Waste Biomass Valor (2013) 4:347–357
DOI 10.1007/s12649-012-9140-8

ORIGINAL PAPER

Utilizing Agricultural Wastes as Substrates for Lipase Production by *Candida rugosa* NCIM 3462 in Solid-State Fermentation: Response Surface Optimization of Fermentation Parameters

Aravindan Rajendran · Viruthagiri Thangavelu

Received: 18 January 2012 / Accepted: 19 June 2012 / Published online: 26 June 2012

Abstract
Purpose Utilizing agricultural wastes for the production of industrially valuable enzymes like lipases in solid state fermentation holds a promising alternative for conventional processes due to the various obvious advantages, especially the cost-effectiveness of the process and sustainability. In this present work, the feasibility of exploiting various agricultural residues for lipase production was evaluated and optimization of the important parameters was done using statistical methods.
Methods *Candida rugosa* NCIM 3462 was used for the production of lipase in solid state fermentation. Agro industrial wastes such as sesame oil cake, groundnut oil cake and coconut oil cake were used for the solid state fermentation. The Energy dispersion spectrum of the solid substrates was used to study the elemental composition of oil cakes. The response surface methodology was employed to optimize the lipase production and to study the effect of temperature and substrate to moisture ratio.
Results The maximum lipase activity of 22.40 U/g substrate was obtained using sesame oil cake which was 1.8 times the maximum activity obtained during the initial screening of variables by 'one-factor-at-time' approach. The optimized temperature and substrate to moisture ratio were found to be 32.3 °C and 1:3.23 g/ml respectively.
Conclusions A predictive model for the combined effects of the independent variables using response surface methodology and artificial neural network was proved to be excellent empirical model for lipase production by *C. rugosa* in solid state fermentation and aid in improving lipase fermentation utilizing agricultural residues as valuable substrates.

Keywords Lipase · Optimization · Response surface methodology · *Candida rugosa* · Protease · Solid state fermentation

Introduction

Lipase [EC 3.1.1.3] has significant industrial importance due to its ability to bring about a range of bioconversions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis [1–3]. *Candida rugosa*, an industrial producer is the potential microbial source for lipase production [4–6]. Interest in *C. rugosa* lipase has been greatly developed due to its enantiospecific and stereoselective properties and its non-specificity towards the different ester bonds in triglycerides. Lipases catalyze complete hydrolysis of triglycerides to glycerol and free fatty acids and also transesterification reaction [1–7], which has found extensive application in the production of biodiesel.

Among processes used for enzyme production, industrially important enzymes have traditionally been obtained from submerged culture because of ease of handling and greater control of environmental factors, such as temperature and pH. Solid state fermentation (SSF) constitutes an interesting alternative, since metabolites so obtained are more concentrated and product costs are much lower due to the efficient utilization and value addition of wastes. Some of the advantages of SSF over conventional submerged fermentation for work involving fungi are simplicity of equipment and low moisture content, which prevents

A. Rajendran (✉) · V. Thangavelu
Biochemical Engineering Laboratory, Department of Chemical Engineering, Annamalai University, Annamalai Nagar, Tamil Nadu 608 002, India
e-mail: aravindraj@gmail.com

bacterial contamination, requires lower capital, reduced energy requirement, simpler fermentation media (agro-industrial residues) and absence of rigorous control of fermentation parameters, uses less water and produces lower wastewater [8–14]. Apart from various advantages with SSF, the major challenges are in the scale up, purification of end products and biomass estimation [14].

Usually SSF is a batch process that utilizes heterogeneous natural residues, which provides essential carbon, nitrogen and mineral sources to the microorganisms. Additional nutrients and mineral salt solution can be supplemented to enhance the production [10]. In recent times, increasing reports on enzyme production by SSF was available in literature since the results have shown that SSF can give higher yields and better product characteristics than submerged fermentation [8–19]. Filamentous fungi are best adopted for SSF and use of unicellular microbes for SSF is sparse [15–18]. Benjamin and Pandey [17] have reported coconut cake as a potent substrate for lipase production by *C. rugosa*. Kamini et al. [20] produced lipase from *Aspergillus niger* using gingelly oil cake. Mahadik et al. [21] have compared the lipase production by *A. niger* in both submerged and SSF and they have established the need for lipid based carbon source for lipase production, although its role in lipase synthesis was not well understood. Oil cakes can serve this requirement of microbes to synthesis lipase. Factors such as fermentation time, temperature, pH, oxygen level, moisture content and water activity significantly affect microbial growth and enzyme secretion [10]. Rao et al. [15, 16] have studied the production of lipase by *C. rugosa* using rice bran as substrate and addition of rice bran oil increased the lipase activity up to 17 %. The screening of several agro-industrial residues in the SSF processes is the most important step and its selection depends upon several factors mainly the cost and the availability. In the SSF process, the solid substrate supplies the essential nutrients to the culture and also serves as an anchorage for the microbial cells.

The effect of SSF conditions were studied by varying one parameter at a time [15] and the interaction effects were studied by response surface methodology [16] in various reports. The 'one-factor-at-time' approach may be useful for estimating suitable operational intervals for important inhibitory or stimulatory variables prior to conducting response surface studies. Response surface methodology (RSM) using central composite design (CCD) was adopted to design the experiments and to study the independent and interaction effects of variables on lipase production, and was widely employed to optimize various bioprocesses [22–24]. Mathematical models play an important role in rational design and optimization of biochemical process. However due to the inherent nonlinearity, complexity and uncertainty of biochemical process, it is usually difficult to obtain an accurate model for a biochemical system. An artificial neural network (ANN) is a modeling technique, which has the ability to 'learn' complex non linear relationship with limited prior knowledge about the process structure and to perform inferences for unknown combinations of input variables [25]. The RSM and ANN approach offers an attractive alternative to fundamental process model development.

In the present study, optimization strategy for lipase production in SSF using *C. rugosa* was done which includes,

1. Screening of various agro industrial residues sesame oil cake, ground nut oil cake and coconut oil cake for lipase production.
2. Elemental analysis of the oil cakes using energy dispersion spectrum by Scanning Electron microscope.
3. Optimization of temperature and substrate to moisture ratio by central composite design using the best substrate.
4. Establishing a mathematical model expressing the relationship between lipase production and fermentation conditions and verification of the model using RSM.
5. Application of Artificial neural network model for the prediction of lipase activity.

Materials and Methods

Microorganism

Candida rugosa NCIM 3462 was obtained from National Chemical Laboratory, Pune, India. The medium components were procured from Himedia Ltd, Mumbai, India. All chemicals used in the experiments were of analytical grade. Spirit blue agar was used for the detection of lipase activity of *C. rugosa*.

