Original Article

Response Surface Methodology for Optimization of Media Components for Production of Lipase from *Bacillus subtilis* KUBT4

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How to cite this article: **Nadaf RD, Nadaf PD, Toragall MM, CT S. Response Surface Methodology for Optimization of Media Components for Production of Lipase from *Bacillus subtilis* KUBT4. Archives of Razi Institute Journal. 2024;79(3):659-668.**
DOI: 10.32592/ARI.2024.79.3.659

Article Info:
Received: 21 January 2023
Accepted: 2 March 2023
Published: 31 August 2024

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ABSTRACT

Lipases are triacylglycerol hydrolases with various potential applications because of their different physical properties. Most lipase producers are extracellular in nature and are created using solid-state fermentation and submerged fermentation methods. The fungal, mycelial, and yeast lipases are produced using various solid substrates through the solid-state fermentation method. This method is cost-effective and widely used in industries to produce lipase using fungi. However, lipases from bacteria are produced using submerged fermentation. The optimization of media is a main requirement for increasing the quantitative yield by the overproduction of enzymes. The optimization of media is a main requirement for increasing the quantitative yield by overproduction of enzymes. Different parameters, such as pH, temperature, agitation speed, inoculum size, incubation time, and carbon and nitrogen sources, have been of great importance for researchers in designing economical media. The optimization by one factor at a time (OFAT) is a one-dimensional approach that is laborious and time-consuming and does not consider interactions between the factors. The limitations of OFAT method can be alleviated by employing some techniques, such as Plackett-Burman design (PBD) and response surface methodology (RSM). The PBD is a method to screen the variables that influence production and remove the non-significant factors to attain a smaller and manageable set of factors. Subsequently, the chosen significant factors are optimized by RSM that assists to study the interactions of different factors. The RSM comprises of central composite design (CCD) to fit a second-order polynomial equation. In this study, the effect of temperature, tryptone, inoculum size, and incubation time on the lipase production were analysed by PBD screening experiments. The experiments were designed using a CCD with four variables as part of RSM, utilizing the Design Expert software. This model predicted optimal activity of lipase at 58.53 U/mL when using 1.5% tryptone, a 10 mL inoculum size, and an incubation period of 48 h at 34°C. This experiment was further validated and optimal activity of lipase of 57.85 U/mL was observed. Thus, RSM model enhanced the production of lipase and can be applied for the maximum yield of lipase.

Keywords: *Bacillus subtilis* KUBT4, Lipase, Plackett-Burman design, Response Surface Methodology.

1. Introduction

Most of the lipase producers are extracellular in nature and are created through solid-state fermentation and submerged fermentation methods (1). The fungal, mycelial, and yeast lipases are produced using various solid substrates through the solid-state fermentation method (2). This method is cost-effective and widely used in industries to produce lipase using fungi. However, lipases from bacteria are produced using submerged fermentation (3). The optimization of media is the main requirement for increasing the quantitative yield by the overproduction of enzymes. Different parameters, such as pH, temperature, agitation speed, inoculum size, incubation time, carbon, and nitrogen sources, have been of great importance for researchers in designing economical media (4). The optimization by one factor at a time (OFAT) is a conventional method that involves changing a single independent factor at a time and keeping other factors constant. The OFAT is a one-dimensional approach that is laborious and time-consuming and does not consider interactions between the factors. The limitations of the OFAT method can be reduced by employing other techniques, such as Plackett-Burman design (PBD) and response surface methodology (RSM). The PBD is a method to screen variables that influence the production and removal of the non-significant factors to attain a smaller and manageable set of factors (5). Subsequently, the chosen significant factors are optimized by RSM, which assists in studying the interactions between different factors. The RSM comprises a central composite design (CCD) to fit a second-order polynomial equation (6). Generally, the type of oil used as a carbon source can influence the production of lipase in the submerged technique (7). Usually, vegetable oil, coconut oil, and almond oil increase the yield of lipase. Moreover, lipases produced by submerged fermentation can easily recover and purify the yield (2, 8). Previously, the optimization of *Bacillus substilis* KUBT4 media was carried out using OFAT method. The present study aimed to optimise the media using RSM and PBD as they allow for the simultaneous optimization of multiple factors.

2. Materials and Methods

2.1. Plackett-Burman design

To investigate the variables that considerably affect the production of lipase, a screening by PBD was performed using the Minitab statistical software (Release 17, PA, USA). Four independent variables were calculated across a range from a level low (-2) to a high level (+2). The factors and levels are presented in table 1. The first-order polynomial model is the basis of PBD.

$$Y = \beta_0 + \sum \beta_i x_i (i = 1,2, \ldots\ldots, k)$$

where Y represents the response (lipase activity), β_0 represents the model intercepts, βi represents the linear coefficient, and X_i represents the level of the independent variable. The four factors selected included temperature, tryptone, inoculum size, and incubation time. As per the design, a set of thirty-one runs was performed. In all experiments, the remaining factors were kept constant. Lipase assay was performed in triplicates, and the mean of the enzyme activity was considered the response. The factors in the response surface regression analysis with a significance level of ≥ 95% (P=0.05) were considered to have a bigger influence on lipase activity and were further improved using CCD.

