Appl Biochem Biotechnol (2009) 159:317–329
DOI 10.1007/s12010-009-8547-6

Multiple Responses Optimization and Modeling of Lipase Production by *Rhodotorula mucilaginosa* MTCC-8737 Using Response Surface Methodology

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Received: 26 August 2008 / Accepted: 20 January 2009 /
Published online: 4 February 2009

Abstract Response surface methodology was employed to optimize culture medium for production of lipase with *Rhodotorula* sp. MTCC 8737. In the first step, a Plackett–Burman design was used to evaluate the effects of different inducers qualitatively. Of all the seven inducers tested, soybean oil showed significant influence on the lipase production. Further, response surface studies were conducted to quantitatively optimize by considering linear, interactive, and quadratic effects of test variables. A novel approach was proposed to optimize the lipase production system by optimizing the responses in terms of yield kinetics rather than optimizing the direct responses like lipase titer and biomass growth. The coefficient of determination (R^2) calculated for $Y_{P/S}$ (0.769), $Y_{P/X}$ (0.799), and $Y_{X/S}$ (0.847) indicated that the statistical model could explain 76.9%, 79.99%, and 84.7% of variability in the response.

Keywords Lipase · Yield kinetics · Central composite design (CCD) · Optimization and modeling · *Rhodotorula* sp.

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids having very wide commercial applications in industries like oleo-chemical, detergent manufacturing, food and dairy, paper and textile, cosmetics, and pharmaceutical. A large number of microorganisms have been reported for lipase production such as bacteria (*Bacillus*, *Pseudomonas*, *Burkholderia*), Fungi (*Aspergillus* sp., *Rhizopus* sp., *Penicillium* sp.), and Yeast (*Candida* sp., *Yarrowia* sp., *Rhodotorula glutinis*) [1].

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Effective lipase production employs various screening and optimization procedures [2]. The classical method of experimental optimization involves changing one variable at a time keeping the others constant. In addition, it is not practical to carry out experiments with every possible factorial combination of the test variables because of the large number of experiments required [3–4]. This does not consider the effect of interactions of various parameters. Besides this, it is a tedious, cumbersome, and time-consuming process especially when a large number of parameters are taken into account. An alternative and more efficient approach is the use of statistical method. Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different process parameters [5]. RSM was applied successfully for optimizing process parameters for various processes in biotechnology [6–12].

Plackett–Burman experimental design [13] is used to screen media components prior to optimization. Determining the effective operating parameters and the optimum operating conditions improves the process development [14].

Since different lipase inducers have their own properties, which may affect the lipase production, it was decided to screen various lipase inducers like soyabean oil, coconut oil, sunflower oil, rice bran oil, castor oil, palm oil, and olive oil by Plackett–Burman design. Further, the production medium components, such as soyabean oil, soyabean meal, and starch on lipase production were studied with the help of statistical design of experiments, and subsequent analysis was done by response surface methodology. This is the first report employing multiple response optimization studies for enhanced lipase production. In this work, direct responses of the experiments were not considered; instead, second-order regression equations were developed by calculating the yield coefficients of the process.

Materials and Methods

Source of the Strain and Inoculum Preparation

The yeast used in this study is a new strain, *Rhodotorula mucilaginosa*, isolated from marine soil samples near oil-extraction platform inside Arabian Sea, beside Mangalore port, Mangalore, India and is deposited in Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh India with accession number MTCC-8737. The strain was cultured in the medium (MYPG) containing (g/l) malt extract, 3; yeast extract, 3; peptone, 5; glucose, 10. After cultivating at 28±2 °C for 48 h, 2 ml of this suspension was used as inoculum to a 250-ml conical flask containing 50 ml of production medium, which contained MYPG (g/l) and oil of olive 3% as lipase inducer. The flasks were incubated for 120 h at 28±2 °C and 150 rpm.

Analytical Methods

Estimation of the biomass: Five milliliter of culture samples was centrifuged at 8,000×*g* for 10 min, and the pellet was washed with distilled water and dried at constant temperature in oven and weighed for the estimation of biomass. *Enzyme assay:* Lipase activity was determined using *p*-nitrophenyl palmitate as the substrate [15]. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per milliliter per minute. *Protein estimation:* Protein content was determined by Lowry method using bovine serum albumin as the reference standard. *Carbohydrate estimation:* Total carbohydrate content was determined according to the phenol-sulfuric acid method [16].

