solid fermented mass (Tunga et al. 1999). Among different leaching conditions, the agitated and recirculation conditions gave slightly better results than the stationary condition. The agitated condition attributes to the reduction of concentration, polarisation and uniform dispersion of the fermented biomass in the solvent. Additional drag force by peristaltic pump facilitates the isolation of maximal lipase during recirculation conditions. The effective temperature for lipase extraction was 30°C, compared to other temperatures, which could have a denaturing effect on the enzyme activity (Palit and Banerjee 2001). Among the different washing conditions, the first wash was most effective than the subsequent washes. Similar enhanced enzyme activities were reported in case of levansucrase, protease and α-amylase recovery from the SSF biomass by selecting the optimal extraction parameters such as extraction time, mechanical agitation, temperature

and liquid to solid ratio by OVAT (One Variable At a Time) approach. A maximum extraction of 10 mL/g biomass was achieved in case of levansucrase recovery from the fermented broth of *B. megaterium* using distilled water as solvent, soaking time of 90 min at 40°C under agitated conditions (160 rpm) (Ahmed 2008). In another study, reversed micelles of sodium di(2-ethylhexyl) sulfosuccinate (AOT) in isooctane was used to extract an extracellular alkaline protease from *Nocardiopsis* sp. fermentation broth (Monteiro et al. 2005). The effect of extraction variables such as the type of solvent, soaking time, temperature, agitation and washing conditions on enzyme recovery was investigated in case of α-amylase extraction from the SSF biomass of *B. circulans* GRS313 (Palit and Banerjee 2001).

Modeling and optimisation studies of lipase extraction using RSM

Statistical analysis of the RSM model

The set of experiments used for developing a non-linear model based on CCD is given in Table 2.

Table 2 - Central composite design for experimental design with the experimental, predicted responses and its R-studentized residuals.

Run order	Input parameters				Response E. A[e] (U/gds)		R-studentized residual
	DMSO[a](%) (A)	BC[b](mM) (B)	ST[c](min.) (C)	Temp[d](°C) (D)	Exp*.	Predict.	
1	20	35	60	30	108.09±0.60	106.195	1.217
2	30	35	60	30	104.45±0.77	106.291	-1.178
3	20	45	60	30	106.23±1.02	107.042	-0.495
4	30	45	60	30	98.77± 0.46	96.048	1.889
5	20	35	140	30	103.36±0.92	102.303	0.649
6	30	35	140	30	92.64±0.82	93.278	-0.388
7	20	45	140	30	109.60±0.44	110.050	-0.272
8	30	45	140	30	88.66±0.92	89.936	0.791
9	20	35	60	40	126.07±0.84	125.735	0.203
10	30	35	60	40	109.84±0.96	108.040	1.149
11	20	45	60	40	131.23±0.84	129.242	1.286
12	30	45	60	40	98.46±0.71	100.457	-1.292
13	20	35	140	40	113.39±0.78	114.762	-0.854
14	30	35	140	40	87.82± 0.98	87.948	-0.077
15	20	45	140	40	126.07±0.85	125.170	0.550
16	30	45	140	40	86.72±1.00	87.265	-0.330
17	20	40	100	35	118.55±0.95	122.092	-2.100
18	30	40	100	35	105.09±0.94	103.187	0.995
19	25	35	100	35	113.49±0.97	114.598	-0.563
20	25	45	100	35	114.15±0.83	114.680	-0.267
21	25	40	60	35	105.05±0.98	109.140	-2.607
22	25	40	140	35	103.05±0.92	100.598	1.323
23	25	40	100	30	104.41±0.98	105.067	-0.331
24	25	40	100	40	112.52±0.92	113.502	-0.498
25	25	40	100	35	112.06±0.87	111.941	0.045
26	25	40	100	35	115.50±0.90	111.941	1.475
27	25	40	100	35	113.17±0.71	111.941	0.473

*Data are mean ±SD for triplicate runs

[a]Dimethylsulfoxide; [b]Buffer concentration; [c]Soaking time; [d]Temperature and [e]Enzyme activity.