Table 1. Experimental levels of four factors tested in PBD.

Factor code	Factors	Min value (-2)	Max value (+2)
A	Temperature (°C)	34	42
B	Tryptone (%)	0.5	1.5
C	Inoculum size (ml)	6	14
D	Incubation time (hrs)	48	64

2.2. Central composite design

To study the variables that significantly affected the production of lipase, variables were screened by CCD using the Minitab statistical software (Release 17, PA, USA). The CCD was applied to fit a second-order model that needed only a minimum number of experiments for modeling. Four independent variables, namely temperature (A), tryptone (B), inoculum size (C), and incubation time (D), were used in the experimental design, as listed in table 2. The variables were evaluated across a range from low (-2) to high (+2), with six replicates at the central points being utilized in the study. In the present study, thirty-one experiments were conducted in 250 mL conical flasks consisting of 100 mL production media prepared as per the CCD of the four factors at five levels (-2,-1, 0,+1,+2) and the remaining parameters were constant. The results were used to fit the second-order polynomial model as expressed by the following equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} AA + \beta_{22} BB + \beta_{33} CC + \beta_{44} DD + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

where Y stands for the predicted response (lipase activity), A, B, C, and D for independent variables, as presented in table 2, β_0 represents model constant, and β_1, β_2, β_3, and β_4 represent linear coefficients, β_{11}, β_{22}, β_{33}, and β_{44} represent the quadratic coefficients, and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} represent cross-product coefficients.

2.3. Verification experiment

The measured lipase activity values for each variable in the experimental design were inserted for statistical and numerical analysis of the model using Minitab software (Release 17, PA, USA). Analysis of variance (ANOVA) was performed to analyze the variables and responses. Fisher's F-value was calculated using the same software. The polynomial model was expressed by the correlation coefficient (R^2) associated with probability (P). The experiment was validated by comparing the actual values

obtained from the fermentation process performed using predicted media components to the predicted results by the design. Quadratic models for each graph were plotted as contour plots and surface plots that signified the validity of the mode.

Table 2. Ranges of variables used in experimental design of RSM.

Symbols	Variables	Range	Code level				
			-2	-1	0	+1	+2
A	Temperature (℃)	34-42	34	36	38	40	42
B	Tryptone (%)	0.5-1.5	0.5	0.75	1.0	1.25	1.5
C	Inoculum size (ml)	6-14	6	8	10	12	14
D	Incubation time (hrs)	48-64	48	52	56	60	64

3. Results

3.1. Screening of the most significant medium components by PBD

The PBD analysis of four factors showed a significant variation in lipase activity from 6.08 U/ml to 50.12 U/ml in thirty-one experimental runs (Table 3). This variation indicated the influence of the factors on the activity of lipase. The analysis of regression coefficients, t-value, and P-value of four factors are provided in table 4. A larger t-value is associated with a lower P-value indicating higher significance of the model. The results from table 4 and the pareto chart (Figure 1) depict that the factors temperature and incubation time exhibited significant effects on the production of lipase (*P*=0.005 and *P*=0.000, respectively). In contrast, tryptone and inoculum size had a positive non-significant effect (*P*=0.363 and *P*=0.728, respectively). Hence, temperature, tryptone, inoculum size, and incubation time were selected in PBD screening.

3.2. Central composite design

PBD screening experiments depicted that the four factors played a significant role in lipase production. The influence of the interaction of the four factors on lipase production was determined by CCD of RSM using Design Expert software. The factors selected by the PBD were studied at five different levels (-2, -1, 0, +1, +2), and thirty-one experiments set with varying factor combinations were performed. Response surface regression analysis using ANOVA was conducted to analyze the significance of model coefficients, and the P-values showed the significance of each coefficient and the strength of interaction among each independent variable. The yield observed and predicted by the model equation are shown in table 5, and the analysis of the optimization study by ANOVA is shown in table 6. The contour plots (Figures 2-7) and surface plots (Figures 8-13) for lipase production showed the interactive effects among the selected factors, respectively. The model F-value of 6.06 from the ANOVA analysis suggested that the model was significant since values of "Prob>F" less than 0.05 designated that model terms were significant. In this case, A, D, AA, BB, CC, DD, and AD were significant model terms. The data was fitted to a second-order polynomial equation after the coefficients of the regression equation were computed. Hence, the lipase activity (i.e., response [Y]) is expressed in the following equation.

Lipase activity (U/ml) = 31.45 - 4.37 A + 1.25 B + 0.47 C - 8.77 D - 3.44 AA - 3.14 BB - 3.43 CC - 1.93 DD + 0.41 AB - 0.44 AC + 5.48 AD + 0.29 BC - 0.11 BD- 0.39 CD

where A represents temperature (°C), B represents tryptone (%), C represents inoculum size (%), and D represents incubation time (hours). The f-value of lack of fit was 302.46, suggesting that the lack of fit was significant compared to the pure error. The p-value of lack of fit for the model was 0.000 (<0.05), implying that the model was suitable. The R^2 value predicted by the model was 0.8414, which is reasonably similar to the adjacent R^2 value of 0.7027. The R-squared value must be in a range of 0 to 1.0, and the data of the present study indicated that the model was good. The interaction effects and optimal levels of the factors were determined by plotting the response surface curves.

