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Research paper

Response surface methodology for optimal immobilization of *Aspergillus niger* ATCC 1015 lipase by adsorption method

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Abstract

Optimization of Vegetable Sponge (*Luffa aegyptiaca*) (VS) - immobilization conditions of *Aspergillus niger* ATCC 1015 lipase on Solid State Fermentation (SSF) was carried out using Response Surface Methodology (RSM). Four independent variables (temperature, pH, enzyme loading and enzyme stability) were optimized using Central Composite Design of RSM for lipase production in a solid rice bran-physic nut cake medium. The optimal immobilization conditions obtained were 45 °C, pH 7.0, 2.5% (w/v) enzyme loading and 32.5% (v/v) enzyme stability (using glutaraldehyde as crosslinking agent) resulted into lipase activity of 98.6 Ug^{-1}. The result demonstrates the potential application of vegetable sponge under SSF system in immobilizing lipase, thus contributed to efficiency of the use of this bio-omatrix as an immobilizing agent. The statistical tools employed predicted the optimal conditions for the production of the immobilized lipase thus revealing the full potential of the support.

Keywords: *Adsorption; Aspergillus niger* ATCC 1015*; Lipase; Immobilization; Optimization; Response Surface Methodology.*

1. Introduction

Application of lipases (tricylglycerol hydrolase EC 3.1.1.3) particularly those produced by microorganisms, in the splitting of oil has completely revolutionized the industrial sector because of their high potency (Osho 2013). The physico-chemical means of lipolysis have been under-studied by biocatalysis using microbial lipases in a wide array of industrial applications such as the production of detergents, oils and fats, dairy-products, emulsifying and favouring agents (McNeil et al. 1991). They are also often used in the processing and preparation of therapeutic agents (Benjamin & Pandey 1998). However, soluble enzymes usually exhibit lower stability than chemical catalysts and often cannot be recovered and reused (Bussamara et al. 2012). This might severely hinders their application in practice. Nevertheless, the problem can be overcome by enzyme immobilization, which usually enhances thermal and operational stabilities, it is easy to handle, and prevent aggregation. Enzyme immobilization may be defined as confining the enzyme molecules to a distinct phase from one in which the substrates and the products are present. Selection of an immobilization procedure greatly influences the properties of biocatalyst (Mateo et al. 2007). The decrement levels in activity and diffusion limitations occurring with immobilization are mainly dependent on the properties of support material and the immobilization method (West & Strohfus 1996). Immobilized lipases on solid support allow recoverability and reuse which can lead to a significant reduction in operational costs of industrial processes (Mateo et al. 2007, Chang et al. 2008, Liu & Chang 2008, Pahujani et al. 2008). The hydrophobic binding of immobilized lipases by adsorption has proved successful due to the enzyme affinity for water/oil interfaces (Deng et al. 2004, Dizge et al. 2008). Experimental statistical techniques such as RSM, provides an effective alternative to the conventional approach in many biotechnological processes thus revealing the correlations between the factors and responses as well as the optimum level of each factor employed (Bussamara et al. 2012). RSM is an empirical modeling system for developing, improving, and optimization of complex processes (Manohar & Divakar 2004). RSM assesses the relationships between the independent variables and the response(s) and defines the effect of each independent variable or combination (Afshin et al. 2008). In this study, an extracellular lipase was immobilized by adsorption onto the Vegetable Sponge (*Luffa aegyptiaca*) (VS) fibres. Response surface methodology was used to determine the optimal conditions of the VS-system in continuous lipid hydrolysis.

2. Materials and methods

2.1. Materials

The lipase-producing fungi *Aspergillus niger* ATCC 1015 was isolated from physic nut cake wastes (*Jatropha curcas*) obtained in garden farms in Ijebu Ode, Ijebu Ode Local Governments, Ogun State, Nigeria. This strain was phenotypically characterized by standard morphological and physiological tests and the identification confirmed by sequencing the genomic DNA by submitting them to the non-reductant nucleotide database with GenBank access number ACJE01000015.1 (Osho 2013). Vegetable Sponge (VS) (*Luffa aegyptiaca*) was locally obtained in a local farm in Olomore, Abeokuta North LGA, Ogun State, Nigeria. The VS was preheated in distilled water for 3 h and soaked in water for a minimum of ten times with continual rinsing, and later dried at 100 °C overnight (West & Strohfus 1996). It was stored at 4 °C prior before use. All chemicals were of analytical grade.

2.2. Methods

2.2.1. Lipase immobilization on Vegetable Sponge (VS) matrix

Immobilization of lipase on VS matrix was enmeshed in SSF medium comprising of rice bran, physic nut cake, soybean flour, and cassava starch (5:5:3:1, w/w) thoroughly mixed in separate glass petri dish with an addition of physic nut oils (2 %, v/w) and sterilized at 121 °C for 15 min according to the method of Osho et al. (2015). The sterilized medium was allowed to cool to below 40 °C and thereafter inoculated with 72 h old spore suspension of *A. niger* ATCC 1015 and incubated at 30 °C for 72 h. The embedded matrix was removed with the aid of forceps and rinsed off the mold bran with distilled water and the enzyme assayed at 24 h interval.

