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RESPONSE SURFACE METHODOLOGICAL APPROACH TO OPTIMIZE THE NUTRITIONAL PARAMETERS FOR LIPASE PRODUCTION BY *Aspergillus japonicus* MTCC 1975 UNDER SOLID STATE FERMENTATION

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ABSTRACT

Lipase production was optimized by *Aspergillus japonicus* MTCC 1975 in solid state fermentation using sugarcane bagasse and wheat bran as the mixed substrate. Response surface methodology was used to achieve the optimization of the experimental conditions for the optimal production of lipase. To study the proposed second order polynomial model, the central composite experimental design with multiple linear regression was used to estimate the model coefficients of the three selected factors. These factors were considered to influence the optimization process. The best yields were obtained at pH of 7, substrate of 10 g, and 80 % of moisture content. This methodology was found to be very efficient and only 20 experiments were needed to assess these conditions. The model adequacy was very satisfactory, as the coefficient of determination was 0.9085.

Key words: Central composite design, lipase, response surface methodology, solid state fermentation and *Aspergillus japonicus.*

INTRODUCTION

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

Solid state fermentation is generally defined as the growth of microorganisms on solid materials in the absence or near absence of free water[4]. Recently, several reports have been published indicating the application of this culture in upgrading food and industrial wastes and in the production of fine chemicals and enzymes. The utilization of by-products and wastes from food and industrial sources has several advantages over submerged fermentation such as superior productivity, simple techniques, reduced energy requirements, low waste water output, improved product recovery and the reduction in production costs, since they supply the microorganisms with some nutritive substances[5].

Most studies on lipolytic enzymes production by bacteria, fungi and yeasts have been performed in submerged fermentation; however, there are only few reports on lipase synthesis in solid state fermentation. In recent years, increasing attention has been paid to the conversion of industrial wastes to lipase by solid state fermentation. There are several reports dealing with extra cellular lipase production by fungi like *Rhizhopus sp., Aspergillus sp., Pencillium sp.* on different substrates[6, 7, 8].

Optimization of medium by the classical method involves changing one independent variable (nutrient, pH, temperature, etc.) while fixing all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables. To overcome this difficulty, experimental factorial design and response methodology can be employed to optimize medium components. The present work focuses on the different factors that affect the lipase activity by *Aspergillus japonicus* MTCC 1975. Our objectives were to better understand relationships between the factors (pH, substrate concentration, and moisture content) and the response (lipase activity) and to determine the optimal conditions for lipase activity by means of RSM.

MATERIAL AND METHODS

Substrate

Sugarcane bagasse and wheat bran from local market were dried at 60 ^{0}C for 72 h to reduce the moisture content, and grinded to the desired size.

Organism

Aspergillus japonicus MTCC 1975 obtained from Institute of Microbial Technology, Chandigarh, India, was used for the present study and was maintained on Malt-Agar medium. Sub culturing was carried out once in every 3 weeks and the culture was stored at 4 ^{0}C.

Inoculum preparation

Ten ml of sterile water was transferred to a sporulated (5 days old) Malt-Agar slant culture, the spores were dislodged using the inoculation needle. 5 ml of this spore suspension was transferred into 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculum medium. The composition of inoculum medium was Malt extract 20 g, Peptone 5 g, Yeast extract 3 g and Sodium chloride 5 g per liter of distilled water. The cells were cultivated in this medium at 28 ^{0}C on a rotary shaker at 120 rpm for 48 h.

Solid state fermentation

Ten grams of substrate was weighed into a 250 ml Erlenmeyer flask and to this a supplementing salt solution was added to the desired moisture level. The composition of the salt solution was as follows (in g/l) : K_2HPO_4 : 1; $MgSO_4.7H_2O$: 0.5; NaCl : 0.1; $CuSO_4.5H_2O$: 0.00004; $ZnSO_4.7H_2O$: 0.0004; $MnSO_4.H_2O$: 0.0002;[9]. The solid substrate medium was sterilized at 121^o C for 1hr. The sterilized solid substrate was inoculated with 2 ml of inoculum. The contents were mixed thoroughly and incubated in a slanting position at the appropriate temperature.

Enzyme extraction

Enzyme extraction was carried out by adding to the remainder of the fermented solids in each beaker containing 50 ml of 50 mM phosphate buffer (pH 7.0), and then shaking the mixture in a rotary shaker (200 rpm) for 60 min at 37 ^{o}C, a temperature high enough to increase the extraction efficiency without causing enzyme denaturation (10). The raw extract was obtained by pressing the mixture and subsequent centrifugation .The supernatant was used to determine enzyme activity[7].

Lipase assay

The activity of lipase was determined as described in the literature[11] with the following modifications: 1 ml of isopropanal containing 3 mg of *p*-nitrophenyl palmitate (*p*NPP) 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*-nitrophenol at 28^{0}C was detected at 410 nm. One enzyme unit was defined as 1 mmol of *p*-nitrophenol enzymatically released from the substrate per minute[12].