Screening of Different Lipase Inducers using Plackett–Burman Design

The influences of seven inducers on lipase yield were investigated using the methodology of Plackett–Burman [13]. The inducers (soyabean oil, coconut oil, sunflower oil, rice bran oil, castor oil, palm oil, and olive oil) were tested at two levels, a high (+1) and a low (−1) level. The +1 and −1 concentration for the variables are 3% and 1% *v*/*v*, respectively. The selected lipase inducers were replaced with olive oil in production medium defined in previous section, whereas other conditions are kept constant at their respective levels. The selected lipase inducers were replaced with olive oil in production medium defined in previous section, whereas other conditions are kept constant at their respective levels. The design is orthogonal in nature and thus gives pure effect of each variable not confounded with interactions among variables. The Plackett–Burman for the screening of lipase inducers resulted in eight experiments. The effect of each variable was determined by the following equation:

$$E(x_i) = 2\left(\sum M_i^+ + M_i^-\right)\Big/N$$

where $E(x_i)$ is the concentration effect of the tested variable; M_i^+ and M_i^- from the trials, where the variable (x_i) measured was present at high and low concentrations, respectively; and N is the number of trials. STATISTICA 6.0 (Stat Soft, Tulsa, OK, USA) software was used for regression and graphical analysis of the data obtained.

Selection of Media Components for RSM Studies

A typical production media for lipase production consists of carbon, nitrogen, and lipase inducers. In the previous section, different lipase inducers were tested, and soyabean oil was found to be better lipase inducer. Hence, further work was carried out using soyabean oil, soyabean meal, and starch using RSM as a tool for the optimization of media quantitatively.

Experimental Design and Multiple Responses Optimization

A multiple responses optimization method was used to increase the yield of lipase and biomass using response surface methodology as a tool. Based on the best results of one at a time approach and by Plackett–Burman design for the screening of lipase inducers, three critical components of the production medium were selected and further evaluated for their interactive behavior by using a statistical approach. The levels of three medium variables, viz., starch, 1% ($x1$); soyabean meal, 1% ($x2$); and soyabean oil 3% ($x3$) were selected, and each variable was coded at five levels, −1.681, −1, 0, 1, and 1.681 by using Eq. 1. For statistical calculations, the center variables Xi have been coded as xi according to the following transformation. The range and levels of the variables in coded units for RSM studies are given in Table 1.

$$x_i = X_i - X_0/\Delta X \tag{1}$$

Where xi is the dimensionless coded value of the variable X_i, X_0 the value of X_i at the center point, and ΔX the step change.

The behavior of the system is explained by the following quadratic model 2.

$$Y = \beta_0 + \sum \beta_{\mathrm{i}} \times x_{\mathrm{i}} + \sum \beta_{\mathrm{ii}} \times x_{\mathrm{i}}^2 + \sum \beta_{\mathrm{ij}} \times x_{\mathrm{ij}} \tag{2}$$

Table 1 Range and levels of the variables in coded units for RSM studies.

Components	−1.681	−1	0	+1	+1.681	Δx
Starch, $x1$, %	0.594	0.75	1	1.25	1.345	0.25
Soyabean meal $x2$, %	0.594	0.75	1	1.25	1.345	0.25
Soyabean oil, $x3$, %	1.68	2	3	4	4.69	1

Δx is step increment in each variable values

Where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} the interaction effect. The full quadratic equation for four factors is given by model 3.

$$Y = \beta_0 + \beta_1 x1 + \beta_2 x2 + \beta_3 x3 + \beta_{11} x1 \times x1 + \beta_{12} x1 \times x2 + \beta_{13} x1 \times x3 + \beta_{22} x2 \times x2 + \beta_{23} x2 \times x3 + \beta_{33} x3 \times x3 \quad (3)$$

Several experimental designs have been considered for studying such models, and central composite design (CCD) was selected [17]. For this study, a 2^3 full factorial design with six star points and six replicates at the central points were employed to fit the second-order polynomial model, which indicated that 20 experiments were required for this procedure. STATISTICA 6.0 (Stat Soft) software was used for regression and graphical analysis of the data obtained.

In order to search for the optimum combination of major components of the production medium, experiments were performed according to the CCD experimental plan given in Table 2. Three responses, viz., biomass (dry cell weight, g/L), lipase activity (U/L), and carbohydrate concentrations were recorded from the experiments, and the results were tabulated (Table 2). Further, yield of biomass with respect to substrate, $Y_{X/S}$, yield of lipase with respect to substrate $Y_{P/S}$, and specific lipase formation with respect to biomass $Y_{P/X}$ were calculated and tabulated. The results of CCD experiments for studying the effect of three independent variables are presented along with the mean predicted and observed responses in Table 3. Each response was analyzed, and a second-order regression model was developed. The model was validated in each case, and a set of optimum values was calculated.