2.2.2. Determination of VS-immobilized lipase activity

The activity of the VS- immobilized lipase (2 g) each was determined according to the modified method of Lin et al. (2008) by adding 8.9 ml vegetable oil in a separate 25 ml conical flask with 0.1 ml Tween 80 which was used as emulsifying agent. The reaction mixture was mixed with vortex mixer for 1 min. It was then incubated in Gallenkamp orbital incubator shaker at 180 rpm at 45 °C for 30 min. The reaction was stopped by adding 25 ml warm ethanol to 1.0 ml of the hydrolysate. The enzyme activity was determined titrimetrically (Lin et al. 2008). One unit of lipase activity was defined as the amount of enzyme, which produced 1 µmol of fatty acid equivalent per minute under the assay conditions. The results were expressed in terms of unit per gram (Ug^{-1} matrix), the activity was calculated from the following equation:

$$\frac{N \times (Vs - Vb) \times MM}{10 \times m}$$

Where
N = NaOH normality,
Vs = volume of base used in sample titration (ml)
Vb = volume of base used in blank titration (ml)
MM = molecular mass of the predominant fatty acid (g)

m =sample mass (g) (De Souza et al. 2010)

Immobilization was evaluated in terms of lipase activity as follows:

$$\text{Lipase Activity } (Ug^{-1} \text{ matrix}) = \frac{\text{Activity of Immobilized Lipase}}{\text{Weight of Support used}}$$ (Yagiz et al. 2007)

2.2.3 Optimization of the four variable factors

The optimization of immobilization was studied using Response Surface Methodology (RSM) and Central Composite Design (CCD). The factors assessed were immobilization temperatures (30 - 60 °C), immobilization pH (5.0 - 9.0), enzyme loading (1.0 - 4.0 %) and enzyme stability (10.0 - 55.0 %). The lipase activity Ug^{-1} of solid matrix was studied as the response (Table 1).

Table 1: RSM Central Composite Design Arrangement for Coded and Actual Values with Experimental Response

		Factor 1		Factor 2		Factor 3		Factor 4		Response (Y)
Std	Run	A: Temp. (ºC)		B: pH		C: Enzyme Loading (%)		D: Enzyme Stability (%)		Lipase Activity (Ug^{-1})
17	1	0.000	45	0.000	7	0.000	2.5	0.000	32.5	98.3
11	2	-1.000	30	1.000	9	-1.000	1	1.000	55	67.4
2	3	1.000	60	-1.000	5	-1.000	1	-1.000	10	33.5
8	4	1.000	60	1.000	9	1.000	4	-1.000	10	20.6
10	5	1.000	60	-1.000	5	-1.000	1	1.000	55	50.4
14	6	1.000	60	-1.000	5	1.000	4	1.000	55	54.4
9	7	-1.000	30	-1.000	5	-1.000	1	1.000	55	65.2
4	8	1.000	60	0.000	7	-1.000	1	0.000	32.5	37.8
15	9	-1.000	30	1.000	9	1.000	4	0.000	32.5	48.6
6	10	1.000	60	-1.000	5	1.000	4	-1.000	10	47.1
1	11	-1.000	30	-1.000	5	-1.000	1	-1.000	10	55.7
5	12	-1.000	30	-1.000	5	1.000	4	-1.000	10	78
16	13	1.000	60	1.000	9	0.000	2.5	1.000	55	52.9
7	14	-1.000	30	1.000	9	0.000	2.5	-1.000	10	75.8
3	15	-1.000	30	0.000	7	0.000	2.5	-1.000	10	83.6
12	16	1.000	60	1.000	9	-1.000	1	1.000	55	49.3
13	17	-1.000	30	-1.000	5	1.000	4	1.000	55	60.6

2.2.4. Effect of the variables on activity of VS-immobilized lipase of *A. niger* ATCC 1015

The effect of each variable on the activity of VS-immobilized lipases was determined at various ranges as stated in section 2.2.3. The enzymatic extract was buffered at appropriate pH value and vegetable oil was used as the substrate. The reaction mixture containing the immobilized enzyme and oil was incubated for 30 min at 180 rpm.

2.2.5. Lipase activity of modified glutaraldehyde cross-linked VS-immobilized lipase

Glutaraldehyde was modified in various aqueous solutions (10 - 40%, v/v) at 64 °C for 20 min (Lee et al. 2006). The VS-immobilized lipase was suspended in 0.05 M phosphate buffer (pH 7) and thereafter, the matrices removed and suspended in modified glutaraldehyde and incubated at 20 °C for 2 h. They were then washed with 0.05 M phosphate buffer (pH 7) and drying for 24 h at room temperature to obtain glutaraldehyde treated immobilized lipase VS-GA polymer. The enzyme activity was determined as illustrated in section 2.2.2.

