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Research Article

STATISTICAL OPTIMIZATION FOR LIPASE PRODUCTION BY *PENICILLIUM DIGITATUM* OBTAINED FROM DAIRY EFFLUENT

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ABSTRACT

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes with an exponential increase in its application in various fields. The active and stable nature of microbial enzymes and the inability of the plant and animal lipases to meet current world demands have been paid more attention in the production of lipase from microbes than from plants and animals. In the present study, the lipase production by *Penicillium digitatum* isolated from diary waste water was optimized by employing the Box-Behnken design of Response Surface Methodology (RSM). The lipase activity obtained from the experiments was very close to the response predicted by the regression model, which proves the validity of the model. RSM design yielded a maximum activity of 81.77µmol at optimum levels of significant variables of temperature (40°C) and pH (7.0) after 24 hrs incubation.

Keywords: Lipase, Optimization, *Penicillium digitatum,* Dairy Effluent, Response surface Methodology.

1. INTRODUCTION

Untreated Dairy effluents are discharged by small scale milk industries and cause dangerous disease leading to serious threat to the environment. One of the major constituents of dairy factory wastewaters is lactose, a low molecular weight sugar that flourishes the fungus growth in sewage waters. Microbial lipases have received a great deal of attention due to enormous industrial potentials. In comparison to animal or plant lipase, extracellular microbial lipase can be produced relatively inexpensively by fermentation and in large quantities [1]. The microorganisms, bacteria and especially fungi, are the tools of choice for commercial production of lipases in lower cost and shorter time. Filamentous fungi are interesting sources of lipases because they produce extracellular enzymes and are better adapted to the low moisture than yeast and bacteria. The most important sources of commercial lipase are *Mucor, Rhizopus, Candida, Geotrichum, Aspergillus, Penicillium and Humicola* [2, 3]. Many genera such as *Penicillium, Rhizopus, Aspergillus* and *Fusarium* have been observed as producers of lipase with desirable properties, which would have potential applications in a number of different areas.

Lipases are versatile enzymes widely used in various industries like detergents, food, chemical, pharmaceutical, cosmetics, leather and paper as it exhibits flexibility in the bioconversion reactions like esterification, transesterification, acidolysis, alcoholysis, aminolysis along with the traditional hydrolysis of triacylglycerides. Optimization of fermentation conditions becomes necessary to optimize the growth parameters to obtain maximum enzyme. Compared with conventional methods, the use of statistics saves more time and cost, making the process highly practical. Response surface methodology (RSM) is an effective statistic technique for optimizing complex processes [4]. RSM is a tool to study the optimal process parameters that produce a maximum, or minimum value of the response and represents the direct and interactive effects of the process parameters through two and three-dimensional plots. Because of their wide-ranging application the optimization of lipase production by the fungus, *P. digitatum* was carried out using the Box-Behnken design of Response Surface Methodology.

2. MATERIAL AND METHODS

2.1. Collection of Samples

Milk waste water samples were collected from various small scale milk industries in and around Coimbatore, Tamil Nadu and stored at 4°C and used as a source for lipolytic fungal strains.

2.2. Isolation and Screening of Lipolytic fungi

Milk waste water samples after serial dilution were spread in petriplates containing the PDA medium, sub-cultured to obtain pure culture of colonies and maintained at 40°C. The pure culture isolated were identified based on their morphology, mycelia structure and spore formation [5-7]. The isolated fungal strains were screened for lipase production using rapid qualitative Tributyrin Phenol Red Agar plate (TPRA) method and plates were incubated at 28°C for 48h.

2.3. Optimization for enhanced Lipase production

Optimal media components for lipase production were reviewed by one factor-at-a-time method, keeping others constant [8]. After the preparation of production media [9], 5g(disc) was inoculated and incubated for their optimum conditions. The enzyme was separated and used for the study [10]. All the experiments were conducted in triplicates and mean values of the data were presented.

2.3.1. Experimental Design for Enzyme Level Optimization

The factors affecting lipase production were evaluated through Response Surface Methodology using Box-Behnken Design. Experiments were conducted involving three variables resulted in a combination of 13 experiments, 3 continuous factors, and 3 replicates at the center point. The variables tested were pH (6.0, 7.0 and 8.0), Temperature (30°C, 35°C, and 40°C) and incubation period (24, 48, and 72 hrs). The effective three level (-1, 0 and +1) design (table 1) of these parameters was selected and 13 runs were conducted with three levels (table 2).