Results and Discussions

Yield coefficients indicate the efficiency of bioprocess system used for the production of primary and secondary metabolites by microbial fermentation. Better yield coefficients signify the higher production from minimum consumption of raw materials. Yield kinetics may be used to compare the performance of the fermentation system at optimum conditions for subsequent use of the data for kinetic model development. A new approach was proposed to optimize the lipase production by optimizing the responses in terms of yield kinetics rather than optimizing the direct responses like lipase production and biomass growth independently.

Screening of Different Lipase Inducers by Plackett–Burman Design

Figure 1 shows the Pareto chart, which graphically summarizes and displays the relative importance of the differences between different inducers for lipase production by *R. mucilaginosa* from most effective to least effective inducers. From Fig. 1, it is evident that

Table 2 Design of experiments by central composite design for RSM studies.

Run no	*x*1	*x*2	*x*3	Lipase (P), U/L	Carbohydrate (S), g/L	Biomass, (*X*), g/L
1	−1	−1	−1	23,572.31	5	0.047
2	1	−1	−1	33,252.16	9.5	0.0827
3	−1	1	−1	29,724.87	4	0.0698
4	1	1	−1	32,488.07	9.5	0.1
5	−1	−1	1	34,389.67	5	0.135
6	1	−1	1	29,819.57	9.5	0.136
7	−1	1	1	56,789	5	0.0892
8	1	1	1	33,138.84	9.8	0.129
9	−1.681	0	0	31,715.77	8	0.117
10	1.681	0	0	48,963	11	0.144
11	0	−1.681	0	24,892.49	7.06	0.0839
12	0	1.681	0	32,853.56	7.22	0.097
13	0	0	−1.681	28,786.89	7.12	0.0366
14	0	0	1.681	38,425.85	8.06	0.134
15	0	0	0	14,000	8.12	0.0707
16	0	0	0	15,698	8.13	0.072
17	0	0	0	17,856	8.56	0.0771
18	0	0	0	18,256	7.98	0.0817
19	0	0	0	17,412	8.56	0.0798
20	0	0	0	16,841	8.45	0.0846

soyabean oil was the most significant lipase inducer with a maximum lipase production of 29,589 U/L, and coconut oil is least significant with reference to less lipase production at 5,468 U/L. The difference in most significant to least significant inducer is too high for lipase production, and this situation might be due to the effect of hydroxyl-substituted fatty acids as well as the lower-chain fatty acids.

Multiple Responses Optimization and Model Building

RSM is an effective, sequential, and stepwise procedure. The lead objective of the RSM is to run rapidly and efficiently along the path of improvement towards the general vicinity of the optimum [4]. It is appropriate when the optimal region for running the process has been

Table 3 Model summary and ANOVA for the quadratic models.

	Measured $Y_{P/S}$	Measured $Y_{P/X}$	Measured $Y_{X/S}$
R^2	0.769	0.799	0.847
Adjusted R^2	0.561	0.618	0.709
Standard error of the estimate	1,532.44	95,835.41	2.577865E−03
Sum of squares: regression	78,132,789.64	3.65E+11	3.670E−04
Sum of squares: residual	23,483,751.97	9.18E+10	6.645E−05
Sum of squares: total	101,616,541.62	4.57E+11	4.335E−04
Mean square: regression	8,681,421.07	4.05E+10	4.078E−05
Mean square: residual	2,348,375.19	9.18E+09	6.645E−06
F value	3.697	4.415	6.136
Significant probability (*p*)	0.027	0.015	0.004

Degrees of freedom (regression) 9; degrees of freedom (residual) 10; degrees of freedom (total) 19