2.2.6. Statistical analysis

The statistical approach using Central Composite Design developed by the Design Expert 9.0.3 software (2014) was used to generate a set of 17 experimental runs. Three different levels, low (−1), medium (0) and high (+1) were used to study the independent variables (temperature, pH, enzyme loading, enzyme stability), and the lipase activity was taken as a dependent variable (Y) (Table 1). A quadratic polynomial regression model was established to describe the relationship between dependent and independent variables.

3. Results

3.1. Effect of the parameters on lipase activity

The experimental data were analysed by the response surface regression (RSREG) procedure to fit the following second-order polynomial equation (Eqn. 1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta i Xi + \sum_{i=1}^{3} \beta ii Xi^2 + \sum \sum_{i<i=1}^{3} \beta ij Xi Xj \quad (1)$$

Where Y is response (Lipase Activity); β (0=intercept, i=linear, ii=quadratic and ij=interaction) and Xi, Xj (i=1, 4; j=1, 4; i ≠ j represent the coded independent variables) are the model coefficients. To understand and optimize the relationship between the tested variables, the obtained experimental data were analysed by second-order polynomial equations of the RSM. The analysis of variance (Table 2) of the quadratic polynomial model showed low p-values and both high determination coefficients (R2) and high adjustment of the determination coefficients (adjusted R2). The low p-value obtained indicates that the model accurately represented the relationship between response and the variables. The p-values obtained from the regression analysis results showed that only one interaction term - X_1X_2 (Temperature-pH) and two quadratic terms - X_1^2 (Temperature^2) and X_4^2 (Enzyme stability^2) are significant model terms that had a significant effect on lipase activity. The Model F-value of 20.77 implies the model is significant. There is only a 4.69% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case Temperature is a significant model term. Values greater than 0.1 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 18.298 indicates an adequate signal. This model can be used to navigate the design space.
Linear term (X_3) as well as quadratic terms X_3^2 were significant at the 10% level. The final response model equation in terms of the variable factors can be written as follows (Equations 2 & 3):
Final equation in term of Coded Factors

$$\text{Lipase Activity} = +98.30 - 11.84x_1 - 5.59x_2 - 0.82x_3 + 2.57x_4 - 2.52x_1x_2 - 0.25x_1x_3 + 4.25x_1x_4 - 5.26x_2x_3 + 0.55x_2x_4 - 4.56x_3x_4 - 33.42x_1^2 - 3.74x_2^2 - 15.89x_3^2 + 4.77x_4^2 \quad (2)$$

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.
Final equation in term of Actual Factors

$$\text{Lipase Activity} = -277.97627 + 12.78747x_1 + 18.07166x_2 + 51.93382x_3 - 0.81430x_4 - 0.084002x_1x_2 - 0.011231x_1x^3 + 0.012601x_1x^4 - 1.75396x_2x_3 + 0.012319x_2x_4 - 0.13519x_3x_4 - 0.14856x_1^2 - 0.93602x_2^2 - 7.06113x_3^2 + 9.43093E\text{-}003x_4^2 \quad (3)$$

The equation in terms of actual factors can also be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation cannot be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the centre of the design space

Table 2: Analysis of Variance (ANOVA) for Response Surface Full Quadratic Model for Lipase Activity

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	5891.93	14	420.85	20.77	0.0469	significant
A-Temperature	1838.78	1	1838.78	90.73	0.0108	
B-pH	177.74	1	177.74	8.77	0.0976	
C-Enzyme Loading	3.04	1	3.04	0.15	0.7357	
D-Enzyme Stability	38.42	1	38.42	1.90	0.3024	
AB	64.75	1	64.75	3.19	0.2158	
AC	0.60	1	0.60	0.030	0.8793	
AD	169.78	1	169.78	8.38	0.1015	
BC	113.15	1	113.15	5.58	0.1419	
BD	1.94	1	1.94	0.096	0.7863	
CD	166.53	1	166.53	8.22	0.1032	
A^2	366.51	1	366.51	18.08	0.0511	
B^2	7.25	1	7.25	0.36	0.6106	
C^2	175.56	1	175.56	8.66	0.0987	
D^2	9.21	1	9.21	0.45	0.5698	
Residual	40.53	2	20.27			
Cor Total	5932.46	16				

3.2. Verification of model

The predicted versus actual plot of lipase production for the second-order model is shown in Fig. 1. The response surface model was validated with additional experiments under the predicted conditions. The experimental value obtained was 97.8 Ug^{-1}-matrix (mean of triplicates), which was very close to the predicted value of 98.3 Ug^{-1}-matrix. Approximately 99.5% of validity was achieved, indicating the model exerted an adequate prediction on lipase activity. A normal probability plot of the residuals (Fig. 2) showed a satisfactory straight line that concludes the empirical model is adequate to describe the lipase activity by response surface.