Culture Maintenance

The *C. rugosa* stock culture was maintained on MGYP agar slants containing (g/l): Malt extract, 3.0; Glucose, 10.0; Yeast extract, 3.0; Peptone, 5.0 and Agar, 10.0. The 48 h old culture, maintained in MGYP agar was used to inoculate the seed culture medium (MGYP broth) in 250 ml Erlenmeyer flask with working volume of 100 ml and incubated at 30 °C for 24 h.

Lipase Production in Solid State Fermentation

The experiments were conducted in 250 ml Erlenmeyer flasks containing 10 g of solid substrate to which the mineral salt solution was added in such a way that the

substrate to moisture ratio is 1:3.5 g/ml for the initial screening of substrates. The mineral salt solution contains (g/l), KH_2PO_4, 4.0; NaCl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.125; $FeCl_3 \cdot 6H_2O$, 0.0019. The medium was sterilized at 15 psig and 121 °C for 15 min. The sterilized medium was then cooled and inoculated with 5 % (v/w) of 24 h grown *C. rugosa*. The contents of the flask were mixed thoroughly and incubated at 30 °C.

Substrates for Solid State Fermentation

Commercially available groundnut oil cake, sesame oil cake and coconut oil cake were used as solid substrates and their effect on the production of lipase was investigated. The oil cakes were collected from a local small scale oil seed processing and pressing industry in Chidambaram, Tamil Nadu, India. The raw materials for this industry comes from the near by places of Chidambaram (Cuddalore District, Tamil Nadu, India). Since the quality of the raw materials does not vary in large with respect to geographic and processing quality, there is not much variation in the quality of the oil cakes we obtained for the production of the hydrolytic enzyme. The results presented in this work are mean of three experiments with oil cakes obtained from different batches. The dry substrates were grounded and sieved to provide uniform particle sizes between 0.21 and 0.42 mm. The solid substrate that produced maximum lipase activity was used for further optimization studies. Samples were taken for every 12 h time interval for the fermentation period of 72 h. The energy dispersion spectrums for elemental analysis of the substrates were done using Scanning Electron Microscope (SEM-JE0L-JSM-5610, Japan with EDS, Oxford, London).

Enzyme Extraction

Hundred millilitre of 50 mM phosphate buffer of pH 7.0 was added to the solid substrate and kept in a rotary shaker at 200 rpm for 2 h at 30 °C for extraction of enzymes. The suspension was filtered and centrifuged at 5,030*g* for 15 min and 4 °C. The supernatant was used for the determination of extracellular enzyme activity [26, 27].

Lipase Activity Assay

Lipase activity was estimated with olive oil emulsion by the procedure of Ota and Yamada [28]. Olive oil emulsion was prepared by homogenizing 25 ml of olive oil and 75 ml of 2 % polyvinyl alcohol solution in a homogenizer for 6 min at 20,000 rpm. The reaction mixture composed of 2 ml olive oil emulsion, 2.5 ml 0.05 M phosphate buffer and 0.5 ml enzyme solution and the reaction mixture was incubated at 37 °C for 15 min. The emulsion was destroyed by addition of 10 ml acetone immediately after incubation and the liberated fatty acid was titrated against 0.05N NaOH. One unit (U) of lipase activity is defined as 1 μmol of free fatty acid liberated per ml of enzyme per minute at 37 °C.

Protease Activity Assay

The protease activity was assayed by modified Anson method [29] using casein as the substrate. 2 ml of 1 % (w/v) casein solution was mixed with 0.5 ml of enzyme solution and incubated at 37 °C for 30 min. 2.5 ml of 0.4 M trichloroacetic acid was added to arrest the reaction. The solution with precipitate was filtered and to the 1 ml of filtrate, 5 ml of 0.4 M Na_2CO_3 and 0.5 ml of folin reagents were added. After 10 min of incubation, the colour density developed was determined at 660 nm. One unit (U) of protease activity is defined as 1 μg of tyrosine liberated per minute by 1 ml of enzyme at 37 °C.

Central Composite Experimental Design and Optimization by Response Surface Methodology

Central composite design (CCD), one of the response surface methodologies usually utilized to obtain data that fits in a full second order polynomial model. The graphical representation of the model equation results in the response surface plots that represent the individual and interactive effects of test variables on the response. A 2^2 full factorial central composite design with five coded levels and five replicates about the center point, making a total of 13 runs [30], were used to study the effect of temperature (X_1) and substrate to moisture ratio (X_2) on lipase production. If the factorial is full factorial then,

$$\alpha = \left[2^k\right]^{1/4} \tag{1}$$

In this study $k = 2$ factors (X_1, X_2) therefore $\alpha = 1.414$. The parameters were tested at five levels, coded -1.414, -1, 0, $+1$ and $+1.414$ for lowest, low, middle, high and highest concentration respectively. The CCD experiment was designed using the MINITAB software package, version 14.0, The Math Works Inc., Natick, Massachusetts, USA. The variables were coded according to the following equation,

$$x_i = \frac{X_i - X_c}{\Delta X_i}, \quad i = 1, 2, 3, \ldots, k \tag{2}$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_c the real value of an independent variable at the center point and ΔX_i is the step change value. The experimental range with the levels of independent variables and experimental plan is shown in

Table 1 Experimental range and levels of the two significant independent variables used in response surface methodology in terms of actual and coded factors

Variables with designate	Coded levels				
	−1.414	−1	0	1	1.414
X_1: temperature (°C)	24.93	27	32	37	39.07
X_2: substrate to moisture ratio (g/ml)	1:1.59	1:2	1:3	1:4	1:4.41

Tables 1 and 2 respectively for medium optimization. The behavior of the system is explained by the following second degree polynomial equation,

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{i<j} \beta_{ij} x_i x_j \quad (3)$$

where Y is the predicted response, β_0 the offset term, β_i the coefficient linear effect, β_{ii} the coefficient squared effect, β_{ij} the coefficient of interaction effect, x_i and x_j the coded level of variable X_i and X_j respectively. Thus, the second order polynomial equation can be presented as follows; Lipase activity $(Y) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 - \beta_{11} x_1^2 - \beta_{22} x_2^2 + \beta_{12} x_1 x_2$. The MINITAB software statistical program package was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. The goodness of fit of the regression model obtained was given by the coefficient of determination R^2. The statistical significance of the model was determined by the application of Fischer's F test. Since coding of the variable enables direct comparison of the partial regression coefficients, their significance was determined by students t test and the associated probabilities. The response surface plots were used to describe the individual and cumulative effects of the variables as well as the mutual interactions between the variables on the dependent variable (lipase activity). The second degree polynomial equation was maximized by a constraint search procedure using the MATLAB software (Version 6.5, The MathWorks, Inc. Natick, USA) to obtain the optimal levels of the independent variables and the predicted maximum lipase activity. The enzyme activity predicted by maximization procedure was compared with the experimental values.