The regression analysis of the model was carried out through the Analysis of Variance (ANOVA) and surface plots were prepared with the help of MINITAB 14 software. The non-linear relationship between the input variables and the response lipase activity (E. A [U/gds]) of *R. oryzae* NRRL 3562 was represented as a non-linear function of the input process parameters in uncoded form as follows (Rounded to 2 decimal places)*:*

$$E.A\ (U/gds) = -209.216 + 4.80143A - 0.868044B + 1.27294C + 13.2188D + 0.0279437A^2 + 0.0269859B^2 - 0.00349205C^2 - 0.106256D^2 - 0.0554500AB - 0.0101333AC - 0.177900AD + 0.00383333BC + 0.0133BD - 0.00786667CD \quad \textbf{Eq. (3)}$$

The significance test of individual model coefficients involved the determination of the *P* and T-values. The T-value (Table 3) represents the significance of the independent variables on the response. The *P*- value was the minimum value for a preset level of significance at which the hypothesis of equal means for a given factor could be rejected. Considering 95% ($\alpha = 0.05$) level of confidence, the significance of different factors and their interaction is given below.

Based on the significance test (Table 3) of individual model coefficients results, the *P*-values of X_1, X_3, X_4, X_3^2, X_1X_2, X_1X_3, X_1X_4, X_2X_3 and X_3X_4 were less than 0.05 and were considered to have a significant impact on the lipase activity. The factors X_2, X_1^2, X_2^2, X_4^2, X_2 X_4 had an insignificant impact on the lipase activity. Thus, the relationship between lipase activity and X_2 (Buffer molarity) X_4 (Temperature) could only be non-linear in nature.

The significance test for the regression model was done by ANOVA and the results for lipase activity presented in Table 4. The P-values were less than 0.05 for all the terms, which indicated the significant contribution of linear, square and interaction terms towards the response but the lack-of-fit could be said as insignificant, which was desirable for the model to fit.

Table 3 - Results of significance test on the non-linear model – coefficients, standard errors, T statistics and P-values for Lipase activity (E.A (U/gds) (coded form).

Sl.no	***Terms***	***Coeff.***	***SE coeff.***	***T***	***P***
1	Const	111.941	1.0021	111.709	0.000
2	X_1	-9.452	0.6409	-14.748	0.000
3	X_2	0.041	0.6409	0.064	0.950
4	X_3	-4.271	0.6409	-6.664	0.000
5	X_4	4.217	0.6409	6.580	0.000
6	X_1^2	0.699	1.6957	0.412	0.688
7	X_2^2	2.699	1.6957	1.591	0.138
8	X_3^2	-7.071	1.6957	-4.170	0.001
9	X_4^2	-2.656	1.6957	-1.567	0.143
10	X_1X_2	-2.773	0.6798	-4.078	0.002
11	X_1 X_3	-2.280	0.6798	-3.354	0.006
12	X_1 X_4	-4.448	0.6798	-6.542	0.000
13	X_2 X_3	1.725	0.6798	2.537	0.026
14	X_2 X_4	0.665	0.6879	0.978	0.347
15	X_3 X_4	-1.770	0.6798	-2.604	0.023
	SS = 2.719		R-Sq = 97.3%		R-Sq (adj) = 94.1%

Table 4 - Results of ANOVA – Lipase activity (E.A (U/gds))**.**

Source	***DF***	***Seq SS***	***Adj SS***	***Adj MS***	***F***	***P***
Regression	14	3174.15	3174.15	226.725	30.66	0.000
Linear	4	2256.72	2256.72	564.181	76.30	0.000
Square	4	289.97	289.97	72.493	9.80	0.001
Interaction	6	627.46	627.46	104.576	14.14	0.000
Residual Error	12	88.73	88.73	7.394		
Lack-of-Fit	10	82.57	82.57	8.257	2.68	0.302
Pure Error	2	6.16	6.16	3.079		
Total	26	3262.88				

The surface plots of Figure 1A – E were curved in nature, which indicated that the interaction terms X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4 were significant on the response lipase activity, showing the non-linear relationship between the input parameters on the response. Thus, the observations of Table 2 matched with those made from Figure 1. The following response behaviours were observed from the above surface plots.

