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Optimization of critical medium components using response surface methodology for lipase production by *Rhizopus delemar*

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ABSTRACT

The production process of a 1,3-position specific lipase from *Rhizopus delemar* was optimized by response surface methodology (RSM) and a Box–Behnken experimental design was used to study the interactive effects of fermentation medium components on lipase activity and microorganism growth. Preliminary batch tests were employed to obtain the favorable conditions for lipase activity analysis and found that sucrose, molasses, yeast extract, sunflower oil, tween-80 have significant influences on the lipase production and microorganism growth. The concentrations of five fermentation medium components were optimized. Among five variables, molasses sucrose and yeast extract were identified as less significant variables for lipase production. The optimum fermentation medium composition for lipase production by *R. delemar* was sucrose concentration 4.19 g/L, molasses sucrose 1.32 g/L, yeast extract 0.53 g/L, sunflower oil 1.11% (v/v), and tween-80 1.80% (v/v). In these conditions, the biomass concentration of 4.52 g/L with a lipolytic activity of 1585 μmol/L min was reached.

Keywords: Lipase; *R. delemar*; Medium optimization; Response surface methodology; Box–Behnken design

1. Introduction

Lipases (glycerol ester hydrolases) catalyze the hydrolysis of acylglycerols to fatty acids, di-acylglycerols, mono-acylglycerols and glycerol. Lipases have the ability not only to hydrolyze ester bonds, transesterify triglycerides, and allow the resolution of rasemic mixtures but also to synthesize ester bonds in non-aqueous media (Costas et al., 2004; Silva et al., 2005; Sun et al., 2009). They also own characteristic properties like substrate specificity, stereospecificity and the ability to catalyze heterogeneous reactions at the interface of water soluble and water insoluble systems. They differ from characteristic esterases in that their instinctive substrates are insoluble in water and their activity is maximum only when the enzyme is adsorbed to the oil–water interface (Kumar et al., 2005; Dominguez et al., 2005). As lipases show unique chemo-, regio-, enantioselectivites, and can catalyze a number of different reactions, they are used widely in medical and industrial applications. Apart from their prevalent use in detergent, pharmaceutical industries, lipolytic enzymes are found in an increasing number of applications within the food industry, in particular, for interesterification of fats and oils, flavour development in dairy products, processing of foods such as meat, and in vegetables, fruits, and baked goods and beer (Sharma et al., 2001; Jaeger and Eggert, 2002; Chen et al., 2004; Li et al., 2005; Aravindan et al., 2007). Among the microorganisms, fungi are widely recognized as the best lipase sources, and are used preferably for industrial applications, especially in the food industry. Using granulation as a new immobilization technology for lipases, it is possible to produce a food-grade, cost-effective immobilized 1,3-regioselective lipase aimed for the interesterification of commercial oils and fats for production of frying fats and margarine components (Malcata et al., 1990; Hasan et al., 2006; Osorio et al., 2009). The lipase obtained from *Rhizopus* sp. has especially 1,3-regioselectivity, so it can selectively catalyze

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the hydrolysis of triacylglycerol to produce some particular industrial products. As the lipase of *Rhizopus* sp. is particularly suited for interesterification of fats and oils and represent maximum activity towards medium-chain fatty acids (C_8–C_{10}), this catalytic characteristic has drawn more interest in recent years (Macrae, 1983; Yang et al., 2005; Li et al., 2006). However, the lipase production by *Rhizopus* sp. has relatively low yield and high cost. Research on lipases is focused especially on production, purification, structural characterization, clarification of action mechanism, kinetics of lipase-catalyzed reactions, sequencing and cloning of lipase genes (Haas et al., 1992; Joerger and Haas, 1993, 1994; Schmidt-Dannert, 1999). Increasing lipase production during the fermentation process is also an important step in industrial application of this enzyme. Various methods were used to optimize the fermentation process to enhance production of lipase. However, few efforts have been made to improve the fermentation process by searching the variation of medium components and interactions among them during the cultivation process.

The regulation of the synthesis of *Rhizopus delemar*-lipase is complex; most of the lipase is produced in the stationary phase and the effects of fermentation conditions can be large. Lipase production depends on several process variables, such as pH, temperature, carbon sources, nitrogen sources, substrate concentration, inoculum level, inducer sources and concentration (Lin et al., 2006). Information about the fermentation process is critical to search optimal operation conditions for enzyme production. The conventional technique for determining these optima is by varying one parameter while keeping the others at an unspecified constant levels. The major disadvantage of this single variable optimization is that it does not include the interaction effects among the variables. Moreover, it does not depict the net effect of the various medium constituents on the enzyme activity, is also time consuming and requires a number of experiments to determine optimum levels. For all of the reasons mentioned above this method does not guarantee the determination of optimal conditions. In order to overcome these limitations of the classical method, optimization can be done by statistical experimental design such as response surface methodology (RSM) (Kaushik et al., 2006; Liu et al., 2006; He and Tan, 2006).

