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RESEARCH ARTICLE

Optimization of *Aspergillus japonicus* lipase production by Response Surface Methodology

A. Jayaprakash and P. Ebenezer
Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai 600 025, Tamil Nadu, India.
aruljaypee@gmail.com; +91 9841378323

Abstract

Central Composite Design (CCD) was used to evaluate *Aspergillus japonicus* lipase production by Response Surface Methodology (RSM). A 2^{3-1} fractional factorial design with 6 star points and 6 replicates at the centre points were employed with 20 experiments. RSM for the first set of experiments yielded 124 U/mL, while the predicted value was 125.41 U/mL. The final validated experiment yielded 127 U/mL. Pig fat favoured lipase production to an appreciable extent influenced by the sucrose and peptone concentrations. The second set of experiments involving pH, temperature and rpm yielded 134 U/mL and an increase in the enzyme production to about 140 U/mL was observed.

Keywords: Central composite design, *Aspergillus japonicus* lipase, pig fat, sucrose, peptone.

Introduction

The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions emerged as viable alternatives to tedious and expensive chemical methods. Enzymes find great use in food, dairy, pharmaceutical, detergent, textile, and cosmetic industries. In the above context, enzymes such as proteases, lipases and amylases flooded the world market owing to their hydrolytic properties with regard to proteins, lipids and carbohydrates. However, on the assessment of the biocatalytic potential of microbial lipases in both aqueous and non-aqueous media in the last one and a half decades, industrial establishments switched over to the use of these enzymes for an array of applications on an industrial scale.

Lipases (Triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and a great deal of industrial potential (Gulati *et al*., 2005). Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Balashev *et al*., 2001). It is in the last decade that lipases have gained importance to a considerable extent over proteases and amylases, especially in the area of organic synthesis. The enantioselective and regioselective nature of lipases has been exploited for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substitutes, biofuels and for synthesis of personal care products and flavour enhancers (Gerhartz, 1990; Priest, 1992). As of today, lipases gained importance as the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists. Most commercially viable lipases are synthesized by fungi and yeasts. Commercial lipolytic enzymes are produced from *Rhizopus delemar* (Espinosa *et al.,* 1990; Cruz *et al.,* 1993; Shimada *et al.,* 1996); *Humicola lanuginosa* (Morinaga *et al.,* 1986, Ivanova *et al.,* 2002) *Penicillium chrysogenum* (Ferrer *et al.,* 2000), *Fusarium heterosporum* (Nagao *et al.,* 1998), *Rhizopus chinensis* (Nakashima *et al.,* 1988) and *Candida rugosa* (Valero *et al.,* 1988; Obradors, 1993).

Fungal lipases are produced mostly by submerged culture (Ito *et al*., 2001) and solid state fermentation methods (Chisti, 1999). Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production by submerged culture. Lipase production is influenced by the nature and concentration of carbon and nitrogen sources, the culture pH, growth temperature, dissolved oxygen concentration etc. (Elibol and Ozer, 2001). Response Surface Methodology (RSM) is a combination of statistical and mathematical algorithms employed for developing, improving, and optimizing processes and products (Raymond and Montgomery, 2002).The method for determining optimal conditions in fermentation processes for enzyme production involves varying one parameter while others are kept constant. This is a time and cost ineffective method that presents also the disadvantage of not including the interaction effects among variables. Optimization using a factorial design and response

surface methodology can help to overcome such drawbacks. A factorial design technique has been successfully put to use to optimize and evaluate the effect of process parameters in the production of enzymes and other metabolites (Muralidhar *et al*., 2001; Haaland, 1989). Owing to their industrial significance, lipases have been widely studied with the main focus on enzyme action, kinetics, sequencing and cloning of lipase genes and structural characterization (Arroyo and Sinisterra, 1995; Beer *et al*., 1998). Nevertheless, studies on applying RSM for validating and optimizing nutritional parameters for lipase production have been inconclusive. Against these backdrops, this study was aimed to optimize the process parameters for *Aspergillus japonicus* lipase production using central composite design.

Materials and methods

Organism

Aspergillus japonicus was isolated from the paper nest of *Ropalidia marginata* collected from a glass house at CAS in Botany, University of Madras.