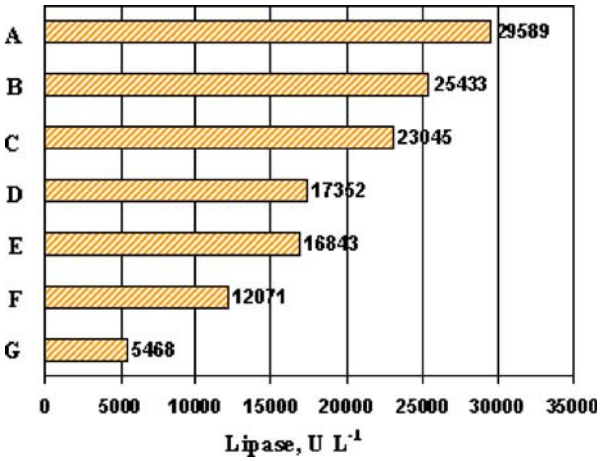


Fig. 1 Pareto chart for the estimation of influence of different lipase inducers on lipase production (U/L) by *R. mucilaginosa*. *A* Soyabean oil, *B* olive oil, *C* palm oil, *D* castor oil, *E* sunflower oil, *F* rice bran oil, *G* coconut oil

identified. The three independent variables starch, soyabean oil, and soyabean meal in the production medium were chosen for optimizing the production of lipase and biomass growth. Experiments were performed according to the given CCD experimental design to obtain optimum combination of components of the medium.

The coefficient of determination (R^2) was calculated for $Y_{P/S}$ (0.769), $Y_{P/X}$ (0.799), and $Y_{X/S}$ (0.847; Table 4), indicating that the statistical model can explain 76.9%, 79.99%, and 84.7% of variability in the response. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model, and the better it predicts the response [5]. In this case, the value of the determination coefficient, R^2, indicates that only 24.10%, 21.1%, and 15.3% of the total variations for $Y_{P/S}$, $Y_{P/X}$, and $Y_{X/S}$, respectively, are not explained by the model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj R^2) for $Y_{P/S}$ (0.561), $Y_{P/X}$ (0. 618), and $Y_{X/S}$ (0.709) is also very high to advocate for a high significance of the model [18–19]. 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. Here in this case, the adjusted R^2 values are 0.561, 0. 618, and 0.709, which are lesser than the R^2 value of 0.769, 0.799, and 0.847, respectively.

By applying multiple regression analysis on each response, the experimental results of the CCD design were fitted with a second-order full polynomial equation. The empirical relationship between $Y_{P/S}$, $Y_{P/X}$, and $Y_{X/S}$ and the three test variables in coded units obtained by the application of RSM is given by Eqs. 4, 5, and 6 respectively.

Yield of lipase ($Y_{P/S}$, U/g):

$$\begin{aligned} Y_{P/S} =& 1,972.442 - 1,181.027 \times x1 + 665.201 \times x2 - 506.033 \times x3 + 1,015.693 \\ & \times x1x1 - 879.305 \times x1x2 - 811.208 \times x1x3 + 955.630 \times x2x2 - 260.769 \\ & \times x2x3 + 1,085.573 \times x3x3 \end{aligned} \quad (4)$$

Specific lipase formation ($Y_{P/X}$, U/g)

$$\begin{aligned} Y_{P/X} =& 215,365.016 - 36,609.142 \times x1 + 24,705.340 \times x2 - 82,549.679 \times x3 \\ & + 28,021.247 \times x1x1 - 43,225.150 \times x1x2 - 26,850.100 \times x1x3 \\ & + 32,319.753 \times x2x2 + 71,552.525 \times x2x3 + 109,802.987 \times x3x3 \end{aligned} \quad (5)$$

Table 4 Model coefficients estimated by multiple linear regressions (significance of regression coefficients).

Variables	Measured $Y_{P/S}$		Measured $Y_{P/X}$		Measured $Y_{X/S}$	
	Beta	p value (significant at <0.05)	Beta	p value (significant at <0.05)	Beta	p value (significant at <0.05)
	1,972.442	0.01023	215,365.016	0.00026	9.287E−03	0.0000012
$x1$	−1,181.027	0.01732[a]	−36,609.142	0.18847	−2.019E−03	0.01602[a]
$x2$	665.201	0.13983	24,705.340	0.36331	1.587E−04	0.82470
$x3$	506.033	0.25043	−82,549.679	0.00978[a]	3.336E−03	0.00074[a]
$x1x1$	1,015.693	0.03068[a]	28,021.247	0.29331	2.045E−03	0.01312[a]
$x1x2$	−879.305	0.13567	−43,225.150	0.23089	2.223E−04	0.81222
$x1x3$	−811.208	0.16521	−26,850.100	0.44650	−1.218E−03	0.21110
$x2x2$	955.630	0.03957[a]	32,319.753	0.22966	1.621E−03	0.03823[a]
$x2x3$	260.769	0.64066	71,552.525	0.06086	−2.523E−03	0.01985[a]
$x3x3$	1,085.573	0.02280[a]	109,802.987	0.00145[a]	9.926E−04	0.17480