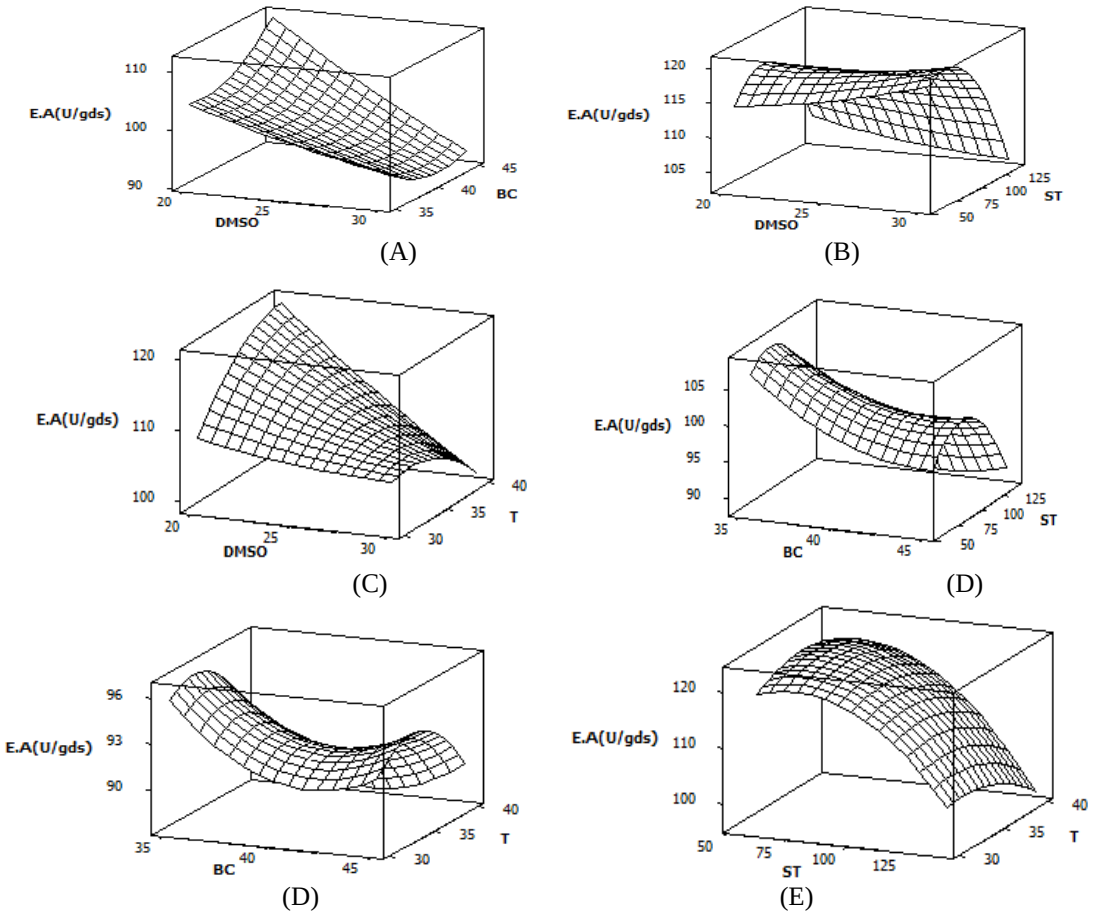


Figure 1 - Surface plots of lipase B 26 activity (E.A (U/gds)) with (A) buffer molarity [BC] and DMSO (B) soaking time [ST] and DMSO (C) temperature [T] and DMSO (D) temperature [T] and buffer concentration [BC] (E) temperature [T] and soaking time [ST].

I. The surface plot of lipase activity with DMSO and buffer concentrations (Fig. 1A) indicated that the lipase activity decreased with increasing DMSO concentration and lipase activity increased with increasing buffer concentration. This was due to the denaturing effect of higher DMSO concentration and stabilising effect of higher buffer concentration.

II. Figure 1B showed that the lipase activity slightly increased with increasing DMSO concentration from nearby midpoint value. An initial increase followed by a sharp decline in lipase activity was observed with increasing soaking time. The increased lipase activity with increasing DMSO concentration and decreasing soaking time was attributed to the maximum penetration of the DMSO into the fermented biomass.

III. Lipase activity continuously decreased with increasing DMSO concentration and initially increased, reaching maximum at mid-value, then decreased towards the end with increasing temperature (Fig. 1C). This indicated that increased interaction between lipase and DMSO was possible with decreased DMSO concentration and increased temperature.