Lipase production medium

Tween-20 broth was prepared by mixing 10.0 g peptone; 5.0 g NaCl and 0.1 g $CaCl_2$ and 10 mL Tween-20 was autoclaved separately and was added to the broth. A number of sterile Erlenmeyer flasks (250 mL) containing 100 mL of the Tween-20 medium were inoculated with 7 mm mycelial discs from seven day old culture of *A. japonicus* and incubated for a period of 7 days. The culture filtrates were used as the enzyme source.

Experimental design of RSM for lipase optimization

Lipase production medium for *A. japonicus* was optimized using Design Expert 7.0 software by Response Surface Methodology (RSM). The optimal levels of six variables such as sucrose, peptone, pig fat, pH, temperature and rpm on lipase production were determined, by two sets of experiments. The first set was carried out by the first three variables like sucrose, peptone and pig fat. A second set of environmental variables pH, temperature and rpm was used with the optimized concentrations of the first set of variables. For this purpose, the response surface approach by using a set of experimental design (central composite design with two coded levels) was performed according to Box *et al*. (1978). For the three factors, this design was made up of a complete 20 factorial design with its eight points augmented with three replications of the center points. A set of 20 experiments was carried out with first set of variables and 20 experiments for the second set of variables. In developing the regression equation, the test factors were coded according to the following equation:

$$x_i = \frac{(x_i - x^x{}_i)}{\Delta x_i}$$

Where *x*i is the coded value of the *i*th independent variable, *Xi* the natural value of the *i*th independent variable, *XXi* the natural value of the *i*th independent variable at the center point, and ΔXi the step change value (ΔXi Sucrose 5g/L, Peptone 5 g/L, and pig fat 5 g/L. For Set-2, pH 7.5, temperature 40°C and 120 rpm for a three-factor system, the model equation is:

$$Y = b_o + \sum_i b_i x_i + \sum_i \sum_y b_{ij} x_i x_j + \sum b_{ii} x^2 i + e$$

Where, Y is the measured response, b_o, intercept term, $b_{i,ij}$, and b_{ii} are, respectively the measures of the effects of variables X_i , X_i X_j , and X_i^2. The variable X_i X_j represents the first- order interactions between X_i and X_j (i<j).

Lipase assay

Lipase activity was assayed quantitatively by using ρ-nitro phenyl palmitate as the substrate (Winkler and Stuckmann, 1979). 10 mL isopropanol containing 30 mg ρ-nitro phenyl palmitate (Sigma) was mixed with 90 mL 0.05 M sodium phosphate buffer (pH 8) containing 207 mg sodiumdeoxycholate and 100 mg gum arabic. A total volume of 2.4 mL freshly prepared substrate solution was prewarmed at 37°C and mixed with 0.1 mL enzyme solution. After 15 min incubation at 37°C, absorbance at 410 nm was measured against a blank. One enzyme unit was defined as 1µmol of ρ-nitrophenol enzymatically released from the substrate in milliliters per minute.

Results and discussion

Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relations between a cluster of controlled experimental factors and the measured responses according to one or more selected criteria (Adinarayana and Ellaiah, 2002). A prior knowledge of the initial factor optimization process is thus a prerequisite for RSM. Several experimental designs have been considered to study such model, and we selected the central composite design proposed by Box *et al.* (1978).

A set of three experimental factors capable of influencing the reaction yield such as sucrose, peptone and pig fat constituted the first set of variables. With optimized first set of variables, a second set of experiments was taken up providing the experimental variables such as pH, temperature and rpm by RSM.

Table 1. The coded and actual values of the factors in CCD.

Factor	Name (g/L)	-1	Centre point	+1
X_1	Sucrose	2.5	5	7.5
X_2	Peptone	2.5	5	7.5
X_3	Pig fat	2.5	5	7.5

Table 2. CCD of factors in coded levels with enzyme activity as response.