Table 1: Variables and Levels of the Box-Behnken Experiment

Variables	Levels of Variables			
	Code	-1	0	+1
pH	A	6.0	7.0	8.0
Temperature (°C)	B	30	35	40
Incubation Period (hrs)	C	24	48	72

Table 2: Experimental Design and Corresponding responses of the Box- Behnken Experiment

Standard Order	A (pH)	B (Temperature)	C (Incubation)	Lipase Activity ((µmol)	
				Experimental Value	Predicted Value
1	6.0	35	72	39.6	34.46
2	7.0	30	24	68.80	65.6
3	7.0	35	48	78.70	78.7
4	6.0	35	24	35.44	39.21
5	7.0	40	24	81.77	79.062
6	8.0	30	48	19.8	20.86
7	6.0	40	48	41.08	42.16
8	6.0	30	48	39.6	39.04
9	7.0	30	72	71.37	68.67
10	8.0	40	48	21.28	21.84
11	8.0	35	24	21.08	23.25
12	7.0	40	72	59.4	62.61
13	8.0	35	72	20.79	17.04

According to Equation (1) the quadratic model for RSM for the prediction of optimal points is expressed. Response surface regression analysis was conducted with the statistical software Design Expert (Stat Ease Inc. Minneapolis, USA). The second order polynominal equation describes the relationship between the dependent and independent variables

$$Y \text{ (Lipase Activity u/ml)} = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

Where, Y is the predicted response, β_0 is the intercept, β_1, β_2, β_3 are the linear coefficients, β_{11}, β_{22}, β_{33} are squared coefficients and β_{12}, β_{13}, β_{23} are the interaction coefficients. The responses of the dependent variables and regression analysis of experimental data were analysed using Eq (1). Analysis of variance (ANOVA) involved Fischer's F test to judge the model's overall significance, associates probability values, and coefficient of determination to measure the regression model's goodness of fit. The fitted polynominal equation was further expressed by varying the levels of two factors while the third one constant in the form of 3 D and the contour plots which depicted the interaction graphically.

2.4. Statistical Analysis

The obtained results showing the maximum residual lipolytic activities were submitted to analysis of variance (ANOVA). The effects of variables that were estimated and the regression co-efficients of the models generated were calculated.

3. RESULTS AND DISCUSSION

3.1. Screening of Fungi for Lipase Activity (Hydrolyzing Zone)

Among the numerous mycoflora isolated from the disposed milk waste water, *Penicillium digitatum* showed maximum hydrolyzing zone. A significantly highest hydrolyzing zone (clearance zone) of 31 mm out of colony diameter of 55 mm was showed by *Penicillium digitatum* followed by *Fusarium monoliformis* 24 mm (out of colony diameter of 46 mm). Results of this investigation are consistent with the previous studies demonstrating that lipase producing microorganisms are widely distributed among different soils and agro-industrial wastes [11, 12]. The present study proved that the different soil types and/or organic residues can be used for searching new and potent lipase producers. The clear zone around fungal isolates exhibited positive results on tributyrine agar plates within 5 days. Only six isolates reported for good zone of lipid hydrolysis in diameter ranged between 13.30 to 35.00 mm [13]. Using *Penicillium verrucosum*, 40 U/g of hydrolytic activity was obtained in optimized conditions [14]. *Penicillium restrictum* grown in media supplemented with olive oil yielded activities of 12.1 U/mL and 17.4 U/g in SmF and SSF, respectively [15].

3.2. Optimization Using Response Surface Methodology

RSM incorporates the interaction effects of variables and aid us in simultaneously optimizing several process parameters within a minimal number of experimental runs. These experimental designs which are statistically assisted can lead to significantly enhanced production. In our investigation, A (Temperature), B (pH) and C (incubation period) which were inferred to be significant media constituents were selected for optimization through the Box-Behnken design. The experimental design set up is shown in Table 3. Second-order polynomial equation describing the empirical relationship between the independent variables and response is given underneath in Equation (2):

$$Y \text{ (Lipase activity u/ml)} = 78.700 - 9.095A + 0.495B - 1.992C - 44.68A^2 - 3.575B^2 - 4.785C^2 - 0.005AB - 1.115AC - 6.235BC \quad (2)$$