3.3. Validation of the model

Validation of the experimental model was verified by performing the experiments under optimized conditions of 1% sucrose, 1.5% tryptone, 1.5% olive oil, 0.2% $CaCl_2.2H_2O$, 0.1% $MgSO_4.7H_2O$, 0.4% of 1% stock solution of $FeCl_3$, and pH 7.5 incubated at 34°C for 48 h with an agitation speed of 150 rpm. The experiments were carried out in triplicates, and the results were compared. The observed lipase activity (57.85 U/ml) from experiments closely matched the predicted response (58.53 U/ml) from the regression model, confirming the validity of the model.

4. Discussion

Sucrose and tryptone are good sources for maximum enzyme production. They showed optimal activity at pH 7.5 and 36ºC. An increase in lipase production was achieved by using 10% inoculum size, incubating for 48 h with agitation at 150 rpm. The most important stages in a biological process are modeling and optimization to improve a system and increase the efficiency of the process without increasing the cost. The classical optimization method (single variable optimization) is not only time-consuming and tedious but also fails to depict the complete effects of the parameters in the process. Additionally, this method ignores the combined interactions between physicochemical parameters, leading to misinterpretation of results (9). However, this technique will help reduce the number of parameters to be analyzed in further optimization processes, such as PBD and RSM (10). Following the fitting of the data to various models (e.g., linear, two factorials, quadratic, and cubic), an ANOVA revealed that a quadratic polynomial model best captured the reactions of PKO and l(+)-lysine.

Table 3. PBD generated for four variables.

Run order	Temperature (℃) (A)	Tryptone (%) (B)	Inoculum size (%) (C)	Incubation time (hrs.) (D)	Lipase activity (U/ml)
1	1	-1	1	1	10.0
2	0	0	0	0	31.31
3	1	1	1	1	8.98
4	0	0	0	0	31.78
5	-1	1	1	1	6.08
6	1	-1	-1	-1	15.0
7	1	1	-1	-1	15.56
8	-1	-1	-1	1	8.98
9	0	0	0	-2	50.12
10	0	0	0	2	10.0
11	-2	0	0	0	33.01
12	0	0	2	0	26.12
13	1	-1	-1	1	9.88
14	0	2	0	0	35.32
15	-1	1	-1	1	6.09
16	1	1	-1	1	8.71
17	0	-2	0	0	15.12
18	0	0	0	0	32.01
19	-1	-1	1	1	7.01
20	-1	1	1	-1	35.71
21	-1	-1	-1	-1	34.51
22	0	0	0	0	30.9
23	2	0	0	0	15.01
24	-1	-1	1	-1	36.34
25	-1	1	-1	-1	30.51
26	1	1	1	-1	14.01
27	1	-1	1	-1	14.23
28	0	0	-2	0	22.0
29	0	0	0	0	31.29
30	0	0	0	0	31.98
31	0	0	0	0	30.89

Table 4. Statistical analysis of PBD showing coefficient value, standard error coefficient value, T and P value for each variable.

Term	Coefficient	SE Coefficient	T-Value	P-Value
Constant	31.45	2.48	12.68	0.000
Temp (°C)	-4.37	1.34	-3.26	0.005
Tryptone (%)	1.25	1.34	0.94	0.363
Inoculum size (%)	0.47	1.34	0.35	0.728
Incubation time (hrs)	-8.77	1.34	-6.54	0.000
Temp (°C)*Temp (°C)	-3.44	1.23	-2.80	0.013
Tryptone (%)*Tryptone (%)	-3.14	1.23	-2.56	0.021
Inoculum size (%)*Inoculum size (%)	-3.43	1.23	-2.79	0.013
Incubation time (hrs)*Incubation time (hrs)	-1.93	1.23	-1.57	0.136
Temp (°C)*Tryptone (%)	0.41	1.64	0.25	0.805
Temp (°C)*Inoculum size (%)	-0.44	1.64	-0.27	0.794
Temp (°C)*Incubation time (hrs)	5.48	1.64	3.34	0.004
Tryptone (%)*Inoculum size (%)	0.29	1.64	0.18	0.860
Tryptone (%)*Incubation time (hrs)	-0.11	1.64	-0.07	0.949
Inoculum size (%)*Incubation time (hrs)	-0.39	1.64	-0.24	0.813