RSM is a collection of mathematical and statistical techniques for designing experiments, building models, searching optimum conditions of factors for desirable responses, and evaluating the relative significance of several affecting factors even in the presence of complex interactions. The RSM has been recently used on modelling and optimization of bioprocesses such as fermentation media, cultivation and process conditions (Gao and Jiang, 2005; Ruchi et al., 2008; Wang et al., 2008), enzyme production (Elibol and Özer, 2002; Burkert et al., 2004), enzyme-catalyzed reaction conditions (Shao et al., 2008), xylanase production (Techapun et al., 2002), extracellular polysaccharide production (Wang and Lu, 2005) as well as waste water treatment such as removal of dye by adsorbents (Kapdan and Kargi, 2002; Ravikumar et al., 2005).

The optimization of lipase production by *Rhizopus* species has not yet been described in full detail. The aim of this study is to analyze different carbon source, nitrogen source concentrations and inducer concentrations, and combined interactions to obtain an enhanced lipase production from *R. delemar* and microorganism growth.

2. Materials and methods

2.1. Microorganism and growth medium

R. delemar obtained from the US Department of Agriculture Culture Collection (NRRL, 2872) was used in the study. An agitated liquid-basal medium comprised the following constituents (g/L): sucrose 5; molasses sucrose 1; K_2HPO_4 0.5; KH_2PO_4 0.5; $MgSO_4{\cdot}7H_2O$ 0.2; yeast extract 2. Molasses included in the growth medium was diluted to desired sucrose concentration. Molasses was supplied from Ankara Sugar Industry (Turkey). Molasses is a thick by-product from the processing of the sugar beet, and is defined as the runoff syrup from the final stage of crystallization. Sugar beet molasses is a solution of sugar, organic, and inorganic matter in water with a solid substance of 84% (w/w). Beet molasses is about 50% (w/w) sugar by dry weight, predominantly sucrose, ash 11% (w/w), and total nitrogen containing compounds (mainly betain) 7–8% (w/w). Molasses also contains biotin, limited extent for cell growth and enzyme production, to a lesser extent invert sugar and rafinose, trace elements such as K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^+, Cl^-, $SO_4{}^-$, $PO_4{}^-$, NO^{3-}, metal oxides, acid anhydrates.

Inducers, sunflower oil and tween-80 (also surfactant) were added to culture broth in concentrations of 0.5 and 1.0% (v/v), respectively. The cells at the end of lag phase were used to develop the inoculum. As the optimum inoculum ratio (volume of inoculum/production volume of bioreactor) was determined as 5/1000, 0.5 mL of growth medium was added aseptically as inoculum to 250 mL Erlenmeyer flasks containing 100 mL of fermentation medium. The fermentation was carried out at 30 °C with orbital shaking at 150 rpm. The pH of the medium was initially adjusted to 8.0, and allowed to follow its natural course. The mycelium was separated from the fermentation medium by filtration and the culture filtrate was used as a source of extra-cellular enzyme. The liquid-basal medium content and lipase fermentation conditions by *R. delemar* were determined in the single variable optimization of culture medium by one-at-a time approach (Açıkel et al., in press).

2.2. Analytical procedure

The amount of fungal biomass was determined by centrifuging mycelia, washing with distilled water and drying to constant weight at 60 °C for overnight. Cell concentration was measured spectrophotometrically at 600 nm and the obtained values were converted to g cell dry wt L^{-1} using a factor previously determined (cell concentration = 0.505 OD). Lipase activity in the culture filtrate was determined spectrophotometrically using p-nitrophenylpalmitate (pNPP) as the substrate (Wonderwülbecke et al., 1992). The substrate solution was prepared by adding solution A (30 mg of pNPP dissolved in 10 mL of propan-2-ol) to solution B (0.1 g of gum arabic and 0.4 g of Triton X-100 dissolved in 90 mL of distilled water) dropwise and under intense stirring. The pH of substrate solution was adjusted to 8.5 by using Tris buffer. The assay mixture consisted of 9 mL of substrate solution and 1 mL of suitably diluted enzyme sample (culture filtrate). The assay mixture was incubated at 37 °C for 30 min in a rotary shaker operating at 150 rpm and the p-nitrophenol released was measured at 410 nm in spectrophotometer. One unit of lipase activity was defined as the amount of enzyme solution

Table 1 – Level and code of independent variables, sucrose concentration, molasses sucrose concentration, yeast extract concentration, sunflower oil concentration and tween-80 concentration, chosen for Box–Behnken experimental design.

Variables	Symbols		Coded	Levels	
	Uncoded	Coded	−1	0	1
Sucrose (g/L)	X_1	x_1	1.00	3.00	5.00
Molasses sucrose (g/L)	X_2	x_2	1.00	3.00	5.00
Yeast extract (g/L)	X_3	x_3	0.50	1.25	2.00
Sunflower oil (%, v/v)	X_4	x_4	0.50	2.25	4.00
Tween-80 (%, v/v)	X_5	x_5	0.50	1.25	2.00

liberating 1 μmol p-nitrophenol per minute under standard assay conditions.

