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OPTIMIZATION OF THE CRITICAL MEDIUM COMPONENTS FOR THE PRODUCTION OF LIPASE BY *Yarrowia lipolytica* NCIM 3589 USING DOEHLERT EXPERIMENTAL DESIGN

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ABSTRACT

Lipase production using *Yarrrowia lipolytica* NCIM 3589 was maximized in stationary batch culture. Response surface methodology with Doehlert experimental design was adopted to evaluate the effect on the lipase activity released in the culture medium of three most important factors, such as nitrogen concentration (1 to 3 (g/l)), carbon concentration (7.5 to 22.5 (g/l)), and salt solution concentration (5 to 15 (%v/v)). The optimal set of conditions for high lipase production were as follows: nitrogen concentration 2 g/l, carbon concentration 15 g/l and salt solution concentration 10 (%v/v). The predicated value was 9.23 U/ml and the actual value was 9.30 U/ml lipase activity.

Key words: Lipase, response surface methodology, optimization, doehlert experimental design and *Yarrrowia lipolytica.*

INTRODUCTION

Lipases (triacylglycerol hydrolases EC 3.1.1.3) are ubiquitous enzymes acting on lipid water interface[1] . They can be easily obtained from microorganisms and have potential applications in many industrial domains[2,3]. There are many reasons explaining this interest using lipases. First, they not only catalyze hydrolysis but also the reverse reaction such as esterification[4] and transesterification[5]. Secondly, they usually retain their structure and activity in organic solvents[6]. Thirdly, they have several advantages over chemical catalysts in term of substrate specificity, region and enatio-selectivity, lower temperature and pressure requirements.

Response surface methodology (RSM) is a useful statistical technique for investigating optimal conditions of fermentation medium so as to produce lipase with maximum activity. The Doehlert design[7] describes a spherical experimental domain and it stresses uniformity in space filling. Although this matrix is neither orthogonal nor rotatable, it does not significantly diverge from the required quality for effective use in the design of experiments[8]. The number of levels is not the same for all variables in this design. This property allows to conduct the experiment with process variables at three, five and seven levels respectively. As a general rule, it is preferable to choose the variable with the stronger effect as the factor with seven levels in order to obtain most information of the system.

The present work focuses on the different factors that affect the lipase activity by using *Yarrowia lipolytica* NCIM 3589. Our objectives were to better understand relationship between the factors (nitrogen concentration, carbon concentration, and salt solution concentration) and the response (lipase activity) and to determine the optimal conditions for lipase activity by applying of RSM.

MATERIALS AND METHODS

Microorganism and growth

In order to obtain the inoculum for lipase production, *yarrowia lipolytica* NCIM 3589 was grown in 250 ml Erlenmeyer flasks with 50 ml basal medium (9) containing (per liter): 1 g kH_2P0_4, 0.5 g $MgS0_4$. $7H_20$, 0.1 g $CaCl_2$, 0.1 g NaCl, 0.5 mg H_3BO_3, 0.2 mg $FeCl_3$. $4H_2O$, 0.4 mg $ZnSO_4$. $7H_2O$, 0.4 mg $MnSO_4$. H_2O, 0.1 mg KI, 0.04 mg $Cuso_4$. $5H_2O$, 200 mg thiamin, and 8 mg biotin. Glucose and urea was taken as carbon source and nitrogen source respectively. The medium without urea and vitamins was sterilized at 121°C for 20 min. After cooling, the vitamins and urea, previously sterilized by micro filtration (0.22 mm), were added to the basal medium. A cell suspension was obtained by addition of 10 ml 0.9% Nacl solution to a previously prepared agar plate, and 1 ml of this suspension was added to the liquid medium. The flasks were incubated in an orbital shaker at 150 rpm and 30°C for 96 h.

Lipase assay

The activity of lipase was determined as described in the literature (10) with the following modifications: 1 ml of isopropanal containing 3 mg of p-nitrophenyl palmitate (pNPP) was mixed with 9 ml of 0.05 M Tris-Hcl buffer, pH 8.0, 40 mg of Triton X-100 and 10 mg of gum Arabic. Liberation of p-nitropherol at 28°C was detected at 410 nm. One enzyme unit is defined as 1 m mol of p-nitrophenol enzymatically released from the substrate per minute[11].