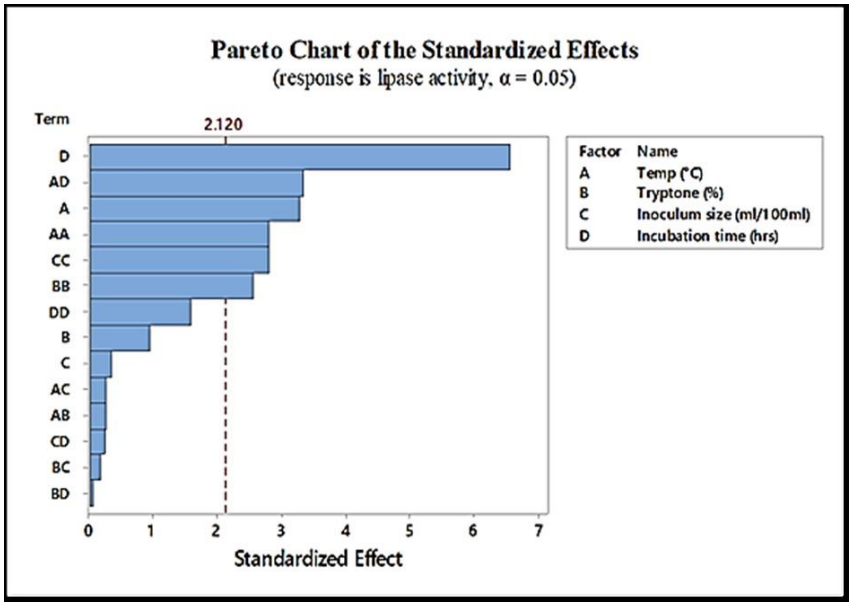

Figure 1. Pareto chart showing the effect of four factors on lipase activity.

Table 5. CCD matrix of four variables (in coded and actual units) with observed and predicted response values.

Temperature (°C) (A)	Tryptone (%) (B)	Inoculum size (%) (C)	Incubation time (hrs) (D)	Lipase activity (U/ml)	
				Observed value	Predicted value
1	-1	1	1	10.0	9.6479
0	0	0	0	31.31	31.45143
1	1	1	1	8.98	13.35375
0	0	0	0	31.78	31.45143
-1	1	1	1	6.08	11.17958
1	-1	-1	-1	15.0	16.51792
1	1	-1	-1	15.56	19.47875
-1	-1	-1	1	8.98	8.679583
0	0	0	-2	50.12	41.26667
0	0	0	2	10.0	6.203333
-2	0	0	0	33.01	26.42333
0	0	2	0	26.12	18.68167
1	-1	-1	1	9.88	10.94875
0	2	0	0	35.32	21.40333
-1	1	-1	1	6.09	9.560417
1	1	-1	1	8.71	13.47958
0	-2	0	0	15.12	16.38667
0	0	0	0	32.01	31.45143
-1	-1	1	1	7.01	9.12375
-1	1	1	-1	35.71	40.67375
-1	-1	-1	-1	34.51	36.16875
0	0	0	0	30.9	31.45143
2	0	0	0	15.01	8.946667
-1	-1	1	-1	36.34	38.18792
-1	1	-1	-1	30.51	37.47958
1	1	1	-1	14.01	20.92792
1	-1	1	-1	14.23	16.79208
0	0	-2	0	22.0	16.78833
0	0	0	0	31.29	31.45143
0	0	0	0	31.98	31.45143
0	0	0	0	30.89	31.45143

Table 6. Analysis of variance (ANOVA) for response surface model-CCD.

Source	DF	Sum of squares	Mean square	F-Value	P-Value
Model	14	3657.62	261.26	6.06	0.000 (Significant)
Linear	4	2345.43	586.36	13.61	0.000
Temperature (°C)	1	458.15	458.15	10.63	0.005
Tryptone (%)	1	37.75	37.75	0.88	0.363
Inoculum size (%)	1	5.38	5.38	0.12	0.728
Incubation time (hrs)	1	1844.16	1844.16	42.81	0.000
Square	4	821.88	205.47	4.77	0.010
Temperature (°C)* Temperature (°C)	1	338.71	338.71	7.86	0.013
Tryptone (%)*Tryptone (%)	1	281.78	281.78	6.54	0.021
Inoculum size (%)*Inoculum size (%)	1	336.25	336.25	7.81	0.013
Incubation time (hrs)*Incubation time (hrs)	1	106.42	106.42	2.47	0.136
2-Way Interaction	6	490.30	81.72	1.90	0.143
Temperature (°C)*Tryptone (%)	1	2.72	2.72	0.06	0.805
Temperature (°C)*Inoculum size (%)	1	3.05	3.05	0.07	0.794
Temperature (°C)*Incubation time (hrs)	1	480.49	480.49	11.15	0.004
Tryptone (%)*Inoculum size (%)	1	1.38	1.38	0.03	0.860
Tryptone (%)*Incubation time (hrs)	1	0.18	0.18	0.00	0.949
Inoculum size (%)*Incubation time (hrs)	1	2.48	2.48	0.06	0.813
Error	16	689.29	43.08		
Lack-of-Fit	10	687.93	68.79	302.46	0.000 (Significant)
Pure Error	6	1.36	0.23		
Total	30	4346.91			