2.3. *Experimental design*

A Box–Behnken design was performed in order to study the effects of 5 medium components: sucrose as carbon source; molasses as carbon, nitrogen, vitamin and trace elements source, yeast extract as organic nitrogen and vitamin source, sunflower oil as inducer, tween-80 as inducer and surfactant. Earlier one-at-a time approach had been followed to identify concentration levels of the parameters having significant effect on lipase production from *R. delemar*. Subsequently, a statistical approach, response surface method was used to study the interaction of these variables. The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL media at 150 rpm and 30 °C. Response was measured in the periodically withdrawn samples.

A Box–Behnken factorial design with five factors and three levels, including six replicates at the centre point, was used for a fitting a second-order response surface. A total of 46 runs were used to optimize the range and levels of chosen variables. The range and the levels of the independent variables investigated using the Box–Behnken experimental design in this study are shown in Table 1. Regression analysis was performed on the data obtained from the design experiments. The lipase activity and microorganism concentration was taken as dependent variables or responses Y_1 and Y_2, these values are the mean of duplicate data from fermentation procedure.

A quadratic polynomial regression model was assumed for predicted response. The model proposed for each response of Y was:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, β_0 is the intercept term, β_i the linear effect, β_{ii} the squared effect and β_{ij} the interaction effect. Design Expert 7.0.0 (trial version, Stat Ease Inc., Minneapolis, USA) computer program was used for determination of the coefficients of Eq. (1) by regression analysis of the experimental data.

3. Results and discussion

3.1. *Effect of fermentation medium components on lipase activity of R. delemar*

Using the Box–Behnken method, a set of 46 experiments with six replicates at the center point was conducted. The design matrix of the variables in coded units is given in Table 2 along with the predicted and experimental values of response (lipase production and biomass concentration).

The model expressed by Eq. (2) represents lipase activity (Y_1) as a function of concentrations of sucrose (x_1), molasses sucrose (x_2), yeast extract (x_3), sunflower oil (x_4) and tween-80 (x_5).

$$\begin{aligned} Y_1(\mu\text{mol/L min}) &= 773.89 - 38.68x_1 + 1.23x_2 - 30.38x_3 + 202.79x_4 + 111.50x_5 \\ &+ 307.35x_1x_2 - 336.66x_1x_3 + 292.68x_1x_4 - 403.42x_1x_5 \\ &- 35.07x_2x_3 + 208.75x_2x_4 + 41.37x_2x_5 + 239.08x_3x_4 \\ &- 397.93x_3x_5 - 341.98x_4x_5 - 72.64x_1^2 + 233.95x_2^2 + 189.42x_3^2 \\ &+ 168.044x_4^2 + 122.26x_5^2 \end{aligned} \quad (2)$$

The statistical significance of Eq. (2) was controlled by *F*-test, and the analysis of variance (ANOVA) for response surface quadratic model is given in Table 3. Values of probability (P) > F less than 0.05 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. The model is highly significant, as is evident from the model *F*-value and a very low probability value (*P*-value < 0.0001). The R^2 value of 0.9773 for lipase production indicates the accuracy of the model. The R^2 value gives a measure of how much variability in the observed response values can be explained by the experimental parameters and their interactions. The closer the values of R^2 to 1, the better the correlation between the experimental and predicted values and the better the model predicts the response. When expressed as a percentage, R^2 implies that the total variation of 97.73% for enzyme activity is attributed to the independent variables and only about 2.27% of the total variation cannot be explained by the model. The predicted R^2 (R^2_{Pred}) of 0.9210 pointed to a good agreement between the experimental and predicted values for lipase production. The predicted R^2 of 0.9210 is also in reasonable agreement with the adjusted R^2 (R^2_{Adj}) of 0.9591. The adjusted R^2 arranges the R^2 values for the sample size and for the number of variables in the model. If there are many variables in the model and the sample size is not very large, the adjusted R^2 may be obviously smaller than the R^2. The object of response surface methodology is to detect which experimental parameters generate signals, which are large in comparison to do noise. Adequate precision measures signal-to-noise ratio, a ratio greater than 4 is desirable. An adequate precision of 27.296 for lipase activity indicated an adequate signal. The lack of fit measures the failure of the model to represent data in the experimental domain at points which are not included in the regression. The value of lack of fit for regression of Eq. (2) is not significant (P = 0.5715). Non-significant lack of fit is good and indicates that the model equation was adequate for predicting the lipase activity under any combination of values of the variables.

The *P*-value serves as a tool for checking the significance of each of the coefficients, which also indicates the interaction strength of each parameter. The low values of *P* of less than 0.05 indicate the more significant correlation of coefficients. The smaller the *P*-values are, the bigger the significance of the corresponding coefficient. It is observed that the coefficients for the linear effect of sucrose concentration, sunflower oil concentration and tween-80 concentration

Table 2 – Box–Behnken design matrix along with the experimental and predicted values of lipase activity and biomass concentration.