Artificial Neural Network Model

A feed forward back propagation algorithm was used in the training of the neural network on the basis of varying input/output pair data sets. Regression-based response surface models require the order of the model to be stated (i.e., second, third or fourth order), while ANN tends to implicitly match the input vector (i.e., medium components) to the output vector (enzyme production) [31]. ANN was applied for the purpose of simulation on the same experimental data (Table 2) used for RSM. A well trained feed forward back propagation neural network with one hidden layer can be employed to overcome the uncertainties typical of biological reactions with no need for prior knowledge of the relationships of the process variables involved [32]. The goodness of fit of the trained neural network to the reference data was determined by the coefficient of determination R^2. The accuracy of the neural network estimation is strongly restricted to the completeness and the preparation method of training patterns as well

Table 2 The central composite design matrix of independent variables used in response surface methodology with corresponding experimental and predicted values of lipase activity

Experiment no.	Temperature (x_1)	Substrate to moisture ratio (x_2)	Observed lipase activity[a] (U/g substrate)	Predicted lipase activity by RSM (U/g substrate)	Predicted lipase activity by ANN (U/g substrate)
1	−1	−1	8.60	10.081	8.599
2	1	−1	8.4	11.597	8.607
3	−1	1	12.87	15.245	12.870
4	1	1	12.5	16.761	12.870
5	−1.414	0	17.17	15.569	17.170
6	1.414	0	21.46	17.713	21.460
7	0	−1.414	8.60	6.554	8.539
8	0	1.414	17.17	13.856	17.170
9	0	0	21.46	21.784	21.732
10	0	0	21.90	21.784	21.732
11	0	0	21.94	21.784	21.732
12	0	0	21.46	21.784	21.732
13	0	0	21.90	21.784	21.732

[a] Data are means of triplicates

as the structure of the neural network. Neural networks consist of many processing elements called neurons interconnected by information channels. In this present work one hidden layer was used with the three neurons. The number of neurons in the input and output layers are given by the number of input and output variables in the process under investigation. The input signals are amplified or dampened by a weight associated with each information channel. The neuron then sums all weighted inputs and passes them through a threshold to determine the activation value (the fired output signal) of this neuron. The inter neuron activity can be modeled by an activation function. In a back propagation neural network, the function is commonly in the form of sigmoid function.

$$y_j = -\left[1 + \exp\left(\sum_{i=1}^{n} a_i w_{ij} + \theta_j\right)\right]^{-1} \quad (4)$$

where a_i—input signal, y_j—fired output signal, w_{ij}—weight associated with the input signal a_i, θ_j—threshold value of neuron j. In back propagation networks, the process is executed according to an error feedback method, by which it will first update activity values of all the neurons corresponding to input data based on current weights and then adjust the weights according to the error between fixed outputs and desired outputs to reduce the error. Let E represent the summation of output errors

$$E = \frac{1}{2}\sum_{j} (y_j - d_j)^2 \quad (5)$$

where d_j is the desired output from neuron j. According to the maximum gradient scheme, a common scheme used for neural network training each connection weight w_{ik} is changed by

$$\Delta w_{ik} = - - \eta \frac{\partial E}{\partial w_{ik}} \quad (6)$$

where η is the positive constant controlling the speed of learning. The Neural network toolbox in MATLAB software was used to construct the ANN topology.

Results and Discussion

Effect of Various Agricultural Residues on Lipase Production

The selection of suitable substrate is the most critical and important factor in any SSF process. The lipase production and protease production in SSF using various agro residues namely sesame oil cake, groundnut oil cake and coconut oil cake as substrates for lipase production by *C. rugosa* is shown in Figs. 1 and 2 respectively. The lipase production was found to be maximum at 12.47 U/(g substrate) for the sesame oil cake as the substrate during 60 h of SSF and was found to decrease later to a certain extent up to 10.28 U/(g substrate) till the end of the SSF of 72 h. Groundnut oil cake and coconut oil cake as substrates gave a maximum lipase production of 8.87 U/(g substrate) at 60 h and 8.58 U/(g substrate) at 48 h respectively. The protease production was found to be maximum at 9.26 U/(g substrate) for the groundnut oil cake as the substrate during the 60 h of SSF and remains almost same at the end of SSF at 72 h. The coconut oil cake and sesame oil cake as substrate gave a maximum protease production of 6.6 U/(g substrate) and 6 U/(g substrate) at 60 h of SSF

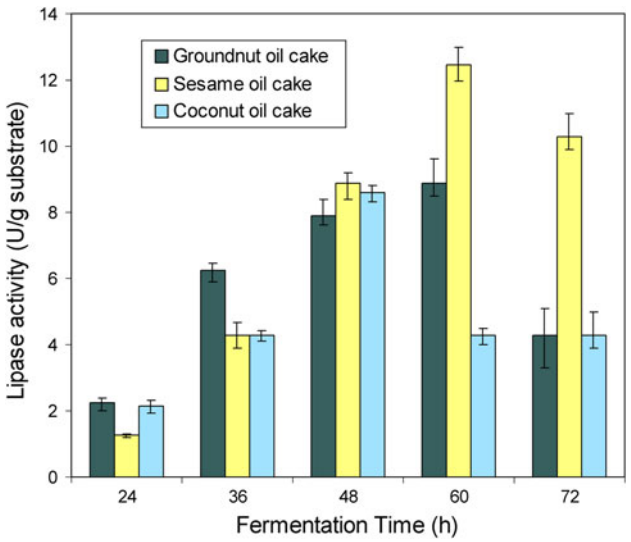

Fig. 1 Solid state fermentative production of lipase by *C. rugosa* using various vegetable oil cakes