The positive regression coefficient indicates that there is a synergistic effect, while the negative coefficient value indicates the effect of an inverse relationship [16]. In the regression equation, it can be seen that linear factors β_1, and β_3 show a negative correlation and β_2 shows the positive correlation. It indicates that the increase in pH causes an increase in the lipase production and vice versa whereas the production decreases when there is an increase in the incubation period and the temperature. The P value serves as a tool for checking the significance of each of the co-efficients and is indicative of the interaction strength of each independent variable. High significance of the corresponding co-efficients are indicated when P shows low value ($P < 0.05$). Here the P value is smaller *i.e.* < 0.001 and larger are the t value (15.66) and F value (9.18) which indicates that the corresponding co-efficient terms are significant. All three linear co-efficients, squared co-efficients, and one interaction co-efficient are significant, as evidenced from low P and high F values. In general, larger t, f and smaller P value indicate that the corresponding co-efficient terms are significant [17].

Table 3: ANOVA for Response Surface Quadratic Model

Source	Coefficient estimate	Df	Std. error	t- value	Pr(>/t/)
Model	78.700	9	5.024	15.665	0.000565
A-Temperature	-9.095	3	1.77	-5.120	0.014420
B-pH	0.495	3	1.77	0.279	0.798600
C-Incubation	0.798600	3	1.77	-1.122	0.343663
AB	-44.680	3	2.512	-13.445	0.000890
AC	-3.575	3	2.512	-1.076	0.360830
BC	-4.785	3	2.512	-1.440	0.245510
A^2	-0.005	3	3.323	-0.002	0.998537
B^2	-1.115	3	3.323	-0.444	0.687203
C^2	-6.235	3	3.323	-2.482	0.089107

Source	Residual	Lack of fit	Pure error
DF	3	3	0
Sum Sq.	75.7	75.7	0.0
Mean Sq.	25.24	25.24	-

Predicted R^2 0.9883, Adjusted R^2 0.9531

The R^2 value for this model is 0.9883 when expressed as a percentage, it implies that a total variation of 96.43% in enzyme activity could be explained by the model. The predicted R^2 is of acceptable agreement with the adjusted R^2 of 0.9531 thus showing the prediction of the experimental data was significant. The value of R^2 was 0.9866 reported a relatively high correlation between experimental and predicted values and 98.66% of the variability in the response was explained by the model [18]. The model processes high reliability, high fitting degree, and deviation with coefficient of determination R^2 = 0.9826 and adequate precision of 22.523 [18]. R^2 value gives a measure of how much variability in the observed response can be explained by the experimental parameters and their interaction [19].

The regression equation is graphically represented in contour plots which depict both, the interactions among the independent variables and their influence on enzyme production. The contour plots might be elliptical mounds, saddle points, or rising ridges [20]. Here, Response surface plots were generated by varying the levels of two factors while the third one constant. The plot between pH ranges and temperature range (fig. 1a), pH ranges and incubation periods (fig. 1b) and temperature ranges and incubation period (fig. 1c) are all elliptical in nature indicating significant interactions between them. In other studies, 72 h was reported as the optimal fermentation time for *Aspergillus niger* lipase grown in solid-state fermentation, with a subsequent decrease in activity seen at 96 h and 120 h [21, 22]. pH is considered to be an important variable in the production of lipase in submerged fermentation by several authors. In the production of *C. cylindracea* an initial pH of 6.5 was used [23]. The effect of pH on the production of lipases by *Rhyzopus chinensis* was found to e between pH 5 and 7and the best results were achieved at pH 5.5 [24].