Runs	x_1	x_2	x_3	x_4	x_5	Lipase activity (μmol/L min)		Biomass	Concentration (g/L)
						Experimental	Predicted	Experimental	Predicted
1	0	−1	0	0	−1	1001	1059	2.84	2.97
2	0	0	1	−1	0	634	659	1.81	1.95
3	0	0	0	−1	−1	401	408	1.15	1.20
4	1	0	1	0	0	502	485	1.43	1.40
5	0	0	−1	0	−1	634	606	1.81	1.72
6	1	0	0	0	1	520	493	1.48	1.44
7	0	−1	1	0	0	1122	1201	3.21	3.41
8	0	0	0	0	0	725	774	2.07	2.21
9	0	−1	0	−1	0	1254	1181	3.61	3.42
10	0	0	0	0	0	871	774	2.42	2.21
11	0	0	0	0	0	742	774	2.21	2.21
12	0	0	−1	1	0	1119	1125	3.20	3.16
13	1	0	0	−1	0	358	335	1.10	1.04
14	1	1	0	0	0	1196	1205	3.39	3.43
15	0	0	0	−1	1	1373	1315	3.92	3.83
16	0	0	1	0	−1	1367	1342	3.90	3.81
17	0	0	0	0	0	721	774	2.06	2.21
18	1	0	0	0	−1	1082	1077	3.09	3.04
19	−1	0	0	1	0	745	818	2.12	2.31
20	1	0	−1	0	0	1148	1219	3.28	3.47
21	0	0	0	1	1	1056	1036	3.01	2.92
22	−1	0	−1	0	0	610	624	1.74	1.77
23	−1	0	1	0	0	1310	1236	3.74	3.53
24	1	0	0	1	0	1309	1326	3.70	3.70
25	0	0	−1	−1	0	1125	1198	3.21	3.46
26	0	0	−1	0	1	1584	1625	4.52	4.64
27	0	1	−1	0	0	1386	1264	3.96	3.61
28	0	0	0	0	0	725	774	2.07	2.21
29	0	0	0	0	0	858	774	2.45	2.21
30	0	1	0	0	−1	976	978	2.78	2.76
31	0	1	0	1	0	1584	1589	4.52	4.51
32	−1	1	0	0	0	609	668	1.70	1.89
33	0	0	0	1	−1	1452	1497	3.98	4.21
34	−1	0	0	−1	0	965	998	2.81	2.87
35	0	−1	0	0	1	1123	1199	3.18	3.40
36	1	−1	0	0	0	612	588	1.71	1.64
37	−1	0	0	0	−1	401	347	1.13	0.98
38	−1	0	0	0	1	1452	1377	4.13	3.93
39	0	1	0	−1	0	749	766	2.15	2.19
40	−1	−1	0	0	0	1254	1280	3.54	3.61
41	0	0	1	0	1	726	769	2.11	2.23
42	0	−1	0	1	0	1254	1169	3.45	3.21
43	0	−1	−1	0	0	1246	1191	3.51	3.34
44	0	0	1	1	0	1584	1543	4.50	4.36
45	0	1	0	0	1	1264	1284	3.60	3.68
46	0	1	1	0	0	1122	1133	3.21	3.22

($P = 0.0364$, $P < 0.0001$, $P < 0.0001$, respectively) for lipase activity is highly significant. The coefficients of the quadratic terms of sucrose, sunflower oil and tween-80 concentrations ($P = 0.0051$, $P < 0.0001$, $P < 0.0001$, respectively) appear to be very significant. The interaction effects between sucrose, sunflower oil and tween-80 with other medium components (all probability coefficients $P < 0.0001$) are also significant. The interaction effects of molasses sucrose with yeast extract and molasses sucrose with tween-80 ($P = 0.3256$ and $P = 0.2480$, respectively) had no significant.

Table 3 – Analysis of variance (ANOVA) for the fitted quadratic polynomial model of lipase activity as a function of concentration levels of medium components.

Source	Sum of squares	DF	Mean square	F-value	Probability (P) > F
Model	5.26×10^6	20	2.63×10^5	53.76	<0.0001
Residual	1.22×10^5	25	4894		
Lack of fit	97,355	20	4868	0.97	0.5715
Pure error	24,995	5	4999		
Corrected total	5.38×10^6	45			

$R^2 = 0.9773$, $R^2_{Adj} = 0.9591$, $R^2_{Pred} = 0.9210$, adequate precision = 27.296