IV. The surface plots of lipase activity with interactions between buffer concentrations, soaking time and temperature were almost similar and non-linear in nature (Fig. 1D and Fig. 1E). The surface plots indicated that the lipase activity initially decreased with increasing buffer concentration and reached maximum at mid-point values of soaking time and temperature, then decreased towards the end with increasing soaking time and

temperature. This type of response behaviour was attributed to the fact that at increased buffer concentration, soaking time and temperature, enzyme structure was distorted.

V. Figure 1E showed that a continuous decreased lipase activity with increasing soaking time reached maximum at mid values of temperature, then decreased with further increase in temperature. This was because the increasing temperature could have some denaturation effect on the enzyme activity.

Validation of the RSM model

The predicted model was validated to test the fitness of the model for further utilisation. The predicted values matched with the experimental values reasonably well (Table 1), which indicated the good prediction accuracy and generalisation ability of the predicted model. The higher coefficient values of multiple regression, R^2 (97.3%) and adjusted R^2 (94.1%) indicated the fitness and adequacy of the model (Myers et al. 2009). The ranges of externally studentised residuals, which were within - 3 to + 2 indicated the model adequacy (Table 1). The adequacy of the optimal point (+1.88) in studentised residuals was in the permissible range of studentised residuals (-3 and +3) (Montgomery and Runger 2002). Xiong et al. (2009) reported the effect of ethanol concentrations, extraction temperature and extraction time on intracellular reduced glutathione recovery from fermentation broth of *Saccharomyces cerevisiae*; an optimal ethanol concentration of 25% (v/v) was obtained using full factorial design study. The extraction conditions such as kind of solvent, the extraction temperature, the solid-liquid ratio, the stirring rate and the extraction time were also investigated through the experimental design approach in case of extraction of xylanase obtained by solid-state cultivation of *B. circulans* BL53 (Heck et al. 2005). Chen et al. (2006) utilised the reverse micelles of isooctane and anionic surfactant AOT for the extraction study of chitosinases produced by *B. cereus* (Chen et al. 2006). A 22-full orthogonal design was employed to optimize the β-xylosidase recovery by reversed micelles of CTAB and the effect of CTAB and butanol concentration on enzyme recovery (Hasmann et al. 2003). Thus, the developed model appeared to be useful for the design, scale-up, and optimisation studies of extracellular lipase extraction from the fermented biomass of *R. oryzae* NRRL 3562 through SSF.

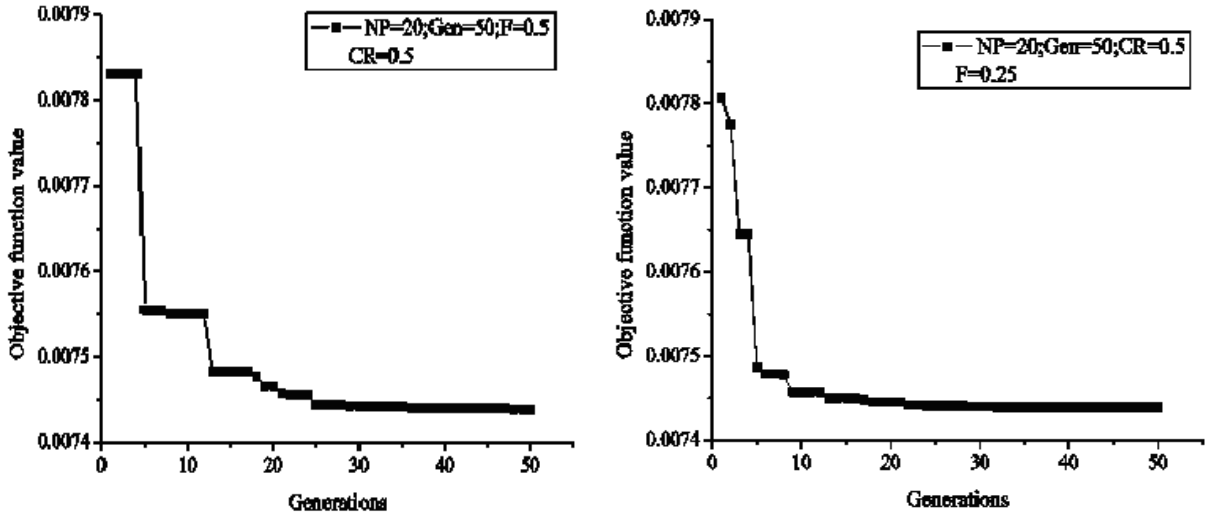
DE based optimisation approach

In the current study, lipase extraction from fermented biomass of *R. oryzae* NRRL 3562 was considered as an optimisation problem for maximising the lipase activity. The maximisation problem was then converted to minimisation problem as given below.

$$Minimize = \frac{1}{Maximize(E.A)} \qquad \textbf{Eq. (4)}$$

subject to $20 \leq A \leq 30$, $35 \leq B \leq 45$, $60 \leq C \leq 140$, $30 \leq D \leq 40$.