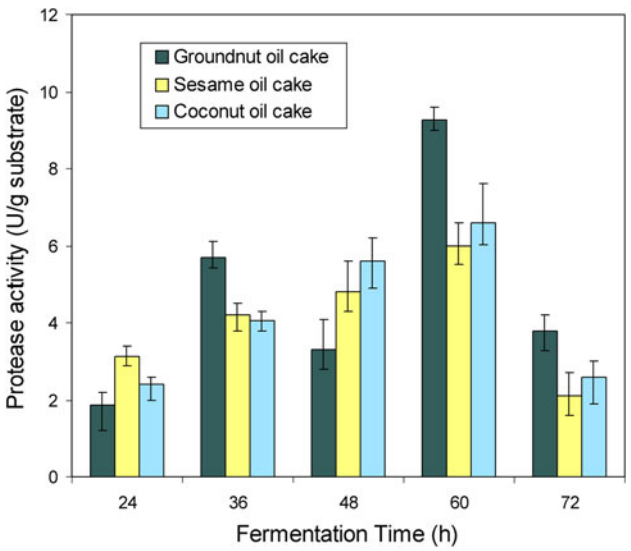

Fig. 2 Protease activity in solid state fermentative production of lipase using *C. rugosa* in various oil cakes

respectively. The increased protease activity in the all the substrates during the later stages of fermentation might be the reason for the reduction in lipase activity after 60 h of SSF. Since sesame oil cake gave a maximum lipase production 12.47 U/(g substrate) it was selected as the best substrate for the further studies in solid state fermentation using *C. rugosa*. A better lipase activity with sesame oil cake might be due to the presence of essential nutrient sources and fatty acid composition. Kamini et al. [20] reported that *A. niger* produced high levels of lipase using sesame oil cake. Lakshmi et al. [33] stated that the presence of high percentage of unsaturated (C18: n) free fatty acid composition in sesame oil was responsible for higher lipase production by *C. rugosa*. Roa et al. [15] have shown the importance of oil content for the increased lipase production. High level lipase activity in sesame oil cake shows the presence of essential nutrient sources for growth and lipase production by *C. rugosa*. However moderate level of lipase production was observed with the other substrates analyzed. Conversely, Benjamin and Pandey [17] have observed coconut cake as best substrate for lipase secretion using *C. rugosa*. A combination of olive oil cake and bagasse was utilized by Cordova et al. [19]. Gombert et al. [9] used babassu oil cake to produce lipase by *Penicillium restrictum*.

Energy dispersion spectrum and the corresponding scanning electron microscope picture and of the various oil cakes exploited for SSF is shown in Fig. 3. The energy dispersion spectrum is used to study the elemental composition of the substrates. The major elements present in sesame oil cake were Ca, K, Cu, Mg and Si. The elements Cu, K, Zn, Cl and Al were present in the coconut oil cake and the elements Cu, K, Zn and Fe were present in the groundnut oil cake significantly. The higher yield of lipase in SSF using *C. rugosa* with sesame oil cake might be attributed to the higher percentage of the elements calcium and magnesium in the sesame oil cake. Coconut oil cake did not contain calcium and gave a less production of lipase. Reports confirmed that the presence of Ca^{2+} and Mg^{2+} stimulated lipase production in *Burkolderia* sp. [34]. Sidhu et al. [35] reported that the presence of Ca^{2+} enhances lipase production by a thermophilic *Bacillus* species RS-12. Lipase production by a thermophilic *Bacillus* species was increased several fold when magnesium, iron and calcium ions were added in the production medium [36].

Time Course of Lipase Production

The time course of lipase production in SSF using sesame oil cake is illustrated in Fig. 1. Lipase activity reaches the maximum of 12.47 U/g substrate at 60 h of fermentation. This is in agreement with the results observed by Rao et al. [15]. Beyond this time period, decrease in lipase activity was observed. This may be due to the decrease in nutrient content in the medium or may be due to the increase in the protease activity [20]. Prolonged incubation after 60 h have not shown any increase in enzyme activity.

Optimization of Fermentation Conditions by RSM

The most important process parameters that affect the solid state fermentation were moisture, nature of solid substrate employed and heat and mass transfer characteristics within the substrate bed [14]. These key parameters are to be addressed for successful scale-up of solid state fermentation [14]. Based on our previous experiments by one factor time at time approach, we found that moisture content and the temperature were the most critical parameters that have significant effect on lipase production by *C. rugosa* NCIM 3462. The moisture level was proved to have a significant effect on various enzyme production processes in SSF [21, 26, 27, 37]. High moisture level results in low substrate porosity precluding mass transfer activities including oxygen penetration, and low moisture levels impede the microbial growth by decreasing the accessibility of nutrients to the microorganism and by a lowering the degree of substrate swelling [21, 26, 27, 37]. Incubation temperature of the process could have an effect on moisture content by deciding the metabolic activities of the microorganisms and also on the water evaporation.

Response surface methodology using central composite experimental design was used to study the effect of significant factors affecting the lipase production in SSF by *C. rugosa*. The individual effects and the interaction effects of these factors were studied. The artificial neural network was trained using the experimental data obtained from CCD and the well trained ANN was used to predict the lipase activity in SSF by *C. rugosa*. The variables fermentation temperature (X_1) and the substrate to moisture ratio (X_2) were optimized to enhance the lipase production in SSF. The CCD experimental plan along with the experimental and predicted values of RSM and ANN are presented in Table 2.

The lipase activity varied markedly in a range of 8.60–21.9 U/g substrate, under the conditions tested. The lowest level of activities was observed when the substrate to moisture ratio was very less (Experiment number 1, 2 and 7 in Table 2). Although when the substrate to moisture ratio was high, lipase activity of 17.17 U/g substrate was observed in experiment number 8 (Table 2), which suggests that higher substrate to moisture ratio also would not favor the increased lipase activity. Maximum lipase activities were observed when sesame oil cake was moistened with mineral solution in 1:3 ratio. There is a decline in the lipase production above this ratio as the porosity of the medium is