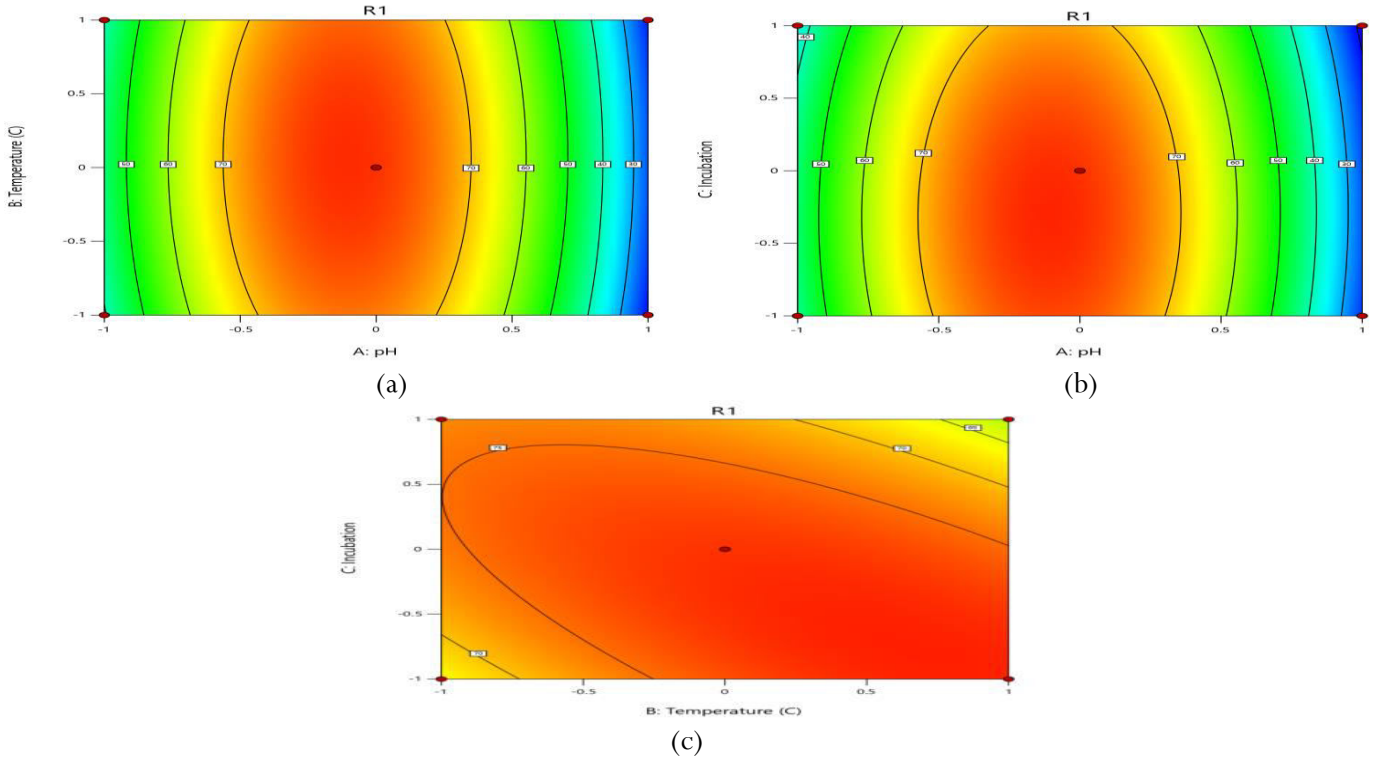

Fig. 1: Contour plots showing the interactive effect of (a) pH and Temperature on lipase production, (b) pH and Incubation period on lipase production, (c) incubation period and Temperature on Lipase production