Experimental design

In the experimental design, the factors are coded according to the following equation.

$$x_i = \left(\frac{X_i - X_{oi}}{\Delta X_i} \right) \alpha_i \qquad \text{.....(1)}$$

Where x_i is the coded value of the i^{th} factor, X_i is the natural value, X_{oi} is the value at the center point, ΔX_i is the step change value, α_i is the maximum value of the coded factor (i.e 1.0, 0.866 and 0.816 for 5 levels, 7 levels and 3 levels respectively).

A three variable Doehlert experimental design involving a total of 15 runs including three replicates at the center point was employed in this study. The nitrogen concentration at 5 levels (1,1.5,2,2.5 and 3 (g/l)), carbon concentration at 7 levels (7.5,10,12.5,15,17.5,20 and 22.5 (g/l)), and salt solution concentration at 3 levels (5,10 and 15 (%v/v)) were selected for the study of lipase activity. The relationship between the variation of the experimental response (lipase activity) and the variation of factors can be represented by a second order mathematical model.

$$Y_c = b_o + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 \qquad \text{...(2)}$$

Y_c is the predicted response, x_1, x_2, x_3 are coded factors, b_0 is offset term, b_1, b_2, b_3 are linear effects, b_{11}, b_{22}, b_{33} are squared effects and b_{12}, b_{23}, b_{13} are interaction terms.

RESULTS AND DISCUSSION

The selection of the factor range is extremely important in the beginning of the experimental design; other wise, after completion of the experimental runs, the optimal conditions obtained by using multiple linear regression may not be found inside the experimental region. For fixing the range of variables, preliminary experiments were conducted and the following range was chosen: nitrogen concentration (1 to 3 (g/l)), carbon concentration (7.5 to 22.5 (g/l)), and salt solution concentration (5 to 15 (%v/v)) as shown in Table-1. Fifteen experiments were performed using different

Table -1: Experimental range and levels

Factors (with levels)	Lower limit	Center point	Upper limit
X_1: Nitrogen concentration (5) (g/l)	1	2	3
X_2: Carbon concentration (7) (g/l)	7.5	15	22.5
X_3: Salt solution concentration (3) (%v/v)	5	10	15

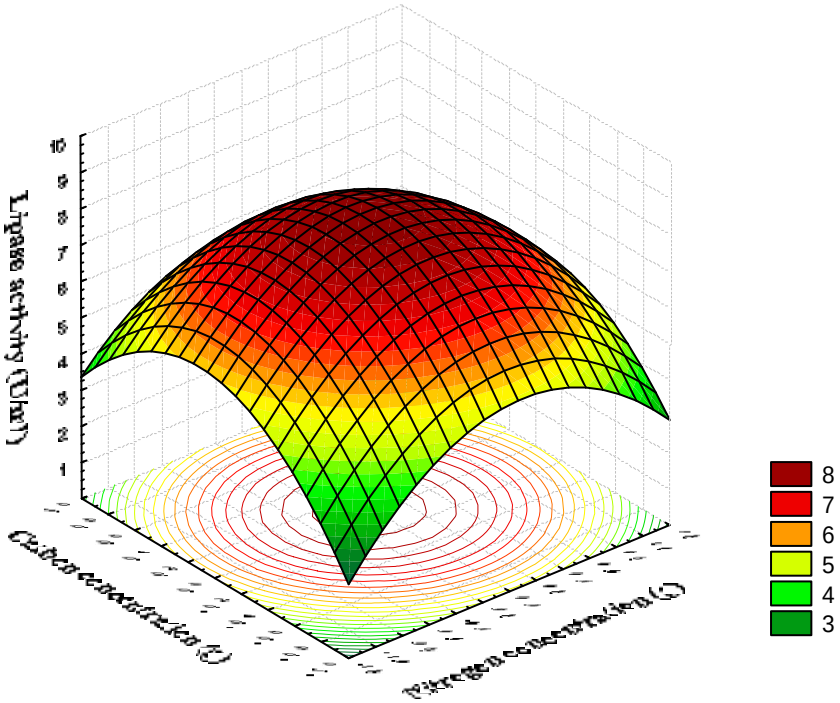

Fig. -1: Response surface plot of carbon concentration and nitrogen concentration on lipase activity