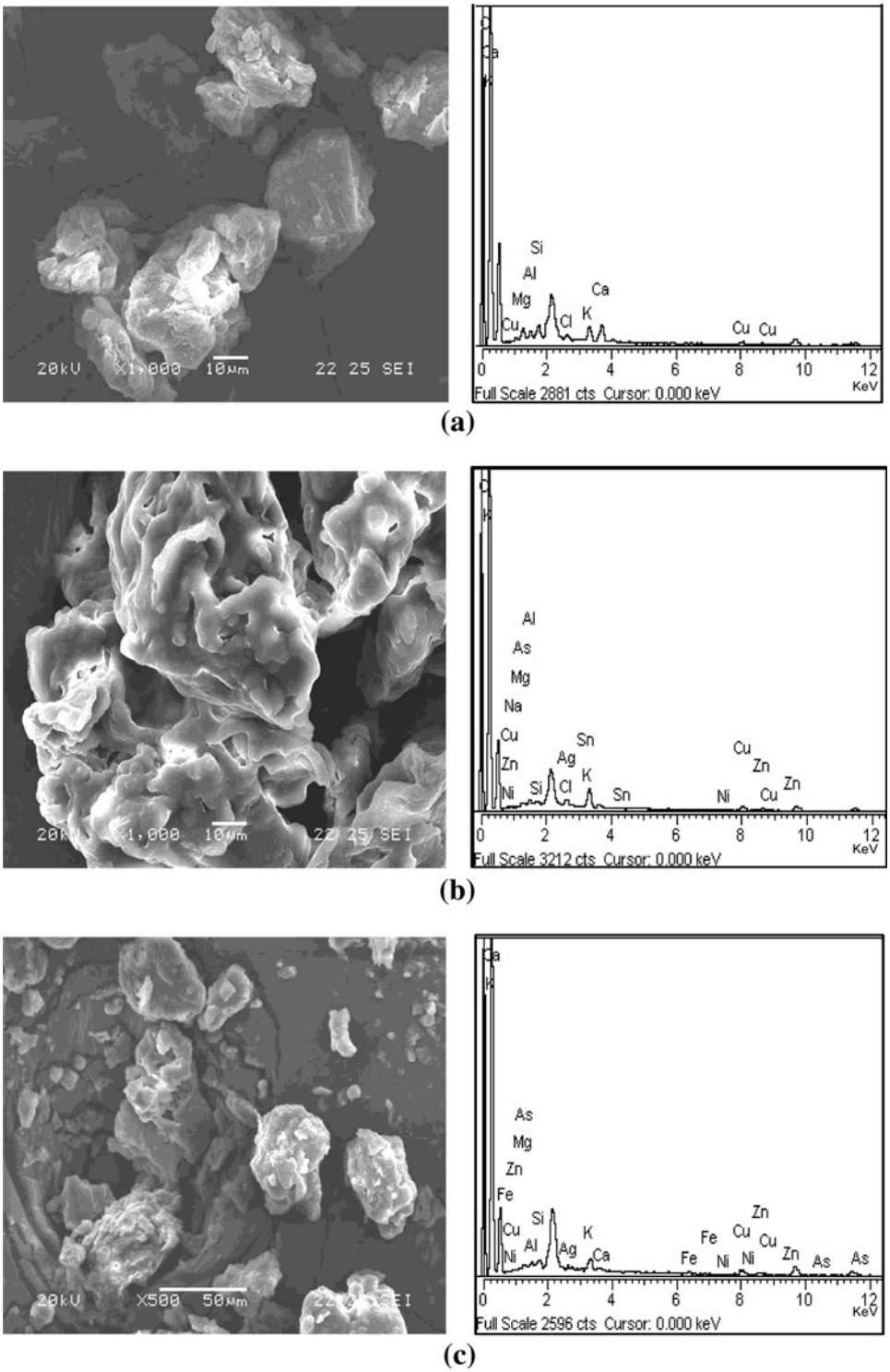

Fig. 3 Scanning Electron Microscope picture and corresponding Energy Dispersion Spectrum of the solid substrates **a** Sesame oil **b** Coconut oil **c** Groundnut oil

decreased [21]. Increase in the lipase activity was observed at the higher temperature ranges experimented in the range of 24.9–39 °C. The elliptical contour obtained as response in Fig. 4 shows the perfect interaction of these variables. Multiple regression analysis of the experimental data gives the following second order polynomial equation,

$$\text{Lipase activity} \quad (Y) = 21.73 + 0.69x_1 + 2.56x_2 - 2.59x_1^2 - 5.8x_2^2 - 0.042x_1x_2 \quad (7)$$

where Y is the lipase activity and x_1 and x_2 are the coded values of the independent variables temperature and substrate to moisture ratio respectively. The correlation

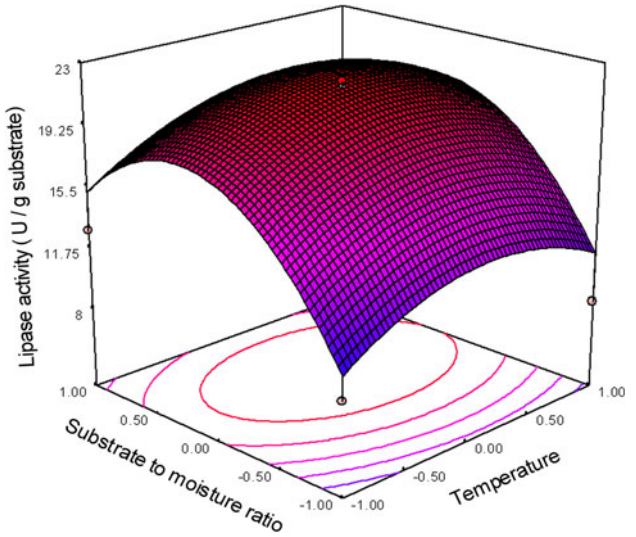

Fig. 4 Response surface plot and contour plots showing the effects of temperature and substrate to moisture ratio on lipase production by *C. rugosa* using the central composite experimental design

measures for testing the goodness of fit of the regression equation are the multiple correlation coefficient, R and the determination coefficient, R^2. The closer the value of R to 1 the better is the correlation between the experimental values and the predicted values by the second order polynomial equation. The value of the determination coefficient R^2 is 0.821 (Fig. 5a), suggests that the model does not explain only about 16.9 % of the total variations. The adjusted determination coefficient (Adj R^2) corrects the R^2 value for the sample size and 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. The adjusted R^2 in this study was 0.70, which was close to R^2 value. Statistical testing of the model was done in the form of analysis of variance (ANOVA), which is required to the test the significance and adequacy of the model. The ANOVA result for the quadratic regression model is given in Table 3. The mean squares are obtained by dividing the sum of squares of each of the two sources of variation, the model and the error variance, by the respective degrees of freedom. The Fisher's variance