[a] Statistically significant at 95% confidence limits of regression analysis

Yield of biomass ($Y_{X/S}$, g/g):

$$\begin{aligned} Y_{X/S} = & 9.287\mathrm{E}-03 - 2.019\mathrm{E}-03 \times x1 + 1.587\mathrm{E}-04 \times x2 + 3.336\mathrm{E}-03 \times x3 \\ & + 2.045\mathrm{E}-03 \times x1x1 + 2.223\mathrm{E}-04 \times x1x2 - 1.218\mathrm{E}-03 \times x1x3 \\ & + 1.621\mathrm{E}-03 \times x2x2 - 2.523\mathrm{E}-03 \times x2x3 + 19.926\mathrm{E}-04 \times x3x3 \end{aligned} \tag{6}$$

The F values of model (Table 3) and values of prob>F (<0.05) indicated that the model terms are significant. Analysis of variance (ANOVA) has been conducted for the second-order response surface model, and the results are given in Table 4. The significance of each coefficient was determined by Student's t-test and p values, which are listed in Table 4. The larger the magnitude of the t value and smaller the p value, the more significant is the corresponding coefficient [4, 5, 17–19]. This implies that the values of factors having p values less than 0.05 are statistically more significant.

For $Y_{P/S}$, $Y_{P/X}$, and $Y_{X/S}$, the values of p less than 0.05 (95% significant) are statistically significant. Therefore, Eqs. 4, 5, and 6 can be reduced by considering significant values of p. The statistically significant models of the optimization studies are given by Eqs. 7, 8, and 9.

Yield of lipase ($Y_{P/S}$, U/g):

$$\begin{aligned} Y_{P/S} = & 1,972.442 - 1,181.027 \times x1 + 1,015.693 \times x1x1 + 955.630 \times x2x2 \\ & + 1,085.573 \times x3x3 \end{aligned} \tag{7}$$

Specific lipase formation ($Y_{P/X}$, U/g)

$$Y_{P/X} = 215,365.016 - 82,549.679 \times x3 + 109,802.987 \times x3x3 \tag{8}$$

Yield of biomass ($Y_{X/S}$, g/g):

$$\begin{aligned} Y_{X/S} = & 9.287\mathrm{E}-03 - 2.019\mathrm{E}-03 \times x1 + 3.336\mathrm{E}-03 \times x3 + 2.045\mathrm{E}-03 \times x1x1 \\ & + 1.621\mathrm{E}-03 \times x2x2 - 2.523\mathrm{E}-03 \times x2x3 \end{aligned} \tag{9}$$

The contour plots are generally the graphical representation of the regression equation for the optimization of $Y_{P/S}$, $Y_{P/X}$, and $Y_{X/S}$. Figures 2, 3, and 4 show the effect of two variables on biomass production, protein concentration, and bioconversion, respectively, while the other three variables are held at a constant level.

Figure 2a–c shows the interaction relationship between the two independent variables, namely, starch/soyabean meal (Fig. 2a), starch/soyabean oil (Fig. 2b), and soyabean meal/soyabean oil (Fig. 2c) and their effects on the response variable, $Y_{P/S}$. It was observed from Fig. 2a–c that the $Y_{P/S}$ was significantly affected by the soyabean meal. An increased trend in $Y_{P/S}$ was observed (16,000 U/g) with increase in soyabean meal and soyabean oil concentrations (Fig. 2b, c). This may be due to the rich source of proteins (40%) and