Where E.A (Eq. 4) indicated lipase activity, and *A*, *B*, *C* and *D* represented the uncoded values of the variables DMSO concentration, buffer molarity, soaking time and temperature, respectively. The important aspect in DE-based optimisation approach is the determination of its three important controlling parameters, namely number of population (*NP*), crossover operator (*CR*) and scaling factor (mutation constant, *F*). In the current optimisation study, the population size was kept equal to 20 (5×*D*), where *D* was the dimension of the problem. Both the crossover rate and mutation factor were varied in the range of [0, 1] and a study was conducted to identify the values of *CR* and *F*, after varying one parameter at a time and keeping the other parameters at a fixed level. Initially, the study was performed to determine the appropriate value of CR, keeping the other parameters fixed at one level (*NP*=20; *Gen*=50; *F*=0.5). The maximum value of lipase activity (that is, minimum value of objective function value) was 134.417, 134.418, 134.420, 134.422 and 134.421 U/gds for different values of *CR*, such as 0.0, 0.1, 0.3, 0.5 and 0.6, respectively. The variation of objective function value (E.A) with the number of generations is shown in Figure 2 A.



(A) (B)

Figure 2 - Variation of objection value with number of generations (A) CR=0.5 (B) F=0.25.

Similarly, the value of *F*, which would give maximum lipase activity was also determined with the help of a similar procedure. In this case, the values *CR*, *NP* and *Gen* were taken as 0.5, which resulted in maximum value of lipase activity/minimum value of objective function, 20 and 50, respectively. The maximum lipase activity obtained were 134.382, 134.423 and 134.421 when the values of mutation constant *F* were fixed at 0.05, 0.25 and 0.5, respectively (Fig. 2B). The optimum values of process variables obtained were 25.01%, 40 mM, 128.52 min and 35^{o}C for DMSO concentration, buffer molarity, soaking time and temperature, respectively. The optimal levels of control parameters *NP*, *Gen*, *CR* and *F* were 20, 50, 0.5 and 0.25, respectively. The maximum value of lipase activity was 134.423 U/gds. The DE-optimised parameters obtained were validated experimentally by conducting the experiment in triplicates. The lipase activity under the DE-stated extraction conditions was 133.36$\pm$0.77 U/gds, which was in close agreement with the maximised DE value of 134.423 U/gds. The usage of DE optimisation approach resulted in a significant improvement (38%) in the extracellular lipase recovery when compared with the OVAT approach (97$\pm$0.85 U/gds). Apart from the utilisation of OVAT and gradient-based statistical approaches, the evolutionary approach such as DE has not been utilised till- date for any enzyme recovery from the SSF biomass.

CONCLUSION

An approach of integrating RSM with DE has been successfully implemented to understand and optimise the different biomass processing aspects on lipase extraction. This approach provided an efficient, reliable method for modeling the lipase extraction process as well as in achieving the significantly enhanced lipase activity (36.28 U/gds than the non-optimized process). The maximum lipase activity under optimized condition obtained was 131.43 U/gds. Lipase being the industrially important enzyme, the present attempt of lipase recovery enhancement from the SSF biomass could have a predominant role on total enzyme recovery as well as on overall economy of process.

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REFERENCES

Adachi S, Kobayashi T. Synthesis of esters by immobilized-lipase-catalyzed condensation reaction of sugars and fatty acids in water-miscible organic solvent. *J Biosci Bioeng.* 2005; 99: 87-94.

Ahmed SA. Optimization of production and extraction parameters of *Bacillus megaterium* levansucrase using solid-state fermentation. *J Appl Sci Res.* 2008; 4: 1199-1204.

Barbosa O, Ortiz C, Torres R, Fernandez-Lafuente R. Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica* in organic media: Enantiospecifc production of atenolol acetate. *J Mol Catal B: Enzymic.* 2011; 71(3-4): 124-132.