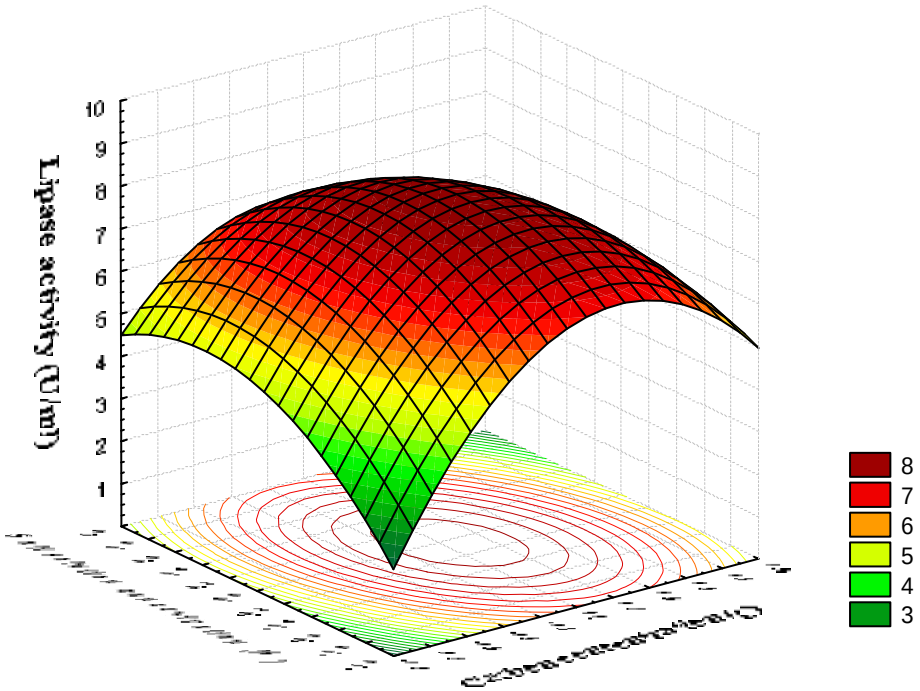

Fig. -2: Response surface plot of salt solution concentration and carbon concentration on lipase activity

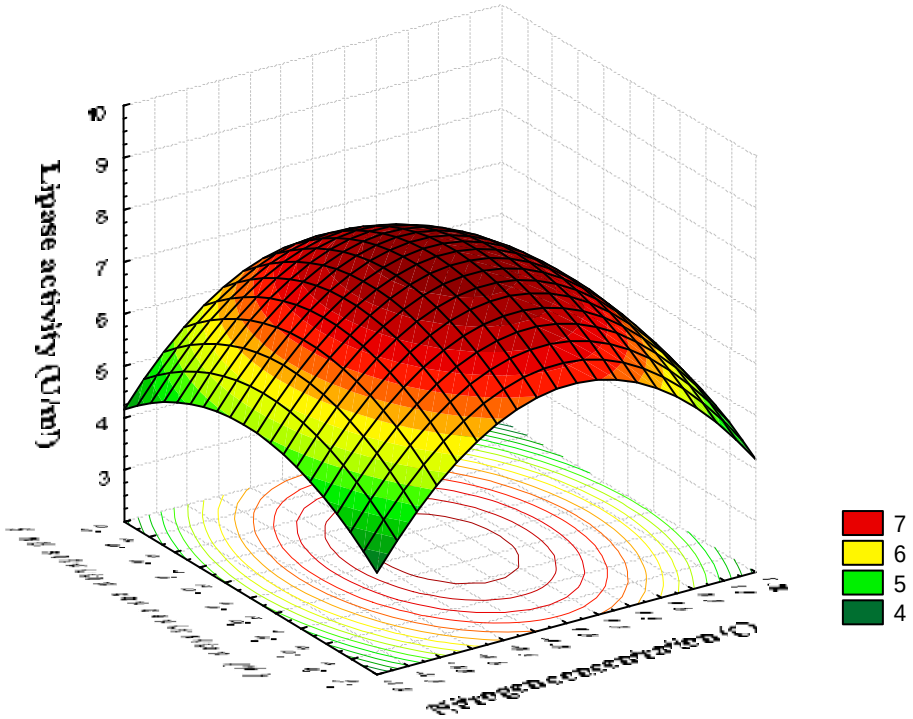

Fig. -3: Response surface plot of salt solution concentration and nitrogen concentration on lipase activity

combinations of the variables as per the Doehlert design as shown in Table -2.