Experimental Design and Optimization

Central composite experimental design CCD[13] was used in the optimization of lipase production. pH (X_1), substrate concentration (X_2,g), and moisture content (X_3, % (v/w)) were chosen for the independent variables shown in Table -1 Lipase activity (Y_c, U/ml) was used as the dependent output variable. For statistical calculations the variables X_i were coded as x_i according to Equation (1)

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

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

Where x_i is the coded value of the ith factor, X_i is the corresponding natural value, X_{oi} is the natural value at the center of the domain, ΔX_i is the increment of X_i corresponding to one unit of x_i.

A 2-^{3}-factorial CCD, with six star points and six replications at the center points leading to a total number of 20 experiments was employed for the optimization of the conditions of fermentation.

Table -1: Experimental range and levels of the independent variables

Variables	Range and levels				
	-2	-1	0	+1	+2
pH, X_1X_3	5	6	7	8	9
Substrate concentration,	8	9	10	11	12
X_2Moisture content,	60	70	80	90	100

The second degree polynomials (Equation (2)) were calculated with the statistical package to estimate the response of the dependent variable.

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

Y_c is the predicted response, x_1, x_2, x_3 are coded variables, 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 are conducted and the following range was chosen:

Table -2: CCD consisting of 20 experiments for the study of three experimental factors in coded units along with experimental and predicted values

Run number	X_1	X_2	X_3	Coefficients assessed by	Lipase activity (U/ml)	
					Experimental	Predicted
1	-1	-1	-1	2^3 factorial design	6.85	7.150511
2	-1	-1	-1		7.3	7.791761
3	-1	1	-1		7.1	7.646761
4	-1	1	1		6.92	7.223011
5	1	-1	-1		6.4	6.964262
6	1	-1	1		7.8	8.120511
7	1	1	-1		7.4	7.775511
8	1	1	1		7.3	7.866761
9	-2	0	0	Star points (six points)	7.1	6.712614
10	2	0	0		7.65	7.170114
11	0	-2	0		6.9	6.495114
12	0	2	0		7.2	6.737614
13	0	0	-2		7.1	6.640114
14	0	0	2		7.78	7.372613
15	0	0	0	Central points	9.8	9.705455
16	0	0	0		9.89	9.705455
17	0	0	0		9.81	9.705455
18	0	0	0		9.84	9.705455
19	0	0	0		9.87	9.705455

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

Factor		Std. Error	t-value	*P*-value
Intercept	9.705455		45.38135	0.000000*
x_1	0.081616	0.095653	0.85325	0.413492
x_2	0.43261	0.095653	0.45227	0.660728
x_3	0.130674	0.095653	1.36613	0.201831
x_1^2	-0.642923	0.099489	-6.46225	0.000072*
x_2^2	-0.718518	0.099489	-7.22208	0.000029*
x_3^2	-0.627804	0.099489	-6.31029	0.000088*
x_1x_2	0.039735	0.095653	0.41541	0.686613
x_2x_3	-0.134344	0.095653	-1.40449	0.190463
x_3x_1	0.064964	0.095653	0.67917	0.512445

* Significant at P < 0.05

pH (5 to 9), substrate concentration (8 to12) and moisture content (60% to100%) as shown in Table -1. Twenty experiments were performed using different combinations of the variables as per the CCD as shown in Table -2.

The significance of each coefficient was determined by student's t-test and *P*-values, which 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[14]. This implies second order main effects of pH, substrate concentration, and moisture content are highly significant as is evident from their respective *P*-values

$$\left(P^2{}_{x_1} < 0.000072, P^2{}_{x_2} < 0.000029 \; and \; P^2{}_{x_3} < 0.000088\right)$$

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

$$Y_c = 9.705455 - 0.642923x_1^2 - 0.718518x_2^2 - 0.627804x_3^2 \quad \ldots\ldots\ldots (3)$$

The fit of the model was checked by the coefficient of determination, R^2, which was found to be 0.9085, indicating that 90.85% 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 7,10 g, and 80%(v/w) for pH, substrate concentration, and moisture content respectively. The model predicated a maximum response of 9.7 U/m1 lipase yield for 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 Figs. -1, 2 and 3. These response surface display the variation of two factors while the third is kept at the optimum level. Thus the present study using central composite 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 *Aspergillus japonicus* MTCC 1975.