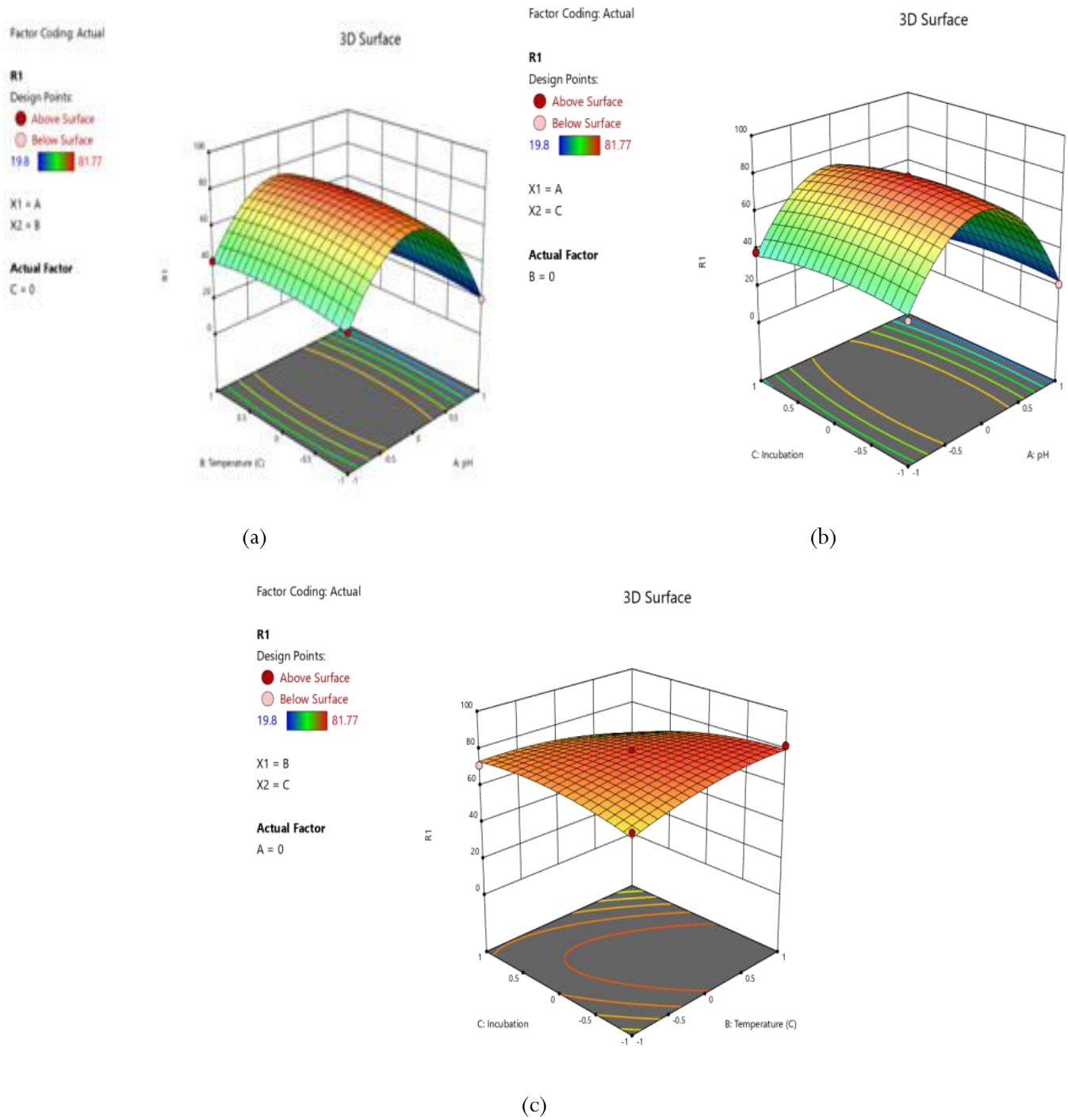

Fig. 2: RSM 3D surface plots presentation the collaborative effects of the medium constituents (a) pH and temperature, (b) pH and Incubation period, (c) Incubation period and Temperature on Lipase production.

The fitted polynominal equation was expressed as 3D surface plots which provided a better visualisation of the relationship between the response and experimental levels of each factor and to determine the optimal conditions. Point optimization method was employed to optimise the level of each variable for the maximum response. The lipase activity obtained from the experiments was very close to the response predicted by the regression model, which proves the validity of the model. RSM design yielded a maximum activity of 81.77µmol. This was obtained in trail no. 5 and the level of the independent variables 7.0 pH, temperature

40°C, and 24 hours of incubation period. Similar optimization with RSM for *Fusarium solani* strain SKWF7 resulted in a maximum lipase production (73.3 U/ml), which increased at 1.7-fold than that obtained in the unoptimized medium [25]. The optimized medium showed the best lipase activity of 2,171 U/mL, which was 16.4% higher than using the initial medium by *Aspergillus niger* [26]. The 3-D response surface corresponding an elliptical contour, reported a significant interaction effect between the two factors and also suggested for well defined optimum operating conditions [27].

3.3. Experimental Validation of the Model

The response surface model predicted a maximum lipase content of 79.06 µmol when the regression equation was solved by the numerical optimization function in the Design Expert software. The optimum levels of significant variables were temperature of 40°C, pH of 7.0 after 24 hrs incubation. To confirm the predicted model, three replicate experiments were performed and the lipase activities of each were determined and finally the average maximum yield reached 81.77µmol. As a result the proposed model was considered to be accurate and reliable for predicting the production of lipase from *P. digitatum*.

4. CONCLUSION

Response surface methodology was effectively performed to optimize the culture conditions for lipase production by *P. digitatum*. Box-Behnken design was employed to evaluate the effects of fermentation period, temperature and pH on production of lipase by *P. digitatum*. Using the above conditions the maximum lipase activity of 81.77 µmol was obtained at a temperature of 40°C, pH of 7.0 and fermentation period of 24h. Based on the present study, it is evident that the use of statistical optimization tools has helped to identify the significant variables and to optimize the factors with minimum number of experiments, effort and time for lipase production.

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