The significance of each coefficient was determined by student's t-test and *P*-values that are listed in Table 3. The larger the magnitude of the t-value and smaller the *P*-value, the more significant is the corresponding coefficient[12]. This implies that second order main effects of nitrogen concentration, carbon concentration and salt solution concentration are highly significant is evident from their respective *P*-values $\left(P^2{}_{x_1} < 0.000087, P^2{}_{x_2} < 0.000015 \, and \, P^2{}_{x_3} < 0.0001\right)$.

Based on *t*--values, linear terms and interaction are considered insignificant. Therefore, the final second order equation for lipase production is

$$Y_c = 9.23 - 0.653941x_1^2 - 0.941623x_2^2 - 0.626355x_3^2 \quad \ldots\ldots\ldots (3)$$

The fit of the model was checked by the coefficient of determination, R^2, which was found to be 0.9864, indicating that 98.64% of the variability in the response could be explained by the model. Since all coefficients of the above equation are all negative, the response surface is suggested to have a maximum point. The optimal concentrations for the three factors as obtained from maximizing the model were found to be 2 g/l, 15 g/l, and 10 % v/v for nitrogen concentration, carbon concentration, and salt solution concentration. The model predicated a maximum response of 9.23 U/m1 lipase yield at this point. The excellent correlation between predicated and measured values of these experiments justifies the validity of the response model and the existence of an optimum point.

The relationship between coded variables and responses can be better understood by examining the series of surfaces plots as shown in

Table -2: Doehlert three factor experimental design along with experimental and predicted values

Experiment number	Coded Variables			Natural Variables			Lipase activity (U/ml)	
	x_1	x_2	x_3	X_1	X_2	X_3	Experimental values	Predicted values
1	1	0	0	3	15	10	6.82	6.626250
2	-1	0	0	1	15	10	6.50	6.693750
3	0.5	0.866	0	2.5	22.5	10	5.85	5.788860
4	-0.5	-0.866	0	1.5	7.5	10	5.43	5.491140
5	0.5	0.866	0	2.5	7.5	10	5.72	5.802390
6	-0.5	-0.866	0	1.5	22.5	10	6.25	6.167610
7	0.5	0.288	0.816	2.5	17.5	15	6.30	6.554973
8	-0.5	-0.288	-0.816	1.5	12.5	5	6.80	6.545027
9	0.5	-0.288	-0.816	2.5	12.5	5	8.50	6.611277
10	0	0.577	-0.816	2	20	5	6.70	6.843696
11	-0.5	0.288	0.816	1.5	17.5	15	6.80	6.688723
12	0	-0.577	0.816	2	10	15	6.70	6.556304
13	0	0	0	2	15	10	9.30	9.230000
14	0	0	0	2	15	10	9.20	9.230000
15	0	0	0	2	15	10	9.19	9.230000

Table -3: Model coefficients estimated by multiples linear regression (significance of regression coefficients)

Factor	Std. Error		t-value	*P*-value
Intercept	9.230000		65.3079	0.000000
x_1	-0.014346	0.052027	-0.2757	0.793778
x_2	0.081324	0.052027	1.5631	0.178788
x_3	-0.017320	0.052027	-0.3329	0.752708
x_1^2	-0.653941	0.056861	-11.5008	0.000087*
x_2^2	-0.941623	0.056870	-16.5575	0.000015*
x_3^2	-0.626355	0.057004	-10.9880	0.000109*
x_1x_2	-0.077273	0.054829	-1.4094	0.217784
x_2x_3	-0.093871	0.054841	-1.7117	0.147636
x_3x_1	0.003132	0.054829	0.0571	0.956665

* Significant at $P < 0.05$

Figs. -1, 2 and 3. These response surface display the variation of two factors while the third is kept at the optimum level.

The present study using Doehlert experimental design enabled us with minimum experimental effort to find the optimum values of the process variables for the production of lipase with maximum activity using *Yarrowia lipolytica* NCIM 3589.

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