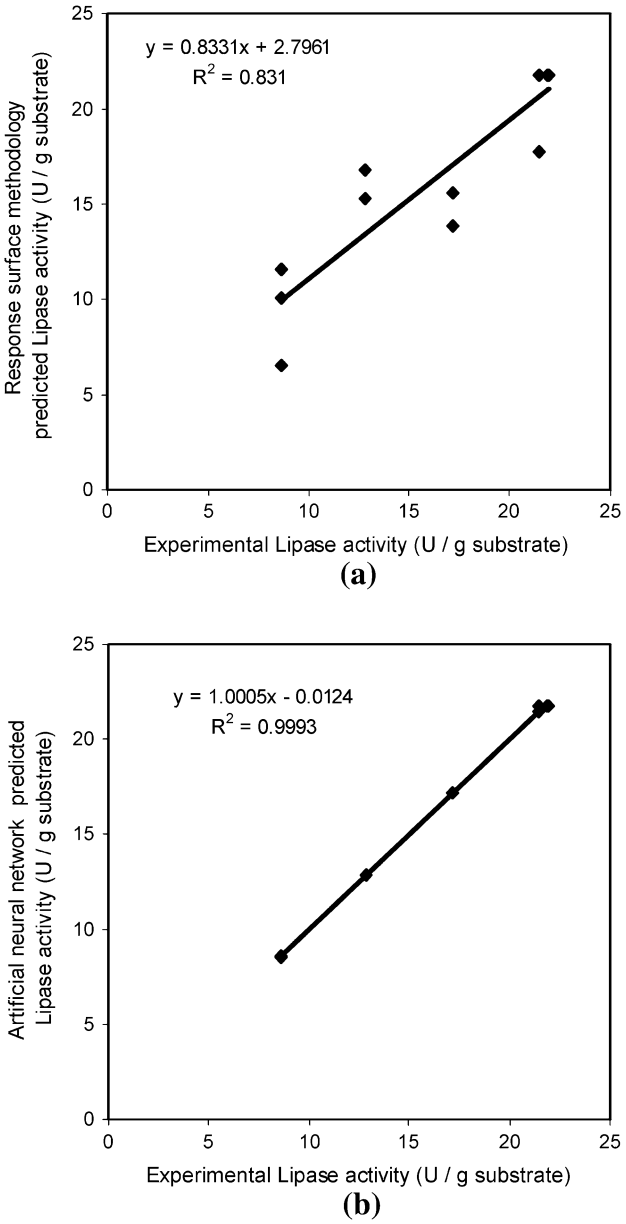

Fig. 5 **a** Plot for the comparison of experimental and response surface methodology model predicted values of lipase activity by *C. rugosa*; **b** Plot for the comparison of experimental and artificial neural network model predicted values of lipase activity by *C. rugosa*

Table 3 Analysis of variance values for the quadratic regression model obtained from central composite design

Source	Degree of freedom	Sum of squares	Mean square	F value	P value
Regression	5	313.98	62.936	6.45	0.015
Linear	2	56.25	28.977	2.89	0.122
Square	2	257.7	128.362	13.23	0.004
Interaction	1	0.007	0.000	0.000	0.979
Error	7	68.179	9.168		
Total	12	382.1			

ratio, the F value $\left(S_r^2/S_e^2\right)$, which is a statistically valid measure of how well the factors describe the variations in the data about its mean which can be calculated from ANOVA, is the ratio of the mean square due to regression to the mean square due to the error. Generally the calculated F value should be greater than the tabulated F value if the model is a good prediction of the experimental results and the estimated factor effects are real [38]. Here the ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the calculated F value ($F_{\text{model}} = 6.45$) and a very low probability value ($P_{\text{model}} > F = 0.0$). Moreover the computed F value is much greater than the tabulated F value ($F_{5,7} = 3.95$ at 5 %) indicating that the treatment differences are highly significant. A well-trained neural network was employed for the prediction of lipase production by *C. rugosa* in SSF. The developed neural network model performed acute satisfactorily when the results were compared with experimental values obtained. A relatively good fit to the experimental data was evident, with an $R^2 = 0.99$ (Fig. 5b) using ANN.

The students t distribution and the corresponding P values, along with parameter estimate were evaluated in MINITAB software and are given in Table 4. The P values are used as a tool to check the significance of each of the coefficients, which in turn may indicate the pattern of the interactions between the variable. The smaller the value of P, the more significant is the corresponding coefficient. The P value signifies that the coefficient for the linear effect of substrate to moisture ratio and squared effect of substrate to moisture ratio are significant. The regression equation was solved by using MATLAB and the optimal values of the test variables in uncoded values were $X_1 = 32.36$ °C, $X_2 = 1{:}3.235$, giving a predicted optimum lipase activity of 22.112 U/g substrate. The shape of the response surface, circular or elliptical, indicates if the interactions between the variables are significant or not. The elliptical nature of the contour plot between the parameters temperature and substrate to moisture ratio indicates that the mutual interaction between these set of

Table 4 Regression results from the data of the central composite designed experiments

Terms in designate	Coefficient	Standard error of coefficient	t value	P value
Constant	21.73	1.396	15.57	0.0
x_1	0.69	1.103	0.623	0.553
x_2	2.56	1.103	2.321	0.053
$x_1\ x_1$	−2.59	1.183	−2.185	0.065
$x_2\ x_2$	−5.8	1.183	−4.902	0.002
$x_1\ x_2$	−0.042	1.56	−0.027	0.979

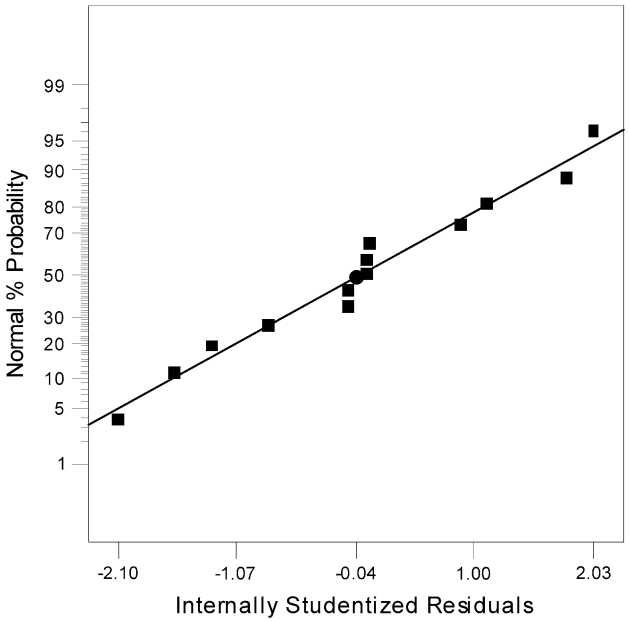

Fig. 6 The Normal probability plot for lipase production model

variables has a significant effect on lipase yield. Checking the adequacy of the model needs information on lack of fit, which is contained is the residuals. The normal probability plot (Fig. 6) of the residuals is an important diagnostic tool to detect and explain the systematic departures from the assumptions that errors are normally distributed and are independent of each other and that the error variances are homogenous. An excellent normal distribution confirmed the normality assumption and the independence of the residuals. The residual plot (Fig. 7) which shows equal