Baskar G, Renganathan S. Optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782 using response surface methodology and artificial neural network-linked genetic algorithm. *Asia-Pac J Chem Eng*. 2010; 7(2): 212-220.

Ben Salah R, Ghamghui H, Miled M, Mejdoub H, Gargouri Y. Production of butyl acetate ester by lipase from novel strain of *Rhizopus oryzae. J Biosci Bioeng*. 2007; 103: 368-372.

Bethea RM, Duran BS, Boullion TL. Statistical Methods for Engineers and Scientists. New York: Marcel Dekker, Inc. 1985.

Castilho LR, Alves TLM, Medronho RA. Recovery of pectolytic enzymes produced by solid state culture of *Aspergillus niger*. *Process Biochem*. 1999; 34: 181-186

Chen YL, Su CK, Chiang BH. Optimization of reversed micellar extraction of chitosanases produced by *Bacillus cereus. Process Biochem.* 2006; 41: 752-758.

Dheeman SD, Henehan GTM, Frías JM. Purification and properties of *Amycolatopsis mediterranei* DSM 43304 lipase and its potential in flavour ester synthesis. *Bioresource Technol.* 2011; 102(3): 3373-3379.

Gupta S, Kapoor M, Sharma KK, Nair LM, Kuhad RC. Production and recovery of an alkaline exo-polygalacturonase from *Bacillus subtilis* RCK under solid-state fermentation using statistical approach. *Bioresource Technol.* 2008; 99: 937-945.

Hama S, Tamalampudi S, Fukumizu T, Miura K, Yamaji H, Kondo A, Fukuda H. Lipase localization in *Rhizopus oryzae* cells immobilized within biomass support particles for use as whole-cell biocatalysts in biodiesel-fuel production. *J Biosci Bioeng.* 2006; 101: 328-333.

Hasan F, Shah A, Hameed A. Industrial applications of microbial lipases. *Enzyme Microb Tech.* 2006; 39: 235-251.

Hasmann FA, Cortez DV, Júnior AP, Roberto IC. Optimization of β-xylosidase recovery by reversed micelles using Response surface methodology. *Elect J Biotechnol*. 2003; 6: 153-160.

Heck JX, Hertz PF, Ayub MAC. Extraction optimization of xylanases obtained by solid-state cultivation of *Bacillus circulans* BL53. *Process Biochem.* 2005; 40: 2891-2895.

Hiol A, Jonzo MD, Rugani N, Druet D, Sarda L, Comeau LC. Purification and characterization of an extracellular lipase from a thermophilic *Rhizopus oryzae* strain isolated from palm fruit. *Enzyme Microb Technol*. 2000; 26: 421-430.

Houde A, Kademi A, Leblanc D. Lipases and their industrial applications: an overview. *Appl Biochem Biotech*. 2004; 118: 155-170.

Jaeger KE, Eggert T. Lipases for biotechnology. *Curr Opin Biotechnol.* 2002; 13: 390-397.

Karra-Châabouni M, Bouaziz I, Boufi S, Botelho do Rego AM, Gargouri Y. Physical immobilization of *Rhizopus oryzae* lipase onto cellulose substrate: activity and stability studies. *Colloid Surf B: Inter.* 2008, 66: 168-177.

Kumari A, Mahapatra P, Garlapati VK, Banerjee R. Enzymatic transesterification of Jatropha oil. *Biotechnol Biofuel*. 2009a; 2:1.

Kumari A, Mahapatra P, Garlapati VK, Banerjee R, Dasgupta S. Lipase mediated isoamyl acetate synthesis in solvent-free system using vinyl acetate as acyl donor. *Food Technol Biotechnol.* 2009b; 47: 13-18.

Kumari A, Mahapatra P, Garlapati VK, Banerjee R. Comparative study of thermostability and ester synthesis ability of free and immobilized lipases on cross linked silica gel. *Bioprocess Biosyst Eng*. 2008; 31: 291-298.

Lee JH, Kim SB, Kang SW, Song YS, Park C, Han SO, et al. Biodiesel production by a mixture of *Candida rugosa* and *Rhizopus oryzae* lipases using a supercritical carbon dioxide process. *Bioresource Technol*. 2011; 102(2): 2105-2108.