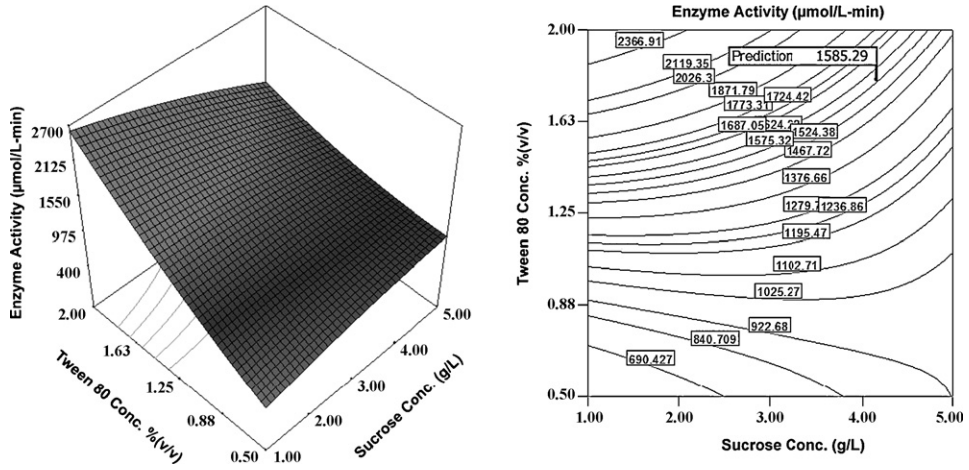

Fig. 1 – (a) Response surface plot described by the model Y_1, which represents the effect of sucrose and tween-80 concentrations and their mutual effects on lipase activity. (b) Response surface contour plot of lipase activity showing interactive effect of sucrose and tween-80 concentrations.

The graphical representations of the regression of Eq. (2), called the response surfaces and the contour plots were obtained using the Design Expert and are presented in Figs. 1–3. The independent variables sucrose, sunflower oil and tween-80 concentrations affected significantly the lipase activity. Inducers, tween-80 and sunflower oil were the more significant parameters than the other variables. The presence of sunflower oil in the fermentation medium affected

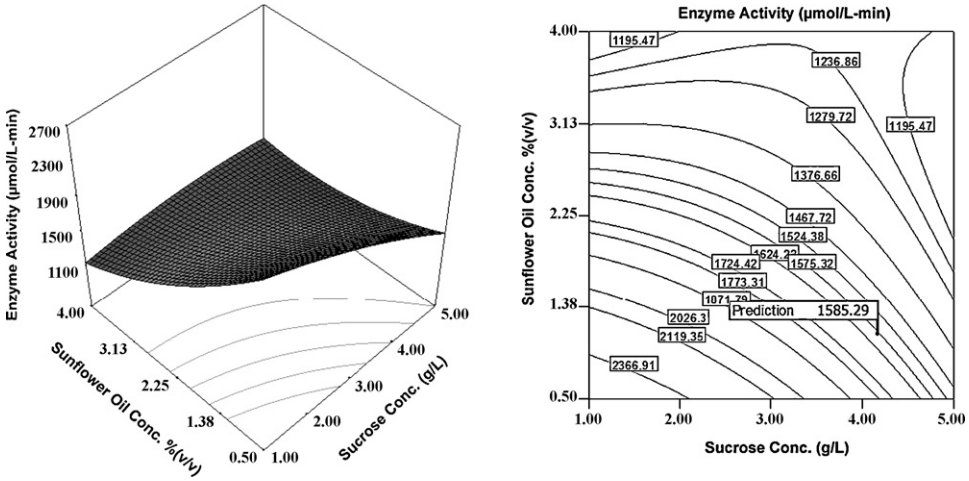

Fig. 2 – (a) Response surface plot described by the model Y_1, which represents the effect of sucrose and sunflower oil concentrations and their mutual effects on lipase activity. (b) Response surface contour plot of lipase activity showing interactive effect of sucrose and sunflower oil concentrations.

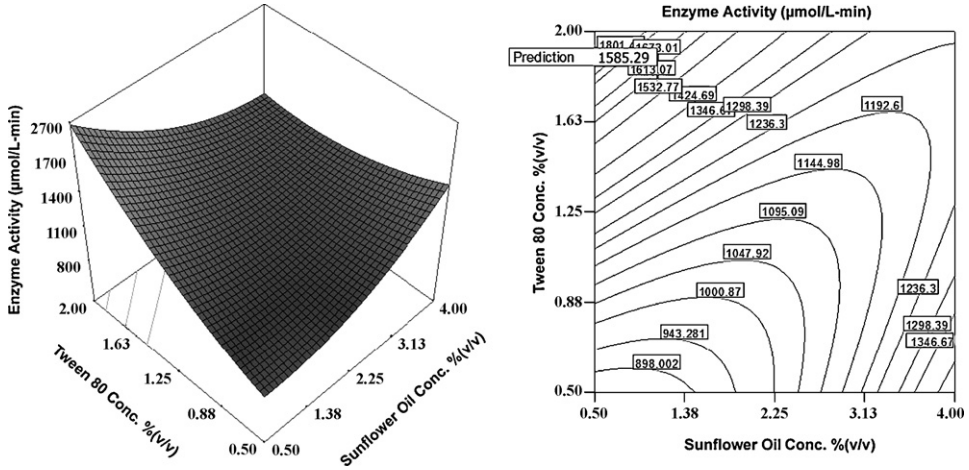

Fig. 3 – (a) Response surface plot described by the model Y_1, which represents the effect of tween-80 and sunflower oil concentrations and their mutual effects on lipase activity. (b) Response surface contour plot of lipase activity showing interactive effect of tween-80 and sunflower oil concentrations.