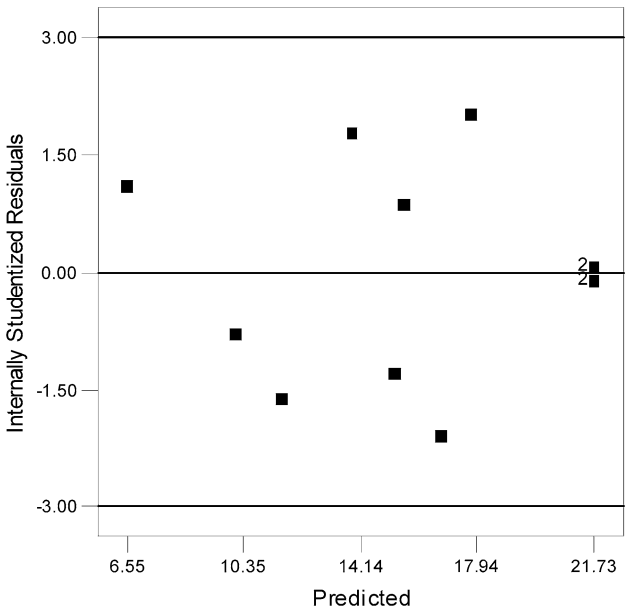

Fig. 7 The residual plot for lipase production model

Table 5 Optimum values of independent variables and the corresponding predicted and experimental lipase activity on analysis of the experimental data by the central composite design

Parameter	Range studied	Optimum value[a]	Lipase activity (U/g substrate)	
			Experimental[b]	RSM predicted[c]
X_1: temperature (°C)	27–37	32.36	22.40	22.11
X_2: substrate to moisture ratio (g/ml)	1:2–1:4	1:3.23		

[a] Values of independent variables obtained by maximizing the second order polynomial equation by a constraint search procedure

[b] Data are means of triplicates experimented at the predicted optimal values of independent variables

[c] Value of the lipase activity predicted by second order polynomial equation

scatter of the residual data above and below the *x*-axis indicates that the variance is independent of the value of the lipase production and thus supporting the adequacy of the least squares fit.

To confirm the predicted optimized condition by RSM, experiment was conducted at the optimum values of the test variables (Table 5) and the maximum lipase activity of 22.4 U/g substrate was obtained at 60 h of cultivation and is illustrated in Fig. 8. The lipase activity was found to reduce after 60 h, which may be due to the increase in protease activity (Fig. 2) at the post exponential growth phase of the microorganism or due to the availability of low level of oil and other essential nutrients in the cultivation broth or may be due to the accumulation of fatty acids [34]. The graphical representations of the regression equation, the response surface plots obtained using MINITAB software package is presented in Fig. 4, which shows the effects of temperature and substrate to moisture ratio on the lipase production while keeping the other factors constant at the middle value.

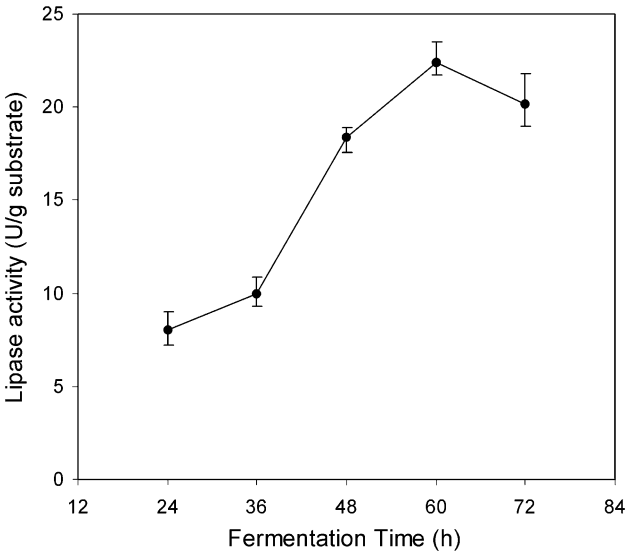

Fig. 8 Experimental time evolution of lipase activity using *C. rugosa* in SSF at the optimized conditions predicted by response surface methodology

Conclusions

The statistical design of experiments offers efficient methodology to optimize the factors with minimum number of experiments. Response surface methodology using central composite design was useful in determining the optimum values of the variables that affect lipase production. Substrate optimization studies showed sesame oil cake as the best substrate for lipase production in SSF. The optimized temperature and substrate to moisture ratio were found to be 32.36 °C and 1:3.23 g ml^{-1} respectively. The experimental results agree closely with the results predicted by response surface methodology confirming that response surface methodology using the statistical design of experiments can be effectively used to optimize the process parameters for lipase production. Both the RSM and ANN models predicted the experimental lipase activity with high R^2 value but ANN provided superior results. We are presently focusing on scaling up the process by developing a novel rotating biological reactor and examining various options to determine the biomass concentration to perform kinetic analysis and modeling of solid state fermentation.

Acknowledgments The authors gratefully acknowledge the Chemical Engineering Department, Annamalai University for providing the facilities to carry out this research work. The authors thank the staffs of Centralized Instrumentation and Service Laboratory (CISL), Dept of Physics, Annamalai University for analyzing the samples in scanning electron microscope.

References

1. Pandey, A., Benjamin, S., Soccal, C.R., Nigam, P., Krieger, N., Soccol, V.T.: The realm of microbial lipases in biotechnology. Biotechnol. Appl. Biochem. **29**, 119–131 (1999)
2. Ghanem, A.: Trends in lipase-catalyzed asymmetric access to enantiomerically pure/enriched compounds. Tetrahedron **63**(8), 1721–1754 (2007)
3. Singh, A.K., Mukhopadhyay, M.: Overview of fungal lipase: a review. Appl. Biochem. Biotechnol. **166**(2), 486–520 (2012)
4. Benjamin, S., Pandey, A.: Optimization of liquid media for lipase production by *Candida rugosa*. Bioresour. Technol. **55**, 167–170 (1996)