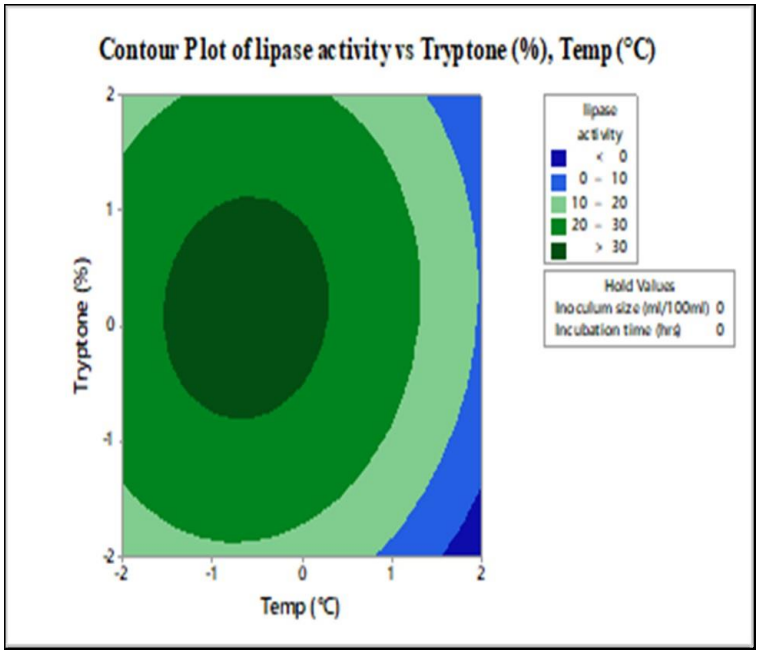

Figure 2. Contour plots for pectinase activity showing the interactive effects of Tryptone (%) v/s Temperature (°C).

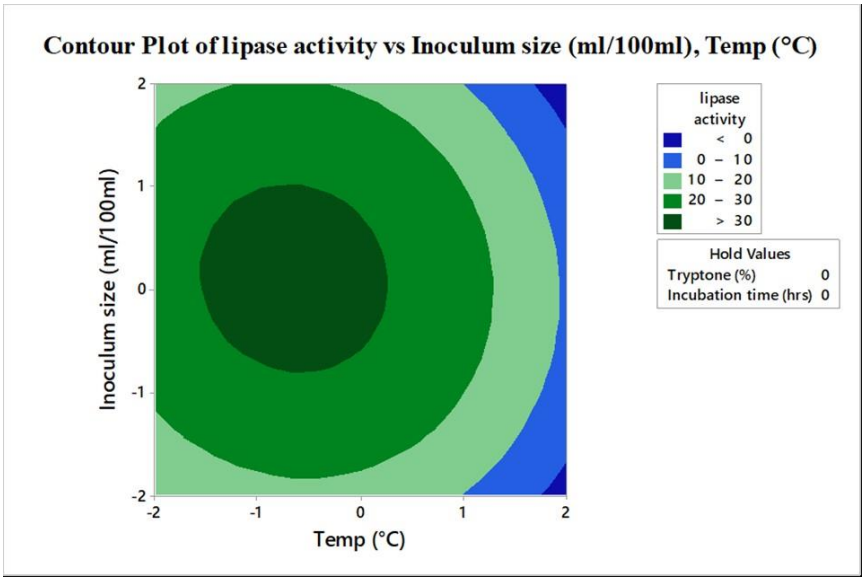

Figure 3. Contour plots for pectinase activity showing the interactive effects of Inoculum size (%) v/s Temperature (℃).

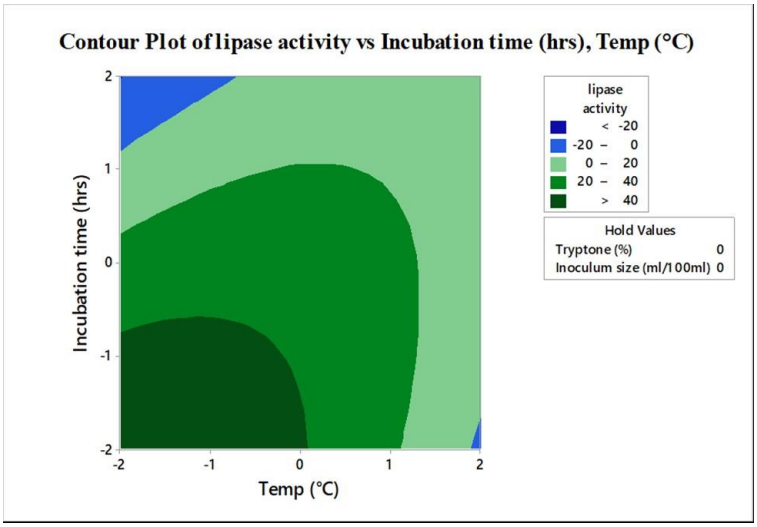

Figure 4. Contour plots for pectinase activity showing the interactive effects of incubation time (hrs.) v/s Temperature (℃).

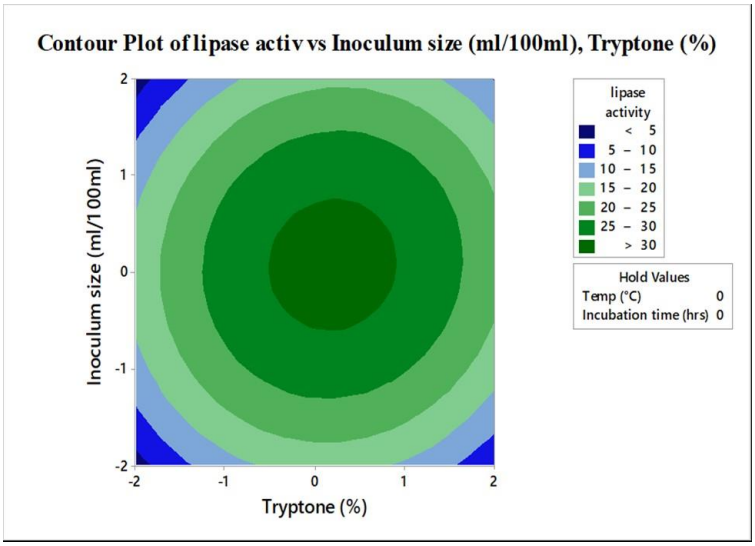

Figure 5. Contour plots for pectinase activity showing the interactive effects of Inoculum size (%) v/s Tryptone (%).