3.3. Effect of operational variables on lipase production

3.3.1. Effect of main variables on lipase production

Fig. 3 presented the influence of the main effect variables on lipase production. The significant factor is temperature according to the statistical analysis of the experimental range studied. The factor pH was least importance. Fig. 3(a) show that the temperature had a positive influence on the activity response as there was steady increase but decreases as the temperature increases. The pH as a single factor does not influence the activity hence insignificant (Fig. 3b). For this reason, an increase of this variable does not modify this response significantly. The enzyme loading slightly affect the activity reaching a peak of 95.4 Ug^{-1}-matrix at 2.3 % as observed in Fig. 3(c) and thereafter decreases at higher level. The influence of enzyme stability was insignificant as it was directly proportional to lipase activity at 25 % (Fig. 3 (d)).
The results of the second order response surface model fitting in the form of ANOVA are given in Table 2. The Model F-value of 20.77 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, A^2, B^2, C^2 are significant model terms. This model presented a high determination coefficient (R2 = 0.9972) explaining 97.5% of the variability in the response. Moreover "Adeq Precision" of 18.298 indicates an adequate signal for the signal noise ratio. A very small value of coefficient of variation (C.V.) 7.82 % clearly indicates a very high degree of precision and a good reliability of the experimental values.
Table 3 shown the significance of each coefficient was determined by confidence interval. The smaller the confidence length the more significant the factor is. Therefore, interactions AB and AC

have influence on enzyme activity as evidenced from lower values of confidence length.

3.3.2. Interpretation of model graph

The 2D contour plots and the 3D response surfaces are the graphical representation of the regression equation (Figures 4-9). The main goal of plot is to establish the optimum values of the variables such that response is maximized. All the plots are delineated as a function of two factors at a time, imposing other factors fixed at zero level. Contour plot is considered as a measure of perfect interactions among independent variables (Muralidhar et al. 2001) and the maximum predicted value is present in the smallest eclipse in the contour diagram (Tanyildizi et al. 2005). Contour diagram is not truly elliptical that indicates the particular optimum point in this interaction is present beyond the experimental setup.

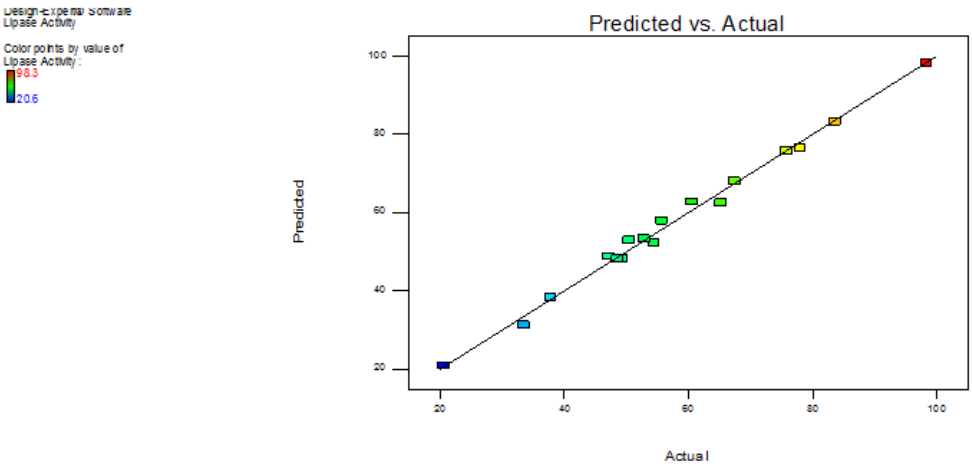


Fig. 1: Predicted Versus Actual Response.