Table 4 – Analysis of variance (ANOVA) for the fitted quadratic polynomial model of microorganism concentration as a function of concentration levels of medium components.

Source	Sum of squares	DF	Mean square	*F*-value	Probability (P) > F
Model	42.28	20	2.11	57.63	<0.0001
Residual	0.92	25	0.037		
Lack of fit	0.75	20	0.038	1.15	0.4796
Pure error	0.16	5	0.033		
Corrected total	43.20	45			

$R^2 = 0.9788$, $R^2_{Adj} = 0.9618$, $R^2_{Pred} = 0.9248$, adequate precision = 28.153

the variables sucrose, molasses sucrose and yeast extract ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively). The variables sucrose and yeast extract ($P < 0.0001$, $P < 0.0001$, respectively) were also influenced by the presence of tween-80 in the fermentation medium. Tween-80 is a good inducer for lipase production because of it being a lipidic surfactant. Tween-80 as a surfactant serves not only as lipase inducer but also as cell permeabilizer resulting in increase extracellular lipase production. Fig. 1 shows the interaction between sucrose and Tween-80, it was obvious that the lipase activity of *R. delemar* increased remarkably as the both sucrose and Tween-80 concentrations were increased. On the other hand, an inverse effect was observed in case of sucrose and sunflower oil, the lipase activity of *R. delemar* increased as the sucrose concentration was increased, it also increased when the sunflower oil concentration was increased up to 1.11% (v/v), but then it began to decrease (Fig. 2). A similar behavior was also observed in case of sunflower oil and tween-80. The lipase activity increased with increase in tween-80 concentration, but decreased with increase in sunflower oil concentration (Fig. 3). The shapes of the contour plots, circular or elliptical, also show if the mutual interactions between the variables are significant or not. If the interactions between the corresponding variables are negligible, a circular contour plot is obtained. If the interactions between the corresponding variables are significant, the nature of the contour plots is elliptical, as in case of sunflower oil and tween-80. The optimal values of the fermentation conditions for obtaining approximately 1585 μmol/L min of lipase activity lie in the following ranges of the tested variables: sucrose concentration = 4.19 g/L; molasses sucrose = 1.32 g/L; yeast extract = 0.53 g/L; sunflower oil = 1.11% (v/v); tween-80 = 1.8% (v/v). Molasses sucrose and yeast extract are less significant parameters for lipase production than the other medium components, however the presence of minimum amounts are necessary as enzyme inductor. Extracellular lipase activity is dependent more on carbon than on nitrogen source concentration.

A lipase activity comparable with that obtained in this study was reported by Elibol and Özer (2002). They investigated the combined effects of initial glucose concentration and corn oil concentration on lipase production by *Rhizopus arrhizus* using response surface methodology (RSM). A 2^2 full-factorial central composite design was employed for experimental design. A lipase activity (tributyrin as substrate) of 370 μmol/L min with a biomass concentration of 2.4 g/L was obtained at the optimum conditions. Rajendran and Thangavelu (2009) used to the Plackett–Burman experimental design to evaluate the medium components for lipase production by *R. arrhizus*. The most significant variables affecting lipase production were reported to be olive oil, peptone, KH_2PO_4, $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$. A maximum lipase activity of 3980 μmol/L min and a maximum cell mass concentration of 5.62 g/L were determined using the optimized medium.

3.2. *Effect of fermentation medium components on growth of R. delemar*

Using the Box–Behnken method, 46 sets of experiments with appropriate combinations of fermentation medium components were conducted. The design matrix of the variables in coded units is given in Table 2 along with the predicted and experimental values of the microorganism concentration. By applying multiple regression analysis on the experimental

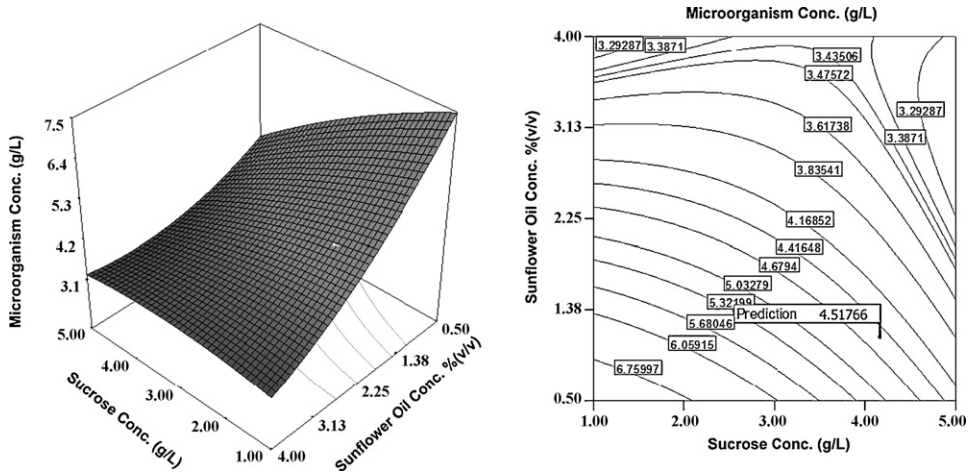

Fig. 4 – (a) Response surface plot described by the model Y_2, which represents the effect of sucrose and sunflower oil concentrations and their mutual effects on microorganism concentration. (b) Response surface contour plot of microorganism concentration showing interactive effect of sucrose and sunflower oil concentrations.