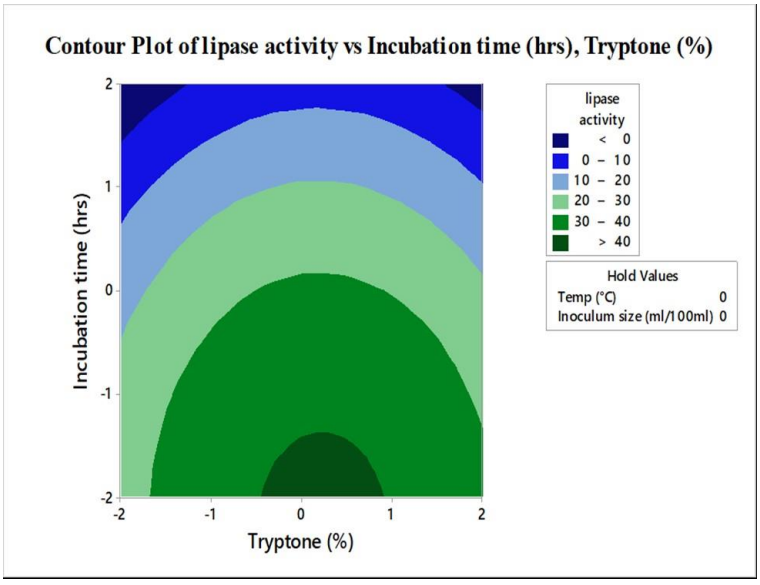

Figure 6. Contour plots for pectinase activity showing the interactive effects of Incubation time (hrs) v/s Tryptone (%).

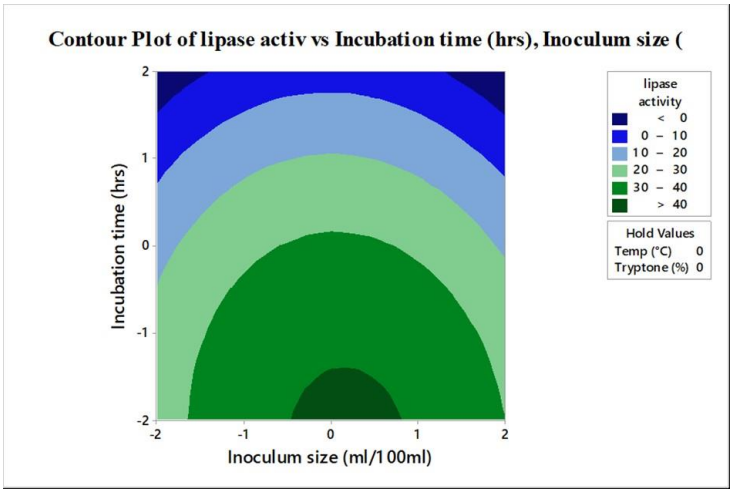

Figure 7. Contour plots for pectinase activity showing the interactive effects of Incubation time (hrs) v/s Inoculum size (%).

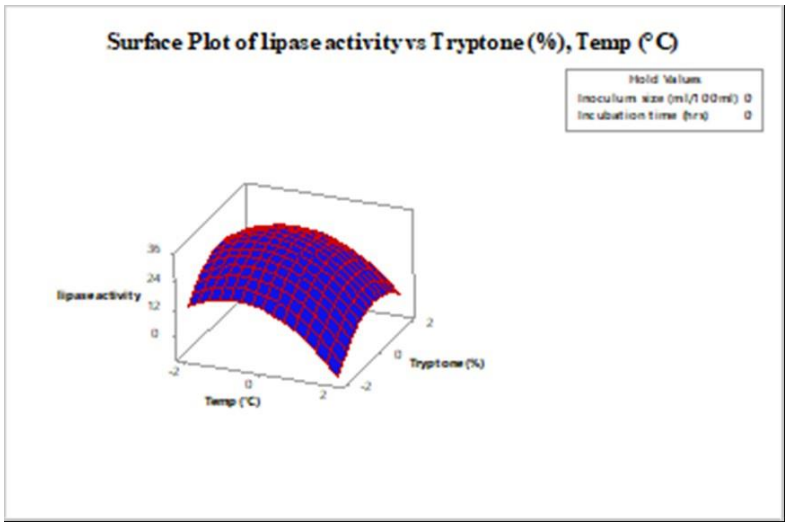

Figure 8. Surface plots for pectinase activity showing the interactive effects of Tryptone (%) v/s Temperature (℃).