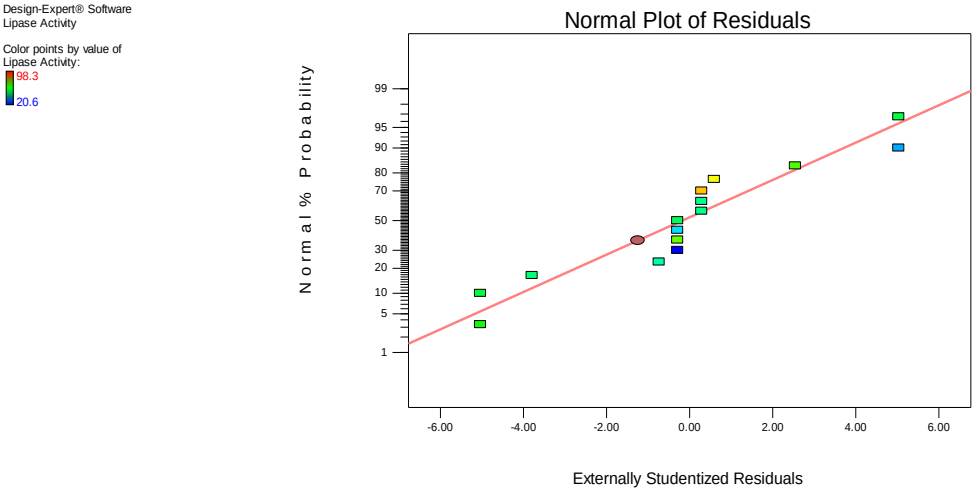
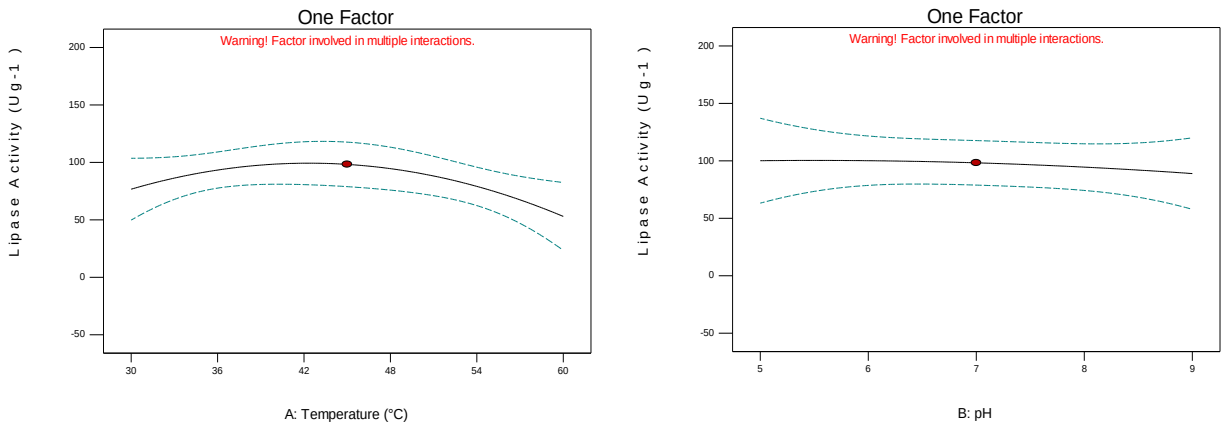
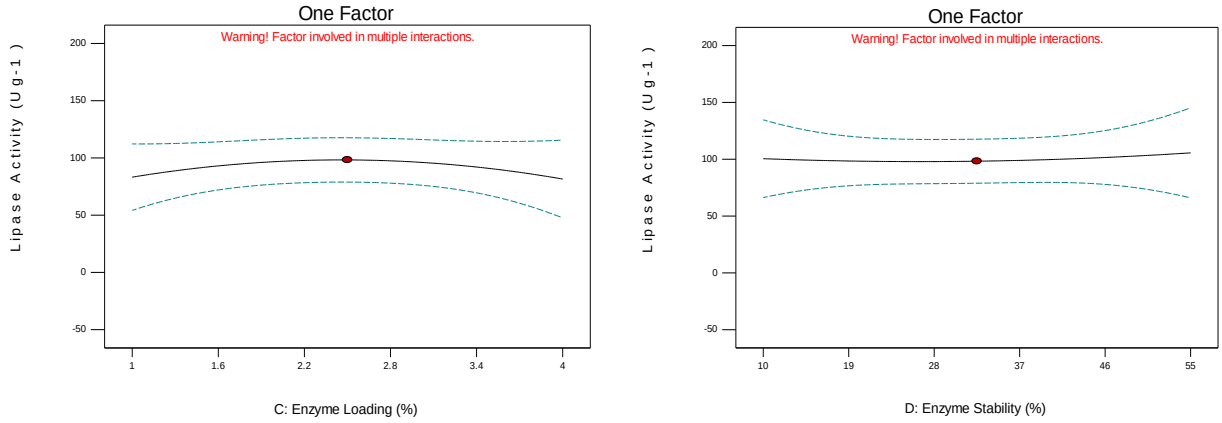


Fig. 2: Normal Probability Plot of the Residuals.



(A) (B)



(C) (D)

Fig. 3: Effects of Main Variables on Lipase Production.