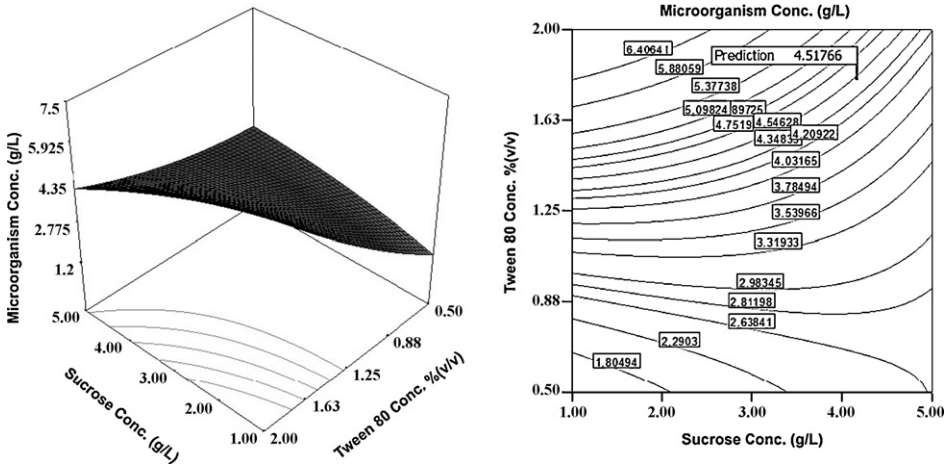

Fig. 5 – (a) Response surface plot described by the model Y_2, which represents the effect of sucrose and tween-80 concentrations and their mutual effects on microorganism concentration. (b) Response surface contour plot of microorganism concentration showing interactive effect of sucrose and tween-80 concentrations.

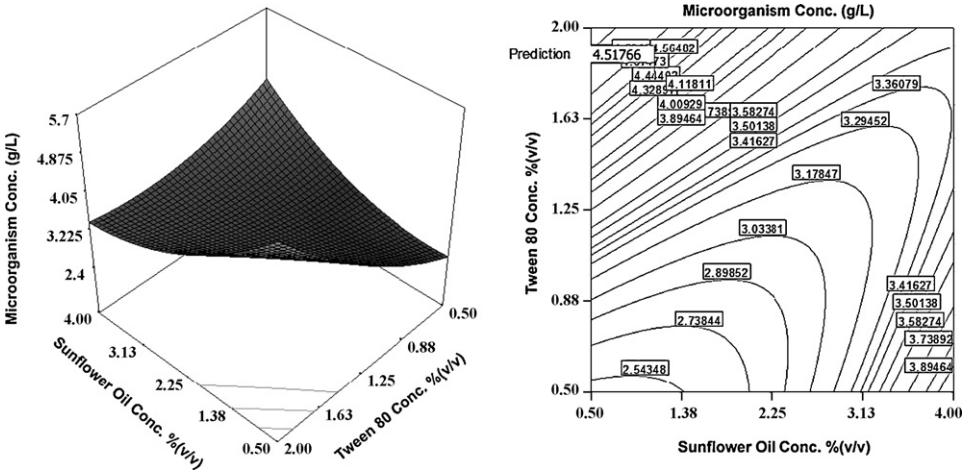

Fig. 6 – (a) Response surface plot described by the model Y_2, which represents the effect of tween-80 and sunflower oil concentrations and their mutual effects on microorganism concentration. (b) Response surface contour plot of microorganism concentration showing interactive effect of tween-80 and sunflower oil concentrations.

data, the second order polynomial equation for the growth of *R. delemar*, as a function of the coded values of sucrose, molasses sucrose, yeast extract, sunflower oil and tween-80 concentrations is as follows:

$$\begin{aligned} Y_2(\text{g/L}) = {} & 2.21 - 0.11x_1 + 0.016x_2 - 0.082x_3 + 0.56x_4 + 0.32x_5 \\ & + 0.88x_1x_2 - 0.96x_1x_3 + 0.82x_1x_4 - 1.15x_1x_5 - 0.11x_2x_3 \\ & + 0.63x_2x_4 + 0.12x_2x_5 + 0.67x_3x_4 - 1.13x_3x_5 - 0.98x_4x_5 \\ & - 0.21x_1^2 + 0.64x_2^2 + 0.54x_3^2 + 0.48x_4^2 + 0.35x_5^2 \end{aligned} \quad (3)$$

A summary of the analysis of variance (ANOVA) for the selected quadratic model is shown in Table 4. The ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the calculated *F*-value (57.63) and a very low probability value ($P < 0.0001$). The determination coefficient ($R^2 = 0.9788$) indicates a high correlation between the experimentally observed and predicted values. The predicted R^2_{Pred} of 0.9248 is in reasonable agreement with the adjusted R^2_{Adj} of 0.9618. The lack of fit *F*-value of 1.15 implies the lack of fit is not significant ($P = 0.4796$) relative to the pure error. There is a 47.96% chance that a "lack of fit *F*-value" this large could occur due to noise. The pure error is very low, indicating a good reproducibility of the experimental data.