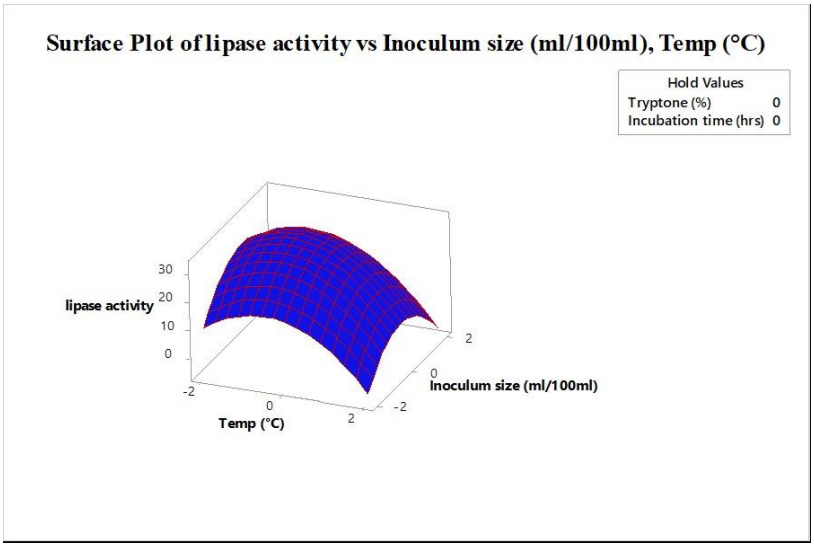

Figure 9. Surface plots for pectinase activity showing the interactive effects of Inoculum size (%) v/s Temperature (℃).

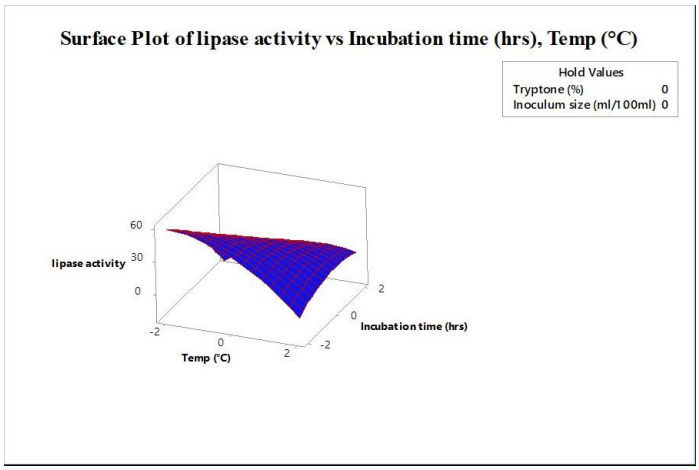

Figure 10. Surface plots for pectinase activity showing the interactive effects of Incubation time (hrs) v/s Temperature (℃).

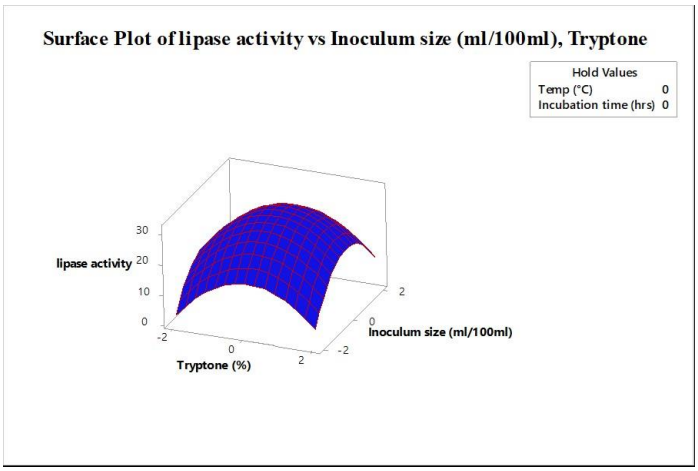

Figure 11. Surface plots for pectinase activity showing the interactive effects of Inoculum size (%) v/s Tryptone (%).

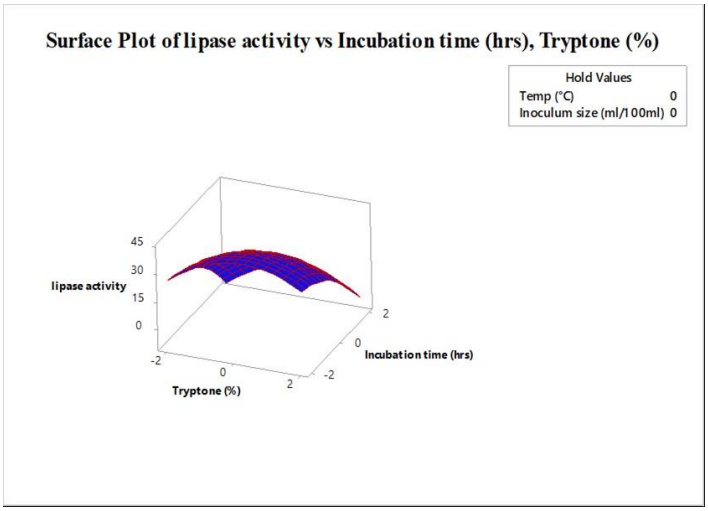

Figure 12. Surface plots for pectinase activity showing the interactive effects of Incubation time (hrs) v/s Tryptone (%).