		Variables			Lipase activity (U/mL)	
Standard	Run	Sucrose (g/L) X_1	Peptone (g/L) X_2	Pig Fat (g/L) X_3	Observed	Predicted
1	13	-1.000	-1.000	-1.000	63	54.08
2	2	1.000	-1.000	-1.000	52	63.04
3	17	-1.000	1.000	-1.000	87	95.06
4	4	1.000	1.000	-1.000	61	59.53
5	10	-1.000	-1.000	1.000	81	83.09
6	12	1.000	-1.000	1.000	103	95.55
7	5	-1.000	1.000	1.000	124	113.57
8	6	1.000	1.000	1.000	72	81.54
9	16	-1.682	0.000	0.000	101	106.76
10	11	1.682	0.000	0.000	94	87.35
11	14	0.000	-1.682	0.000	78	80.21
12	7	0.000	1.682	0.000	106	102.90
13	9	0.000	0.000	-1.682	43	38.11
14	19	0.000	0.000	1.682	77	81.00
15	15	0.000	0.000	0.000	122	116.35
16	18	0.000	0.000	0.000	122	116.35
17	1	0.000	0.000	0.000	118	116.35
18	20	0.000	0.000	0.000	106	116.35
19	8	0.000	0.000	0.000	108	116.35
20	3	0.000	0.000	0.000	122	116.35

Table 3. ANOVA for Response Surface Quadratic Model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	11088.58	9	1232.065	13.1713	0.0002	Significant
X_1-Sucrose	454.359	1	454.359	4.8572	0.0521	
X_2-Peptone	620.9779	1	620.9779	6.6385	0.0276	
X_3-Pig fat	2221.522	1	2221.522	23.7490	0.0006	
X_1X_2	990.125	1	990.125	10.5848	0.0087	
X_1X_3	6.125	1	6.125	0.0654	0.8032	
X_2X_3	55.125	1	55.125	0.5893	0.4604	
X_1^2	670.9209	1	670.9209	7.1724	0.0232	
X_2^2	1107.829	1	1107.829	11.8431	0.0063	
X_3^2	5811.53	1	5811.53	62.1277	< 0.0001	
Residual	935.4161	10	93.54161			
Lack of Fit	660.0828	5	132.0166	2.3973	0.1796	not significant
Pure Error	275.3333	5	55.06667			
Cor Total	12024	19				

Set-1 Optimization of sucrose, peptone and pig fat

The central composite design experiments for studying the effects of three independent variables such as sucrose, peptone and pig fat on lipase production were carried out. For this study, 2^{3-1} fractional factorial design with six star points and six centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots (Khuri and Cornell, 1987). The experiments were conducted and average values of lipase yields were tabulated, as given Table 2, under column, observed response. The results of central composite design experiments for studying the effects of first set of three independent variables such as sucrose (X_1), peptone (X_2) and pig fat (X_3), the experiments were conducted (Table 1).

The ANOVA of quadratic regression with a very low probability value (P *model* > F=0.0002) (Table 3) demonstrate a very high significance for the regression model (Akhnazarova and Kafarov, 1982). The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.9222) indicates about 92.2% of the fitness of the model. The value of the adjusted determination coefficient (Adj. R^2 = 0.8521) is also very high, which indicates a high significance of the model. The "Lack of Fit F-value" of 0.1796 implies the lack of fit is not significant relative to the pure error. The pure error was also very low indicating good reproducibility of the experimental data. Hence, the quadratic model was suitable to represent the real relationship among the selected factors. The coded model was used to generate response surfaces for the analysis of variable effects on selective partitioning of lipase. At the same time a relatively lower value of the coefficient of variation (CV= 10.51) indicates improved precision and reliability of the conducted experiments (Box and Wilson, 1951). The three-dimensional response surface curves were then plotted (Fig. 1-3).

Final equation in terms of actual factors:

Lipase activity = -105.289 + 8.404914 * Sucrose + 13.86278* Peptone + 19.31593 * Pig fat - 0.445 *Sucrose * Peptone + 0.035 * Sucrose * Pig fat - 0.105 * Peptone * Pig fat - 0.27293 * Sucrose^2 - 0.35071* Peptone^2 - 0.80326 * Pig fat^2

The validation of the optimal components (2.5 g/L sucrose, 7.16 g/L peptone and 5.61 g/L pig fat) after RSM was carried out by ρ-nitrophenyl palmitate assay. The observed value for extracellular lipase production was found to be 124 U/mL. The predicted value was found to be 125.41 U/mL by RSM. The final validated experiment yielded 127 U/mL (data not shown). These concentrations were subsequently used for next experiment.