The *P*-values were used to check the significance of each coefficient. The low values of *P* of less than 0.05 indicate the more significant correlation of coefficients. It is observed that the coefficients for the linear effect of sucrose, sunflower oil and tween-80 concentrations ($P = 0.0335$, $P < 0.0001$, $P < 0.0001$, respectively) for *R. delemar* concentration is highly significant. The coefficients of the quadratic terms of sucrose, sunflower oil and tween-80 concentrations ($P = 0.0033$, $P < 0.0001$, $P < 0.0001$, respectively) appear also to be very significant. The coefficient of the linear effect of yeast extract concentration ($P = 0.0984$) is slightly significant on the microorganism growth. The coefficient of the linear effect of molasses sucrose concentration ($P = 0.7372$) was the least significant. The coefficients of the interactive effects among the variables ($P < 0.0001$) seem also to be very significant except for the interactions between molasses sucrose and yeast extract ($P = 0.2512$), molasses sucrose and tween-80 ($P = 0.2218$). The significance of these interaction effects between the variables would have not been determined if the experiments were performed by one-at-a time approach.

Based on these results the model was utilized to generate response surfaces for the analysis of the variable effects on

the microorganism growth. The response surfaces and corresponding contour plots in Figs. 4–6 were obtained using Eq. (3). A maximum microorganism concentration of 4.52 g/L within the studied range was obtained working with 4.19 g/L sucrose, 1.32 g/L molasses sucrose, 0.53 g/L yeast extract, 1.11% (v/v) sunflower oil and 1.8% (v/v) tween-80, at the optimum conditions obtained maximum lipase activity. Fig. 4 shows the effect of sucrose and sunflower oil concentrations on microorganism growth by keeping the concentrations of molasses sucrose, yeast extract and tween-80 at the optimum values. From Fig. 4., it can be seen that the microorganism concentration increased with increase in sucrose concentration, but decreased with increase in sunflower oil concentration. The response surface in Fig. 5 which gives the microorganism concentration as a function of sucrose and tween-80 concentrations at fixed values of the concentrations of other variables shows that concentration of *R. delemar* increased with increasing concentrations of sucrose and tween-80. The response surface of the biomass production with respect to the concentrations of sunflower oil and tween-80 at fixed levels of sucrose, molasses sucrose, and yeast extract were also drawn (Fig. 6). The biomass production increased with an increase in the concentration of tween-80 up to 1.8% (v/v) and sunflower oil up to 1.11% (v/v). A further increase in concentration of sunflower oil decreased the biomass production. The above observations were confirmed by the contour plots. The carbon requirement of the microorganism was supplied mainly from sucrose. Although molasses was taken into account as a carbon source, few amount of carbon requirement was seen to be provided from molasses. Molasses was mainly used as nitrogen, vitamin and trace elements source and affected as enzyme inducer.

To confirm these results, lipase fermentation by *R. delemar* was carried out under the optimum conditions. In this analyze, lipase activity and biomass concentration were found to be 1590 μmol/L min and 4.60 g/L, respectively, and these were very closer to the predicted values. The well correlation between predicted and experimental value justifies the validity of the response model and the existence of an optimum point. The lipase activity was approximately 3.14 times higher than that obtained by the single variable optimization of culture medium in which the lipase activity was 505 μmol/L min. As a result, controlling the culturing conditions and modifying the composition of the medium dramatically enhanced the production of the lipase of *R. delemar*.

4. Conclusion

The improvement of extracellular lipase production by *R. delemar* was considered in the present work. The effects of concentrations of sucrose, molasses sucrose, yeast extract, sunflower oil and Tween-80 on the membrane-bound lipase were investigated by Box–Behnken experimental design and response surface analysis. The results obtained suggested that sucrose, sunflower oil and tween-80 play an important role both in the lipase biosynthesis and microorganism growth. In view of the results reviewed, the production of lipase is mostly inducer-dependent. The coefficients of determination (R^2) of the proposed models for the lipase activity (Y_1) and microorganism concentration (Y_2) was 0.9773 and 0.9788, respectively. Probability values for the lipase activity (P-value < 0.0001) and microorganism growth (P-value < 0.0001) demonstrate a very high significance for the regression models. The composition of optimum culture medium was sucrose concentration 4.19 g/L, molasses sucrose 1.32 g/L, yeast extract 0.53 g/L, sunflower oil 1.11% (v/v), and tween-80 1.80% (v/v). In this study, it was also shown that using medium optimization by RSM, the lipase activity of *R. delemar* could be enhanced from 505 μmol/L min in unoptimized medium to 1585 μmol/L min giving 3.14-fold increase in lipase activity.

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