Table 3: Confidence Interval (CI) of Model Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	9 5% CI High	VIF
Intercept	98.30	1	4.50	78.93	117.67	
A-Temperature	-11.84	1	1.24	-17.18	-6.49	1.22
B-pH	-5.59	1	1.89	-13.72	2.53	2.42
C-Enzyme Loading	-0.82	1	2.12	-9.96	8.31	2.88
D-Enzyme Stability	2.57	1	1.86	-5.45	10.58	2.40
AB	-2.52	1	1.41	-8.59	3.55	1.37
AC	-0.25	1	1.47	-6.57	6.07	1.38
AD	4.25	1	1.47	-2.07	10.57	1.47
BC	-5.26	1	2.23	-14.84	4.32	2.94
BD	0.55	1	1.79	-7.16	8.27	2.05
CD	-4.56	1	1.59	-11.41	2.29	1.31
A^2	-33.42	1	7.86	-67.24	0.39	2.87
B^2	-3.74	1	6.26	-30.69	23.20	4.78
C^2	-15.89	1	5.40	-39.11	7.34	4.40
D^2	4.77	1	7.08	-25.71	35.25	6.12

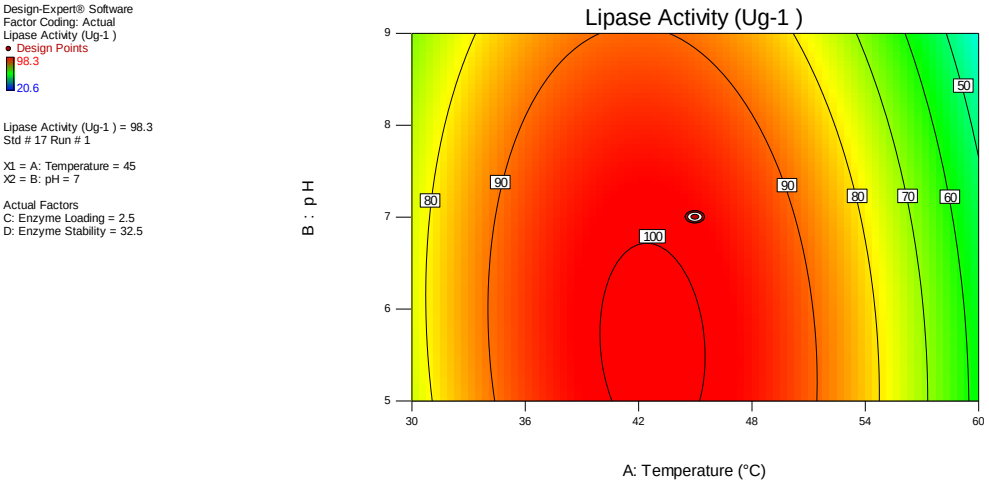


Fig. 4: Interactive Contour Plot Showing Effect of Temperature and pH on Lipase Production with other Variables Constant.

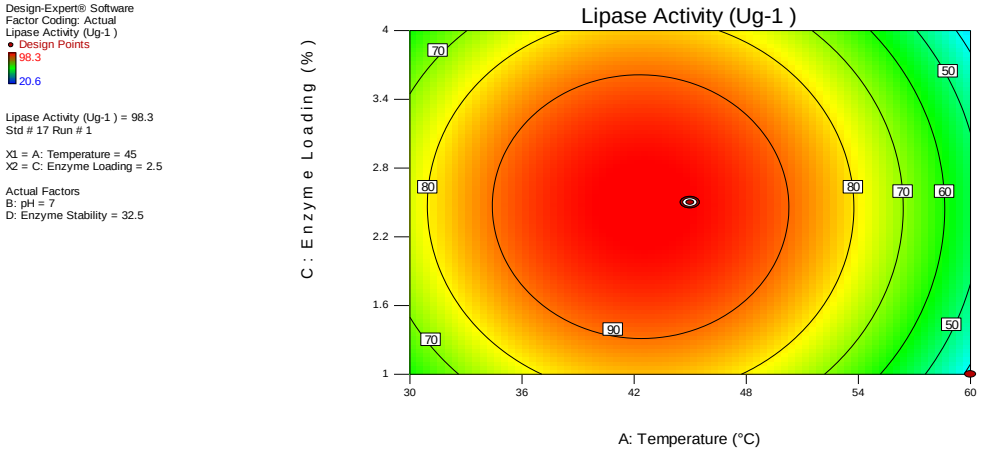


Fig. 5: Interactive Contour Plot Showing Effect of Temperature and Enzyme Loading on Lipase Production with other Variables Constant.