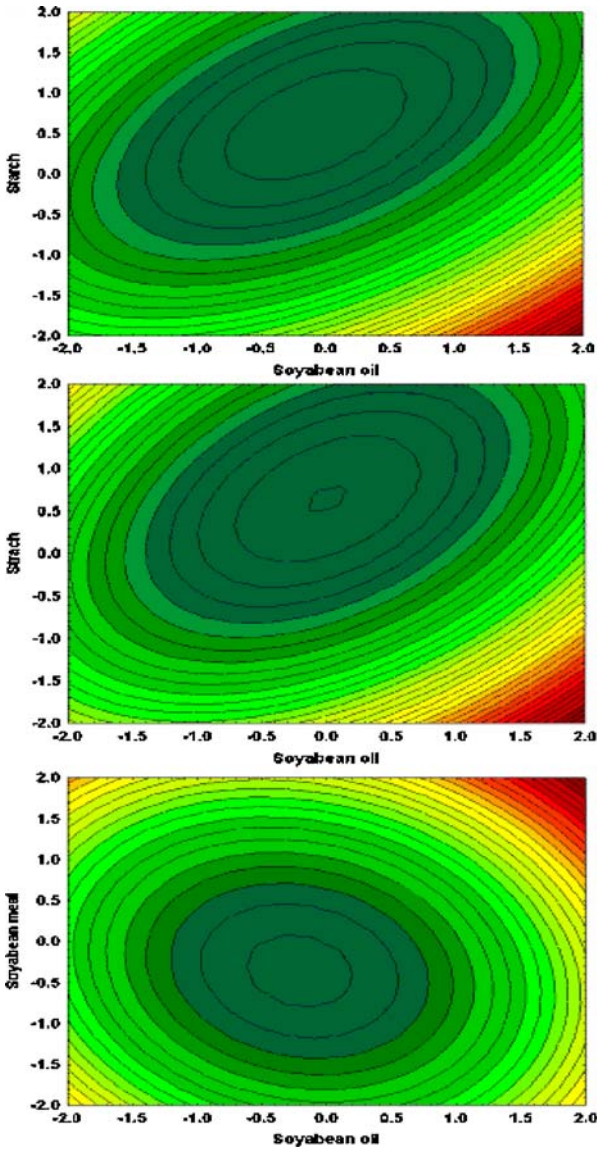


Fig. 2 (**a**–**c**) contour plot of $Y_{P/S}$. the effect of two variables while the other two are held at 0 levels

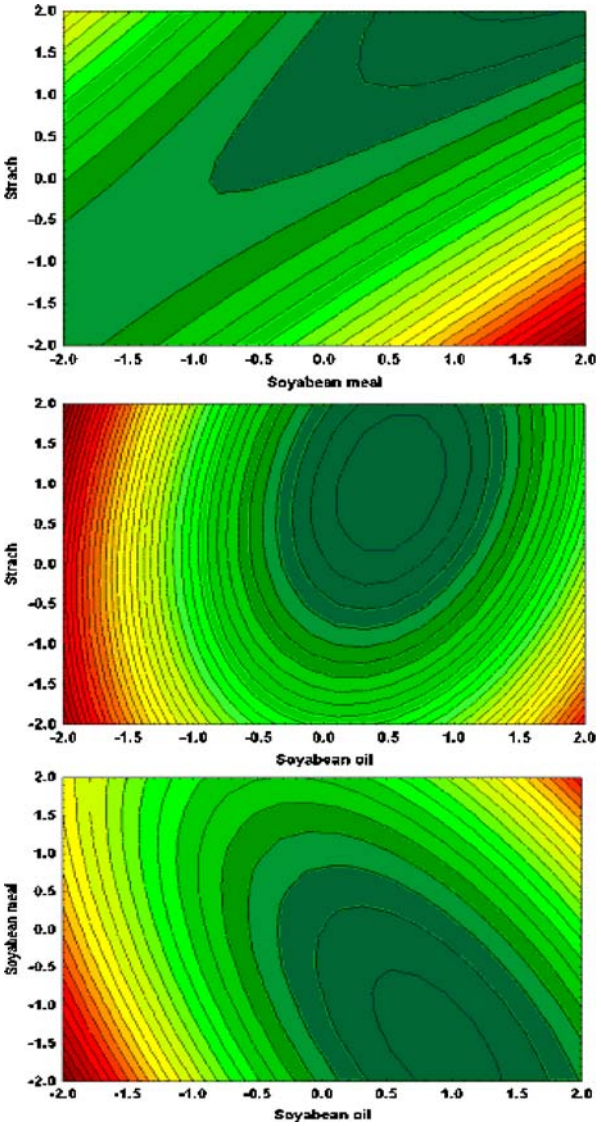


Fig. 3 (**a**–**c**) contour plot of $Y_{P/X}$. the effect of two variables while the other two are held at 0 levels

carbohydrates (35%) available in the soyabean meal and unsaturated fatty acids and triglycerides in the soyabean oil.

The $Y_{P/X}$ was estimated from the experiments, and the data were analyzed by regression equations. The regression equations are represented by contour plots (Fig. 3a–c), and soyabean oil is found to have significant role on the yield. Maximum of 700,000 U/g of $Y_{P/X}$ was predicted when the soyabean meal is high and starch was at its lower concentration (Fig. 3a), and at all concentrations of starch and with lower concentrations of soyabean oil, the $Y_{P/X}$ value predicted was 900,000 U/g (Fig. 3b). Figure 3c shows that a maximum of 1,000,000 U/g of $Y_{P/X}$ could be achieved with lower concentrations of soyabean meal and lower concentration of soyabean oil.