Fig.1. Response from sucrose and peptone for lipase production.

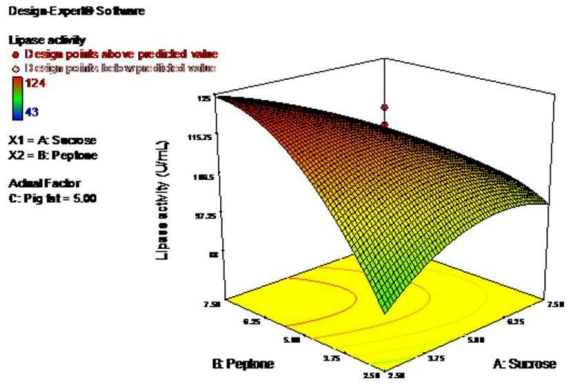


Fig.2. Response from sucrose and pig fat for lipase production.

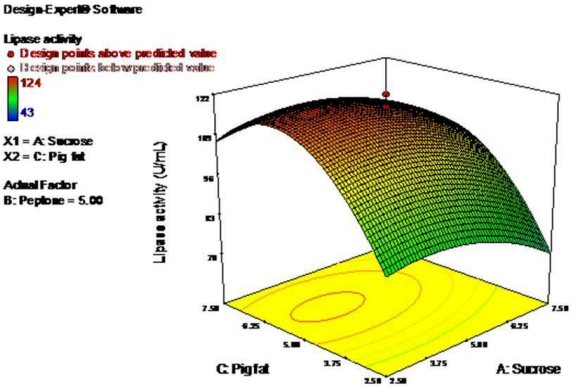
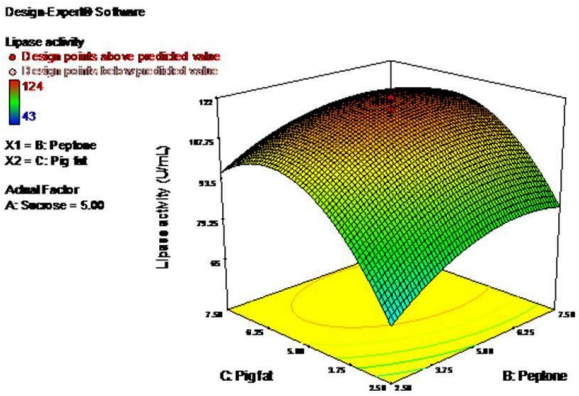


Fig.3. Response from peptone and pig fat for lipase production.



Set-II Optimization of pH, temperature and orbital speed (rpm)

The central composite design experiments for studying the effects of three independent variables such as pH, temperature and rpm on lipase production were carried out using the validated components in the previous experiment. For this study, 2^{3-1} fractional factorial design with six star points and six centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure. The optimum values of the selected variables were obtained by solving the regression equation and also by

analyzing the response surface contour plots (Khuri and Cornell, 1987). The experiments were conducted and average values of lipase yields were tabulated, as given Table 5, under column, observed response. The results of central composite design experiments for studying the effects of second set of three independent variables such as pH (X_1), temperature (X_2) and rpm (X_3), the experiments were conducted (Table 4). The ANOVA of quadratic regression with a very low probability value (P *model* > F=0.0002) (Table 6) indicate a very high significance for the regression model (Akhnazarova and Kafarov, 1982). The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0.9261) about 92.61% indicating the good fit of the model. The value of the adjusted determination coefficient (Adj. R^2 = 0.8596) is also very high, which indicates a high significance of the model. The "Lack of Fit F-value" of 2.23 implies the lack of fit is not significant relative to the pure error.

The pure error was also very low indicating good reproducibility of the experimental data. Hence, the quadratic model was found to be suitable to represent the real relationship among the selected factors. The coded model was used to generate response surfaces for the analysis of variable effects on selective partitioning of lipase.

Table 4. The actual values of the factors in CCD.

Factor	Name	-1	Centre point	+1
X_1	pH	7	7.5	8
X_2	Temperature	35	40	45
X_3	rpm	110	120	130

Table 5. Central composite design of factors in coded levels with enzyme activity as response.