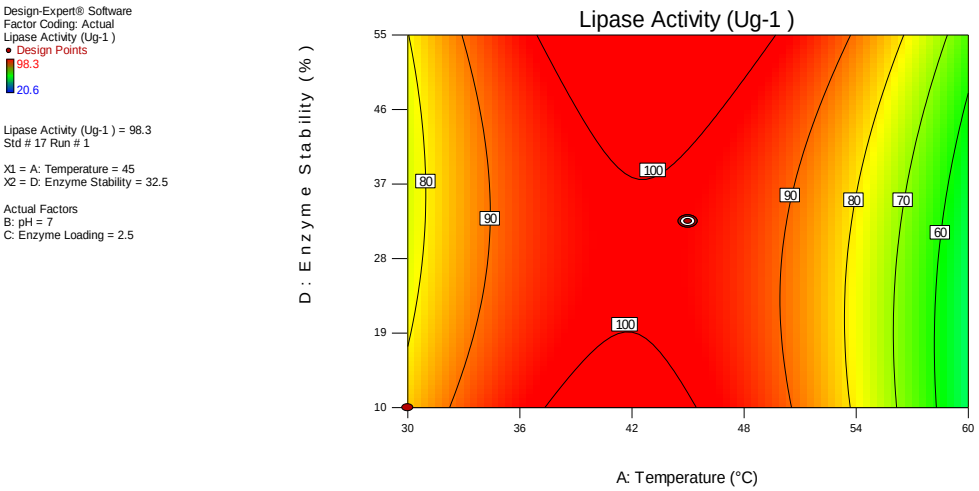


Fig. 6: Interactive Contour Plot Showing Effect of Temperature and Enzyme Stability on Lipase Production with Other Variables Constant.

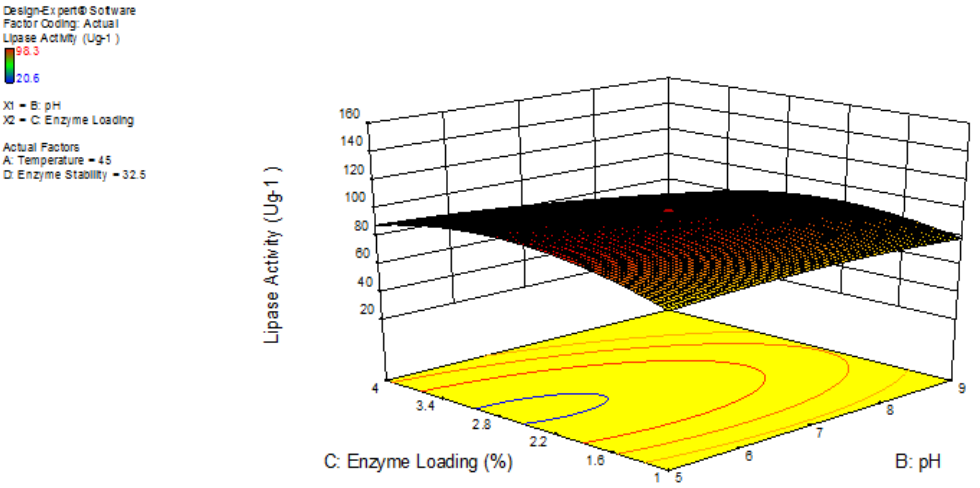


Fig. 7: 3D Response Surface Plot Showing Effect of pH and Enzyme Loading on Lipase Production with Other Variable Constant

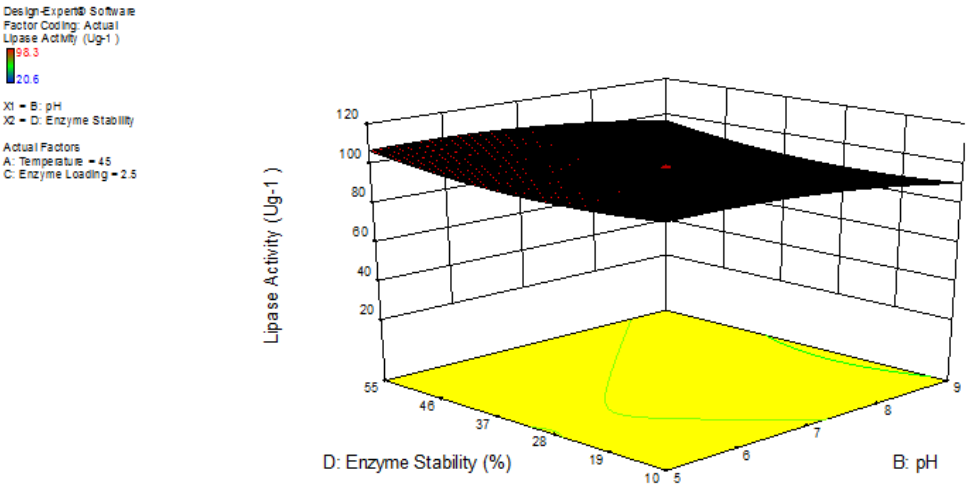


Fig. 8: 3D Response Surface Plot Showing Effect of pH and Enzyme Stability on Lipase Production with other Variable Constant.