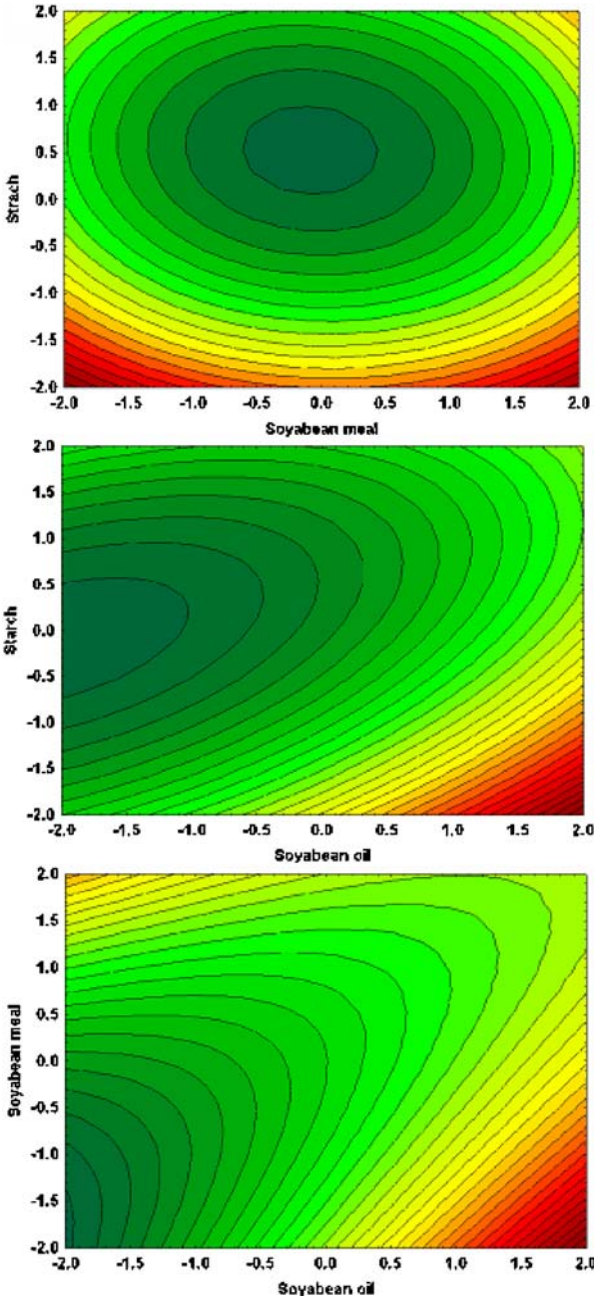
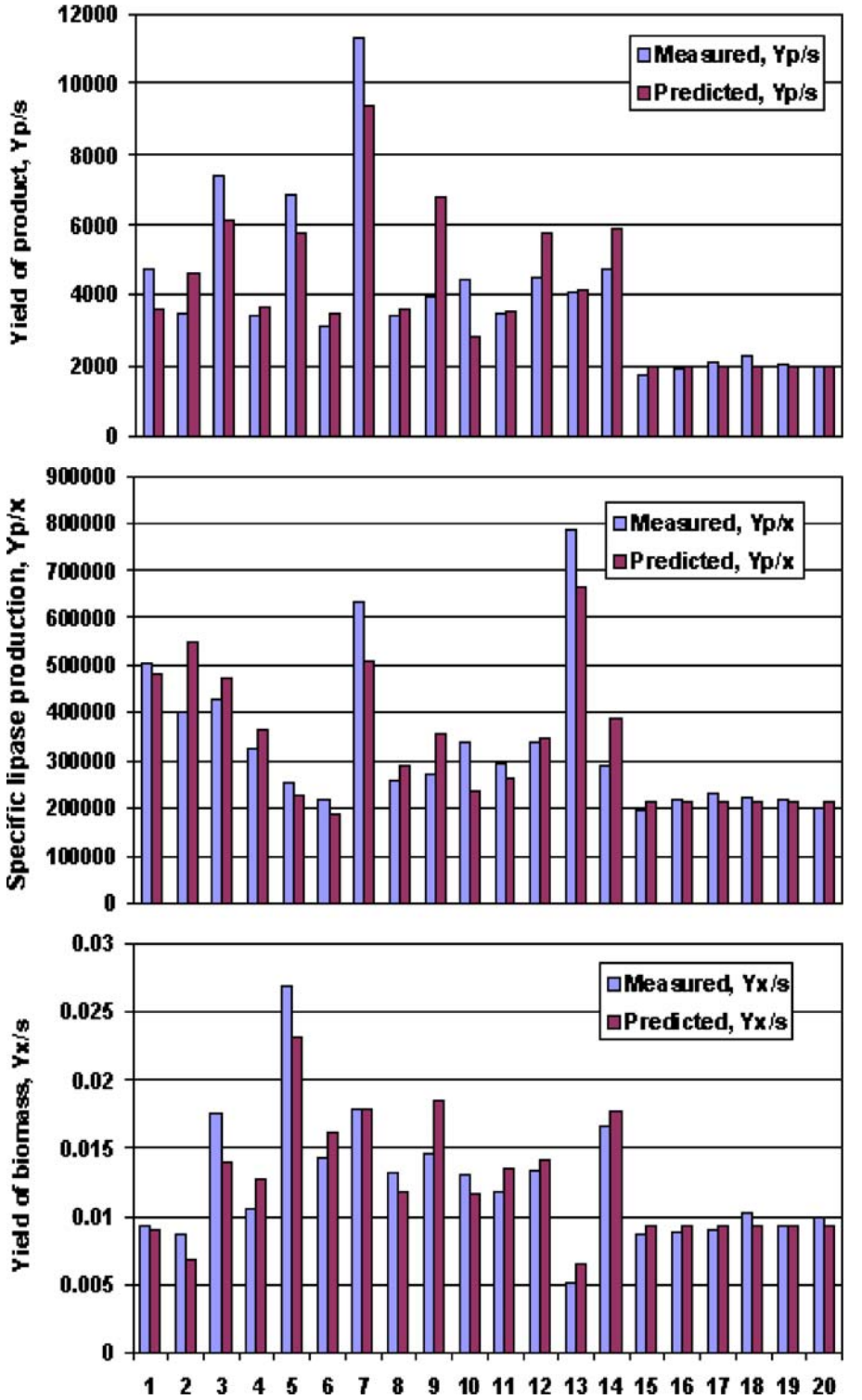