		Variables			Lipase activity (U/mL)	
Std.	Run	pH X_1	Temperature X_2	rpm X_3	Observed	Predicted
1	7	-1.000	-1.000	-1.000	69.00	60.31
2	9	1.000	-1.000	-1.000	88.00	92.51
3	2	-1.000	1.000	-1.000	73.00	73.99
4	1	1.000	1.000	-1.000	67.00	67.68
5	11	-1.000	-1.000	1.000	79.00	72.51
6	10	1.000	-1.000	1.000	109.00	102.00
7	17	-1.000	1.000	1.000	97.00	86.68
8	4	1.000	1.000	1.000	75.00	77.88
9	19	-1.682	0.000	0.000	54.00	65.77
10	5	1.682	0.000	0.000	89.00	85.44
11	18	0.000	-1.682	0.000	84.00	91.58
12	8	0.000	1.682	0.000	82.00	82.63
13	13	0.000	0.000	-1.682	75.00	73.69
14	15	0.000	0.000	1.682	83.00	92.52
15	20	0.000	0.000	0.000	134.00	126.77
16	3	0.000	0.000	0.000	132.00	126.77
17	16	0.000	0.000	0.000	128.00	126.77
18	12	0.000	0.000	0.000	116.00	126.77
19	14	0.000	0.000	0.000	120.00	126.77
20	6	0.000	0.000	0.000	132.00	126.77

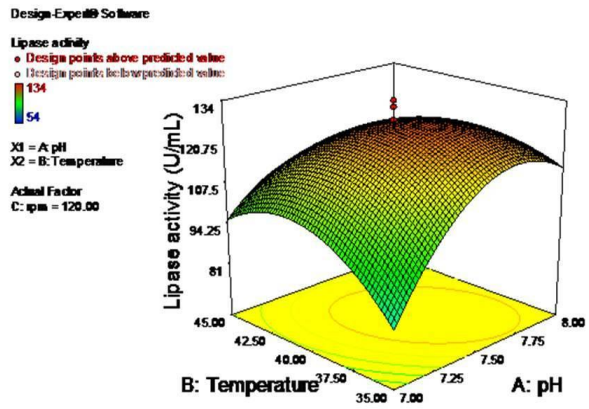
At the same time a relatively lower value of the coefficient of variation (CV= 9.90) indicates improved precision and reliability of the conducted experiments (Box and Wilson, 1951). The three-dimensional response surface curves were then plotted (Fig.4-6).

Final equation in terms of actual factors:

Lipase activity = 126.77 + 5.85*pH - 2.66*Temperature + 5.60*rpm 9.62*pH* Temperature-0.62*pH*rpm + 0.13*Temperature*rpm-18.09*pH 14.02*Temperature^2 - 15.44*rpm^2

The validation of the optimal components (2.5 g/L sucrose, 7.16 g/L peptone, 5.61 g/L pig fat, pH 7.6, temperature 39°C and 120 rpm) after RSM was carried out by ρ-nitrophenyl palmitate assay. The observed value for extracellular lipase production was found to be 134 U/mL. The predicted value was found to be 137.89 U/mL by RSM. The final validated experiment yielded 140 U/mL (Fig. 7).

Fig.4. Response from pH and temperature for lipase production.



Conclusion

Although many experimental designs are available to study enzyme optimization process, the central composite design as proposed by Box *et al.* (1978) was chosen for the study. A 2^{3-1} fractional factorial design with six star points and six replicates at the centre points was employed to fit the second order polynomial model which indicated that 20 experiments were required to be carried out for this procedure. The RSM for the first set of experiments yielded 124 U/mL, while the predicted value was 125.41 U/mL. The final validated experiment yielded 127 U/mL. Pig fat favoured lipase production to an appreciable extent and this was again influenced by the sucrose and peptone concentrations. Sucrose and peptone are therefore, the key nutrients which seem to regulate the biosynthesis of lipase. It is interesting to note that at higher concentrations both nutrients inhibit lipase synthesis.

Fig.5. Response from pH and rpm for lipase production.