5. Del Rio, J.L., Serra, P., Poch, M., Sola, C.: Reaction scheme of lipase production by *Candida rugosa* growing on olive oil. Biotechnol. Lett. **12**(11), 835–838 (1990)
6. Valero, F., Ayats, F., Lopez-Santin, J., Poch, M.: Lipase production by *Candida rugosa*: fermentation behaviour. Biotechnol. Lett. **10**(10), 741–744 (1988)
7. Ghosh, P.K., Saxena, R.K., Gupta, R., Yadav, R.P., Davidson, S.: Microbial lipase: production and applications. Sci. Prog. **79**(2), 85–90 (1996)
8. Ortiz-Vaque, E., Granados-baeza, M., Rivera-Munoz, G.: Effect of culture conditions on lipolytic enzyme production by *Penicillium candidum* in a solid state fermentation. Biotechnol. Adv. **11**, 409–416 (1993)
9. Gombert, A.K., Pinto, A.L., Castilho, L.R., Freire, D.M.G.: Lipase production by *Penicillium restrictum* in solid state fermentation using babassu oil cake as substrate. Process Biochem. **35**, 85–90 (1999)
10. Raimbault, M.: General and microbial aspects of solid state fermentation. Electron. J. Biotechnol. **1**(3), 1–15 (1998)
11. Nagy, V., Tőke, E.R., Keong, L.C., Szatzker, G., Ibrahim, D., Omar, I.C., Szakács, G., Poppe, L.: Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation. J. Mol. Catal. B Enzym. **39**, 141–148 (2006)
12. Treichel, H., Oliveira, D., Mazutti, M.A., Luccio, M.D., Oliveira, J.V.: A review on microbial lipases production. Food Bioprocess Technol. **3**(2), 182–196 (2010)
13. Salihu, A., Alam, Md. Z., AbdulKarim, M.I., Salleh, H.M.: Lipase production: an insight in the utilization of renewable agricultural residues. Resour. Conserv. Recy. **58**, 36–44 (2012)
14. Reeta, R.S., Patel, A.K., Soccol, C.R., Pandey, A.: Recent advances in solid-state fermentation. Biochem. Eng. J. **44**(1), 13–18 (2009)
15. Rao, P.V., Jayaraman, K., Lakshmanan, C.M.: Production of lipase by *Candida rugosa* in solid state fermentation. 1: determination of significant process variables. Process Biochem. **28**, 385–389 (1993)
16. Rao, P.V., Jayaraman, K., Lakshmanan, C.M.: Production of lipase by *Candida rugosa* in solid state fermentation. 2: medium optimization and effect of aeration. Process Biochem. **28**, 391–395 (1993)
17. Benjamin, S., Pandey, A.: Coconut cake—a potent substrate for the production of lipase by *Candida rugosa* in solid state fermentation. Acta Biotechnol. **17**, 241–251 (1997)
18. Virupakshi, S., Gireesh Babu, K., Gaikwad, S.R., Naik, G.R.: Production of a xylanolytic enzyme by a thermoalkaliphilic *Bacillus* sp. JB–99 in solid state fermentation. Process Biochem. **40**, 431–435 (2005)
19. Cordova, J., Nemmaoui, M., Ismaili-Alaoui, M., Morin, A., Roussos, S., Raimbault, M., Benjilali, B.: Lipase production by solid state fermentation of olive cake and sugarcane bagasse. J. Mol. Catal. B Enzym. **5**, 75–78 (1998)
20. Kamini, N.R., Mala, J.G.S., Puvanakrishnan, R.: Lipase production from *Aspergillus niger* by solid-state fermentation using gingelly oil cake. Process Biochem. **33**, 505–511 (1998)
21. Mahadik, N.D., Puntambekar, U.S., Bastawde, K.B., Khire, J.M., Gokhale, D.V.: Production of acidic lipase by *Aspergillus niger* in solid state fermentation. Process Biochem. **38**, 715–721 (2002)
22. Mayerhoff, Z.D., Roberto, I.C., Franco, T.T.: Response surface methodology as an approach to determine the optimal activities of xylose reductase and xylitol dehydrogenase enzymes from *Candida mogii*. Appl. Microbiol. Biotechnol. **70**, 761–767 (2006)
23. Souza, M.O., Roberto, I.C., Milagres, A.M.F.: Solid-state fermentation for xylulose production by *Thermoascus aurantiacus* using response surface methodology. Appl. Microbiol. Biotechnol. **52**, 768–772 (1999)
24. Kunameni, A., Kumar, K.S., Singh, S.: Response surface methodological approach to optimize the nutritional parameters for enhanced production of α-amylase in solid state fermentation by *Thermomyces lanuginosus*. Afr. J. Biotechnol. **4**(7), 708–716 (2005)
25. Glassey, J., Montague, G.A., Ward, A.C., Kara, B.: Artificial neural network based experimental design procedures for enhancing fermentation development. Biotechnol. Bioeng. **44**, 397–405 (1994)
26. Mahanta, N., Gupta, A., Khare, S.K.: Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using Jatropha curcas seed cake as substrate. Bioresour. Technol. **99**(6), 1729–1735 (2008)
27. Gutarra, M.L., Cavalcanti, E.D., Castilho, L.R., Freire, D.M., Sant'Anna, G.L.: Lipase production by solid-state fermentation: cultivation conditions and operation of tray and packed-bed bioreactors. Appl. Biochem. Biotechnol. **121–124**, 105–116 (2005)
28. Ota, Y., Yamada, K.: Lipase form *Candida paralipolytica* part I. Anionic surfactants the essential activator in the systems emulsified by polyvinyl alcohol. Agric. Biol. Chem. **30**, 351–358 (1966)
29. Anson, M.L.: The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. **22**, 79–89 (1938)
30. Cochran, W.G., Cox, G.M.: Experimental Designs, 1st edn. Wiley, New York (1957)
31. Dutta, J.R., Dutta, P.K., Banerjee, R.: Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. Process Biochem. **39**, 2193–2198 (2004)
32. Linko, S., Luopa, J., Zhu, Y.H.: Neural networks as 'software sensors' in enzyme production. J. Biotechnol. **52**, 257–266 (1997)
33. Lakshmi, B.S., Kangueane, P., Abraham, B., Pennathur, G.: Effect of vegetable oils in the secretion of lipase from *Candida rugosa* (DSM 2031). Lett. Appl. Microbiol. **29**, 66–70 (1999)
34. Rathi, P., Goswami, V.K., Sahai, V., Gupta, R.: Statistical medium optimization and production of a hyperthermostable lipase from *Burkholderia cepacia* in a bioreactor. J. Appl. Microbiol. **93**, 930–936 (2002)
35. Janseen, P.H., Monk, C.R., Mogan, W.H.: A thermophilic, lipolytic *Bacillus* sp., and continuous assay of its *p*-nitrophenyl-palmitate esterase activity. FEMS Microbiol. Lett. **120**, 195–200 (1994)
36. Sidhu, P., Sharma, R., Soni, S.K., Gupta, J.K.: Effect of culture conditions on extracellular lipase production by *Bacillus* sp. RS-12 and its characterization. Indian J. Microbiol. **38**, 9–12 (1998)
37. Chaari, F., Kamoun, A., Bhiri, F., Blibech, M., Ghorbel, R.E., Chaabouni, S.E.: Statistical optimization for the production of lichenase by a newly isolated *Bacillus licheniformis* UEB CF in solid state fermentation using pea pomace as a novel solid support. Ind. Crop. Prod. **40**, 192–198 (2012)
38. Aravindan, R., Viruthagiri, T.: Sequential optimization of culture medium composition for extracellular lipase production by *Bacillus sphaericus* using statistical methods. J. Chem. Technol. Biotechnol. **82**, 460–470 (2007)