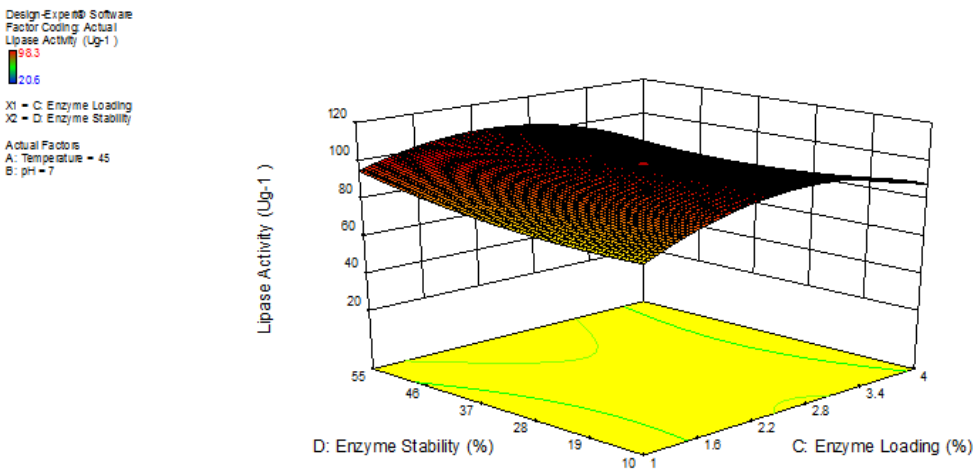


Fig. 9: 3D Response Surface Plot Showing Effect of Enzyme Loading and Enzyme Stability on Lipase Production with other Variable Constant

3.3.3. Effect of interactive variables on lipase production

Higher production of lipase was observed at pH 7 and temperature of 45 °C (Fig. 4). When interaction of Enzyme loading and temperature was studied, higher production of lipase was achieved at Enzyme loading 2.5 % as same temperature was maintained (Fig. 5).

The influence of interactive variables on lipase production was shown in Fig. 4-9. The temperature-pH interaction at temperature 30 °C and 60 °C attained 60.6 Ug^{-1} and 54.4 Ug^{-1} respectively indicating that low pH (Fig. 4) favours lipase activity. The activity was quite low as compared with pH-enzyme stability (Fig. 5) and enzyme loading-enzyme stability (Fig. 6) which had maximum activity of 76.1 Ug^{-1}. The low enzyme loading had significant increase in lipase activity at optimum pH 7 than high enzyme loading at the same pH. Moreover, in the temperature-pH interaction, low pH had significant increase in lipase production at optimum temperature of 45 °C than high pH at the same temperature. Further increase in temperature reduces the activity. To illustrate that several optimal combinations are able to produce the highest lipase activity, contour and response surface plots were drawn at constant value of 45 °C and 60 °C (Temperature), 2.5 % and 3.97 % v/v (Enzyme loading), 7 (pH) and 25 % v/v (enzyme stability) respectively. The responses corresponding to the contour plots of second order predicted model indicated that, an increase in temperature till 45 °C led to maximum lipase activity with enzyme loading at 2 %. Further increase in temperature reduced the activity. Maximum lipase activity was therefore, obtained for large enzyme loading followed by temperature due to the fact that these variables were most significant with negative effect. However, at higher pH, there seemed to be less effect on lipase activity as temperature increases (Fig. 4). Each figure represents effect of two variables on lipase activity. From the analysis of the response surface plots, the optimum conditions were pH 7.0, temperature 45 °C, enzyme loading 2.5 % and enzyme stability 32.5 %.

4. Discussion

The R2 value obtained indicates that the variation in lipase activity correlated with 94.24 % of the independent variables and the obtained adjusted R2 value indicates a 78.11 % correlation between the independent variables which could be explained by the model of Shabbiri & Adnan (2011). The adequate precision which measured the signal- to-noise ratio was 9.809, greater than 4 is desirable. This ratio indicates an adequate signal and this model can be used to navigate the design space satisfactory (Shabbiri & Adnan 2011, Gupta et al. 2007, Acıkel et al. 2010). The enzyme loading slightly affect the activity reaching a peak of 95.4 U/g-matrix at 2.0 % as observed and thereafter decreases at higher level. If the inoculum size is too small, insufficient biomass lead to reduced level of secreted lipase whereas higher inoculum size may cause insufficiency of total dissolved oxygen and nutrient supply in the culture media resulting in poor product yield (Baharum et al. 2003). However, it is outstanding that in these systems, the methodology used for enzyme immobilization was adsorption, which is a methodology where the forces involved in enzyme support bounding, are lower than those present in covalent bond or entrapment (Morais et al. 2013). This may likely be responsible for low relative activity as compared with latter methods adopted by Morais et al. (2013) when amylase immobilized in copolymers of methacrylate-acrylate acid that preserved 95% of initial activity after five cycles. The activity loss observed during operational storage stability of VS-immobilized lipase of *A. niger* ATCC 1015 could probably occurred during drying procedure, when the evaporation of water molecules induced rearrangements of the polypeptide chain. These rearrangements may occur indifferent patterns for lipase molecules as function of the specific surrounding environment.

Conclusively, the conformational changes of the lipid as well as the interaction of the substrate with the bio-matrix brought about the catalytic potency.

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