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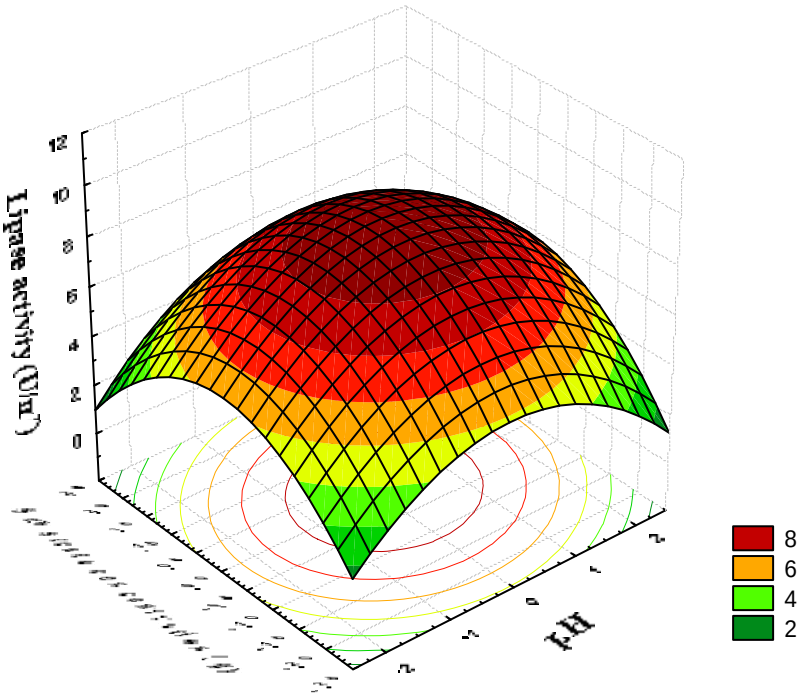

Fig. - 1: Response surface plot of pH and substrate concentration on lipase activity

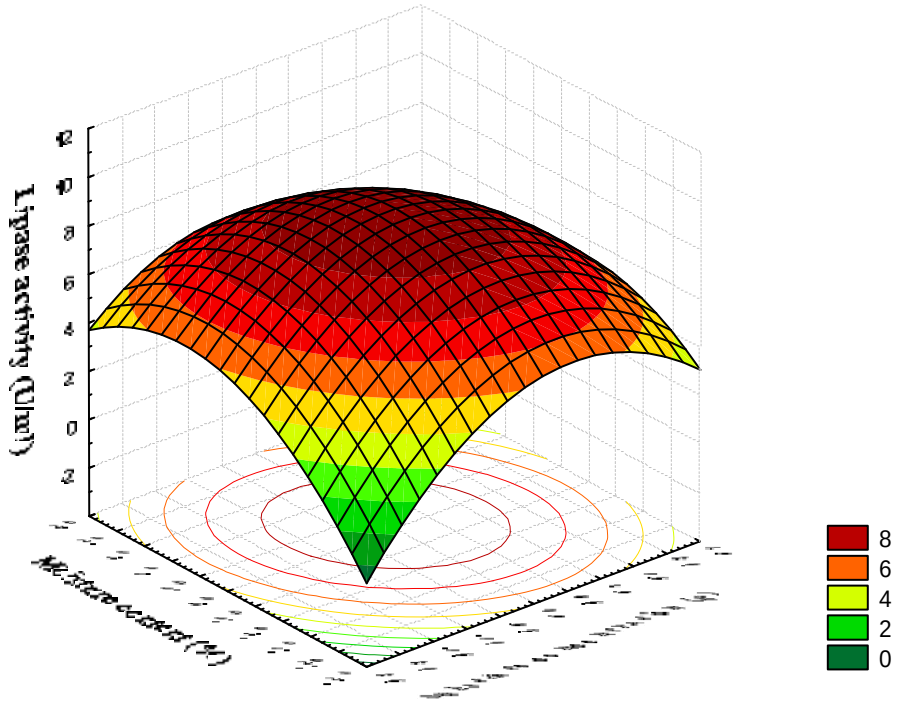

Fig. - 2:Response surface plot of substrate concentration and moisture content on lipase activity

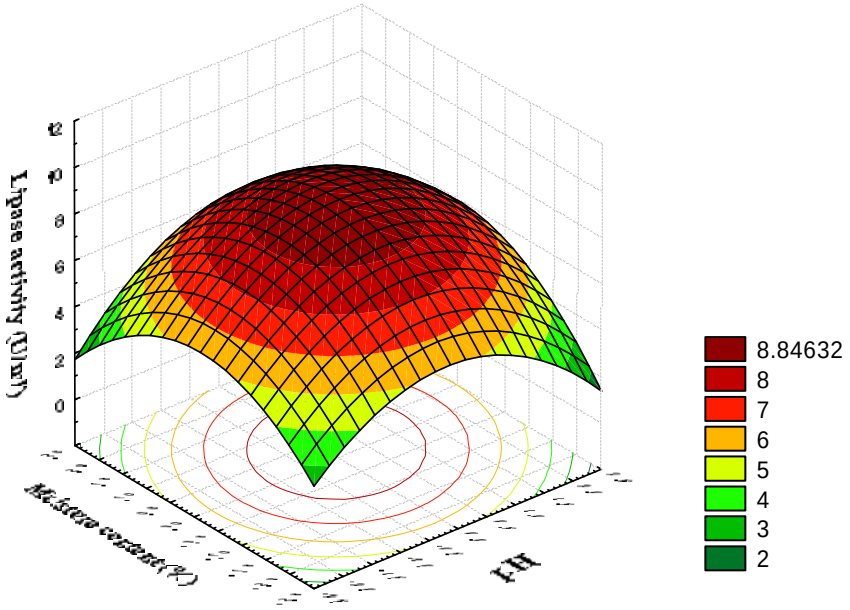

Fig. - 3: Response surface plot of pH and moisture content on lipase activity

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