Fig. 4 (**a**–**c**) contour plot of $Y_{X/S}$. the effect of two variables while the other two are held at 0 levels

The $Y_{X/S}$ was estimated from the experiments, and the data were analyzed by regression equations. The regression equations were represented by contour plots as shown in Fig. 4a–c. From the figure, it is evident that soyabean oil has significant role on $Y_{X/S}$. Maximum of 0.035 g/g of $Y_{X/S}$ was predicted when the soyabean meal is high and soyabean meal and

Fig. 5 The predicted and measured yield calculations during the CCD experimentation


Yield of product, Yp/s
12000
10000
8000
6000
4000
2000
0
Measured, Yp/s
Predicted, Yp/s
Specific lipase production, Yp/x
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
Measured, Yp/x
Predicted, Yp/x
Yield of biomass, Yx/s
0.03
0.025
0.02
0.015
0.01
0.005
0
Measured, Yx/s
Predicted, Yx/s
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

starch were at their lower concentrations (Fig. 4b, c). Figure 4a shows that soyabean meal had significant effect on $Y_{X/S}$ with respect to starch concentration, which resulted in a maximum of 0.028 g/g of $Y_{X/S}$.

Figure 5 shows the predicted and measured yield calculations during the CCD experimentation. Full factorial central composite design was efficient in optimizing the responses. Two responses were observed and recorded from the experiments carried out according to a fractional factorial central composite design. Regression analysis was carried out for $Y_{P/S}$, $Y_{P/X}$, and $Y_{X/S}$. Earlier workers had studied only the direct responses obtained from the RSM for lipase production [7, 20], but in the present study, direct responses of the experiments were not considered, instead, second-order regression equations were developed by calculating the yield coefficients for multiple response optimization for the first time using response surface methodology as a tool along with studying of contour diagrams. The results also indicated that three media components had significant effect on $Y_{P/S}$, $Y_{P/X}$, and $Y_{X/S}$. Hence, maximum lipase production could be achieved with a relatively limited number of experimental runs using the appropriate statistical design and optimization technique.

Acknowledgment The authors are thankful to Dr. J.S. Yadav, Director, Indian Institute of Chemical Technology, Hyderabad, India for his encouragement. One of the authors, Subhakar Chennupati, thanks CSIR, New Delhi, India for SRF fellowship.

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