Mahapatra P, Kumari A, Garlapati VK, Banerjee R, Nag A. Enzymatic synthesis of fruit flavor esters by immobilized lipase from *Rhizopus oligosporus* optimized with Response surface methodology. *J Mol Catal B: Enzymatic*. 2009a; 60: 57-63.

Mahapatra P, Kumari A, Garlapati VK, Banerjee R, Nag A. Kinetics of solvent-free geranyl acetate synthesis by *Rhizopus oligosporus* NRRL 5905 lipase immobilized on to cross-linked silica. *Biocatal Biotransfor*. 2009b; 27: 124-130.

Maron SH, Prutton CF. Principles of physical chemistry. New Delhi: Oxford and IBH publishing Co.Pvt. Ltd.; 1965.

Monteiro TIRC, Porto TS, Carneiro-Leao AMA, Silva MPC, Carneiro-da-Cunha MG. Reversed micellar extraction of an extracellular protease from *Nocardiopsis* sp. fermentation broth. *Biochemical Eng J*. 2005; 24: 87-90.

Montgomery DC. Design and Analysis of Experiments. New York: Wiley; 1997.

Montgomery DC, Runger GC. Applied statistics and probability for engineers. Singapore: John Wiley & Sons (Asia) Pvt. Ltd.; 2002.

Myers RH, Montgomery DC, Anderson-Cook CM. Response surface methodology: process and product optimization using designed experiments. New Jersey: John Wiley & Sons, Inc. 2009.

Palit S, Banerjee R. Optimization of extraction parameters for recovery of α-amylase from the fermented bran of *Bacillus circulans* GRS313. *Braz Arch Biol Technol.* 2001; 44: 107 -111.

Pereira R, Antunes SC, Goncalves AMM, Marques SM, Gonclaves F, Ferreira F. The effectiveness of a biological treatment with *Rhizopus oryzae* and of a photo-Fenton oxidation in the mitigation of toxicity of a bleached kraft pulp mill effluent. *Water Res.* 2009; 43: 2471-2480.

Price KV, Storn RM, Lampinen JA. Differential evolution - A practical approach to global optimization. Heidelberg: Springer-Verlag; 2005.

Rajendran A, Thangavelu V. Statistical experimental design for evaluation of medium components for lipase production by *Rhizopus arrhizus* MTCC 2233. *LWT- Food Sci Technol*. 2009; 42: 985-992.

Reetz MT. Lipases as practical biocatalysts. *Curr Opin Chem Biol.* 2002; 6(2): 145-150.

Sayari A, Frikha F, Miled M, Mtibaa H, Ben Ali Y, Verger R, et al. N-terminal peptide of *Rhizopus oryzae* lipase is important for its catalytic properties. *FEBS Lett*. 2005; 579: 976-982.

Storn R, Price KV. Differential evolution – A simple and efficient heuristic for global optimization over continuous spaces. *J Global Optim.* 1997; 11: 341-359.

Sun SY, Xu Y, Wang D. Novel minor lipase from *Rhizopus chinensis* during solid-state fermentation: biochemical characterization and its esterification potential for ester synthesis. *Bioresource Technol.* 2009; 100: 2607-2612.

Tunga R, Banerjee R, Bhattacharya BC. Some studies on optimization of extraction process for protease production in SSF. *Bioprocess Eng*. 1999; 20: 485-489.

Volken de Souza CF, Rodrigues RC, Heck JX, Ayub MAC. Optimization of transglutaminase extraction produced by *Bacillus circulans* BL32 on solid-state cultivation. *J Chem Technol Biotechnol.* 2008; 83: 1306-1313.

Xiong, ZQ, Guo MJ, Guo YX, Chu J, Zhuang YP, Zhang SL. Efficient extraction of intracellular reduced glutathione from fermentation broth of *Saccharomyces cerevisiae* by ethanol. *Bioresource Technol.* 2009; 100(2): 1011-1014.

Yasuda M, Ogino H, Kiguchi T, Kotani T, Takakura S, Ishibashi T, et al. Purification and characterization of lipase from *Rhizopus chinensis* cells. *J Biosci Bioeng*. 1999; 88: 571-573.

Yu XW, Wang LL, Xu Y. *Rhizopus chinensis* lipase: Gene cloning, expression in *Pichia pastoris* and properties. *J Mol Catal B: Enz.* 2009; 57: 304-311.

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