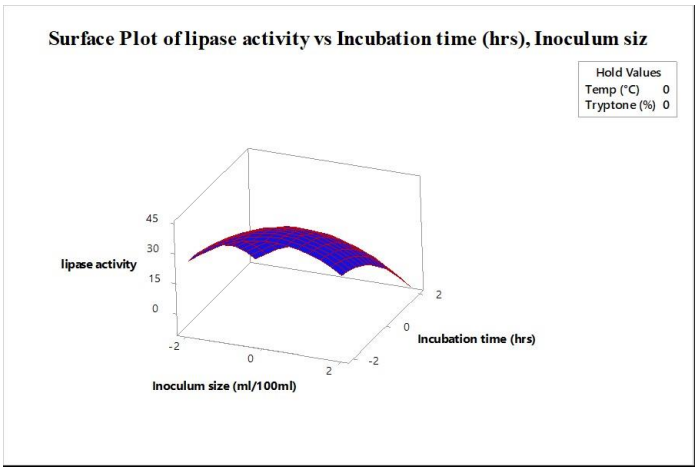

Figure 13. Surface plots for pectinase activity showing the interactive effects of Incubation time (hrs) v/s Inoculum size (%).

The model was inferred to be significant by the model's F-values of 3.69 and Prob > F values of 0.050. The model was found to be suitable for accurately capturing the genuine relationship between the elements, as indicated by the coefficient of determination (R^2=0.775) (11). The R^2 value obtained from Bidin et al.'s model was higher than the values reported by Kiran et al. (12) for the lipase-catalyzed synthesis of stearoyl (R^2=0.565) and palmitoyl (R^2=0.720) lactic acid ester, which ranged from 0.565 to 0.720 (13). The results of a study by Osorio et al. (14) demonstrated that the R^2 value of 0.566 was produced by the novozyme-catalyzed transesterification of palm oil stearin with soybean oil. The model was appropriate to reflect the experimental data based on the insignificant lack-of-fit test results (F-value=4.29). Generally, increasing the reaction temperature made the substrates more soluble by removing mass transfer constraints and increasing the enzyme's accessibility to the substrates (15). However, the high polarity of the solvent used in this reaction (tert-butyl alcohol) greatly enhances the solubility of both substrates by increasing their interaction, which had a direct impact on the reaction and the enzyme. Therefore, even though low temperature was utilized, it was still possible to achieve large yields thanks to the support of raised impeller speed, high molar ratios, and enzyme employment. Similar profiles for the lipozyme-catalyzed synthesis of amino acid esters and isoamyl isobutyrate were described by Soo et al., respectively (16). The authors hypothesized that the operational stability of the enzyme had decreased due to high temperatures. Additionally, applying low temperatures offers advantages, such as reducing power costs and maintaining enzyme stability over prolonged periods of use. However, raising the impeller speed from 175.00 to 325.00 rpm with a constant fixed enzyme amount of 5.75 g resulted in a yield difference of 18.60%. In contrast, keeping a fixed impeller speed of 175.00 rpm led to a yield difference of only 5.20%. This is because the reaction yield will drop when the impeller speed exceeds the ideal level, probably owing to the shearing effect on the molecules of the enzyme (17). The quadratic polynomial model was extremely significant and sufficient to describe the actual link between the response (% yield) and the significant factors, with a very modest P-value (0.0001) from the analysis of ANOVA and an acceptable coefficient of determination (R^2=0.995). Incorporating caproic acid into rapeseed oil and quantification of di-incorporated

structured triacyl glycerol as a function of factors were both successfully predicted using quadratic response models. The optimization of lipase-catalyzed synthesis for biodiesel (soybean oil methyl ester) and kojic acid monolaurate was determined by Shieh et al. (18), using a similar model. The researcher also checked the lipase activity of *S. hasta*, which did not show a significant activity (19). Additional steps involving the isolation of bacteria and purification of lipase can be taken to synthesize nanoparticles, following the procedures outlined by Ali et al. and Peidaei et al. (20, 21). Comparison of predicted and experimental values revealed good correspondence between them, implying that empirical models derived from RSM can be used to adequately describe the relationship between the factors and responses in media to produce lipase from *Bacillus substilis* KUBT4. These models can then be used to predict lipase yield under any given conditions within the experimental range. We have demonstrated that the optimum synthesis of lipases can be successfully predicted by RSM.

Acknowledgment

Authors sincerely acknowledge the Post Graduate Department of Studies in Biotechnology and Microbiology, Karnatak University, Dharwad, and KAHER's Dr. Prabhakar Kore Basic Science Research Centre, Belagavi.

Authors' Contribution

Rubeen D. N. and Parveen D. N. conceived, designed the experiments, performed experiments, analysed the data, and wrote the article. Makhadumsab M. T. edited the manuscript, and Shivasharana C. T. designed and reviewed the manuscript. All authors commented on the manuscript and approved the final manuscript.

Ethics

This article contains no studies with human participants or animals performed by any of the authors.

Conflict of Interest

Rubeen D. N., Parveen D. N., Makhadumsab M. T., and Shivasharana C. T. declare that they have no conflicts of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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