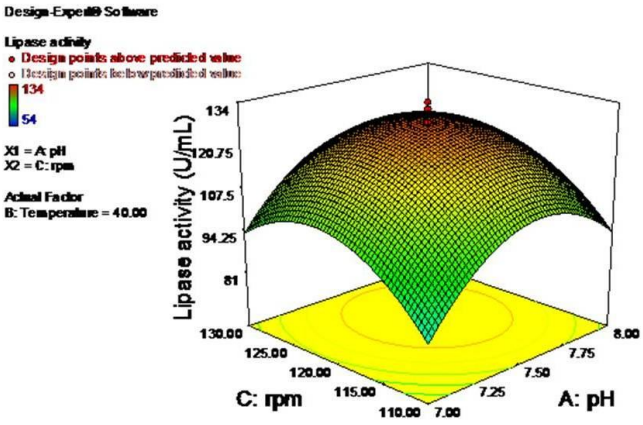
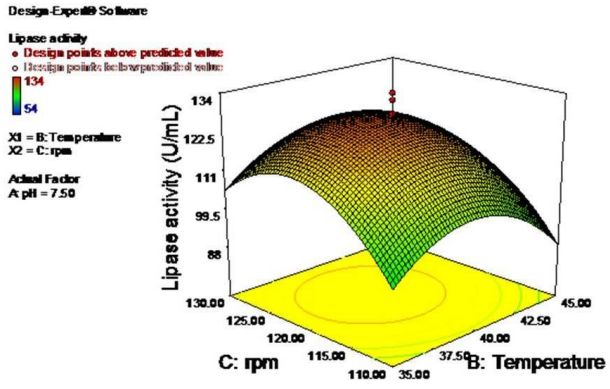


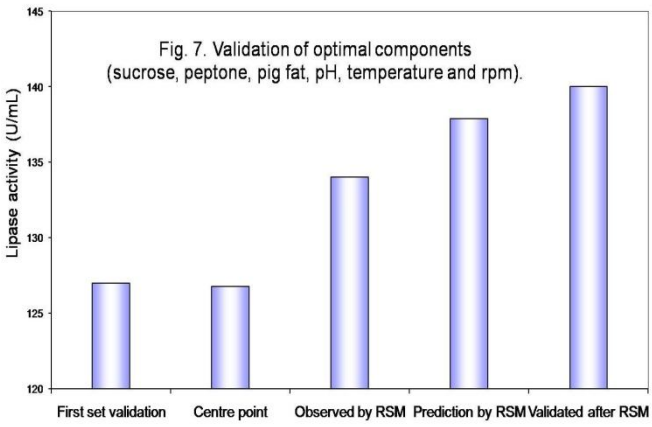
Table 6. ANOVA for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	10932.07	9.00	1214.67	13.93	0.0002	significant
X_1-pH	467.02	1.00	467.02	5.35	0.0432	
X_2- Temperature	96.82	1.00	96.82	1.11	0.3168	
X_3- rpm	428.01	1.00	428.01	4.91		
X_1X_2 741.12	1.00	741.12	8.50	0.0154		
X_1X_3 3.13	1.00	3.13	0.036	0.8537		
X_2X_3 0.13	1.00	0.13	1.433E-003	0.9705		
X_1^2 4714.61	1.00	4714.61	54.06	<0.0001		
X_2^2 2833.23	1.00	2833.23	32.49	0.0002		
X_3^2 3433.58	1.00	3433.58	39.37	<0.0001		
Residual	872.13	10.00	87.21			
Lack of Fit	602.13	5.00	120.43	2.23	0.1997	not significant
Pure Error	270.00	5.00	54.00			
Core Total	11804.20	19.00				

Fig.6. Response from temperature and rpm for lipase production.



The second set of experiments involving pH, temperature and rpm yielded 134 U/mL. The modelled values indicated that the maximum output of the enzyme that could be realized by providing the above optimized concentrations of the variables was 137.89 U/mL. Verification of the predicted results was accomplished by providing the optimized conditions. An increase in the enzyme production to about 140 U/mL was observed. This trend corroborates the predicted values and the effectiveness of the model. RSM hence proved to be an effective alternative for lipase optimization compared to traditional optimization processes.



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