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Enhancement of Alkaline Protease Production from Newly Isolated Strain *Bacillus paramycoides* Using Potato Peel and Mustard Oil Cake as Substrate

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ABSTRACT

Alkaline proteases have received a lot of attention from industrial point of view because of their versatile applications in various industries such as food, detergent, pharmaceuticals, tannery and many others. The aim of the present study was to optimize the fermentation conditions for the production of alkaline protease from a previously isolated strain of *Bacillus* viz., *Bacillus paramycoides* using response surface methodology (RSM). The interaction between most significant factors (pH, temperature and production time) was analyzed using central composite design (CCD). After statistical optimization, there was a significant increase of alkaline protease activity (5.3-fold), compared to basal medium. It was found that the final optimized conditions of pH (9.29), temperature (40.63°C) and production time of 78.96 h showed the maximum alkaline protease production with an activity of 2958.32 U/ml. The results showed the successful implementation of statistical tool for enhancing the production of alkaline protease. Moreover, the high activity of alkaline protease after optimization showed the potential of the isolated micro-organism to be used as an industrial candidate for the production of enzyme.

Key words : Alkaline protease, central composite design, optimization, *Bacillus*, response surface methodology

INTRODUCTION

Proteases are one of the significant groups of industrial enzymes researched most widely (Naveed *et al.*, 2021). Proteases account for one of the third largest group of industrial enzymes of which two-third are produced by micro-organisms commercially (Bhandari *et al.*, 2021). The main role of proteases is to hydrolyze proteins. Of all the proteases, recently alkaline proteases (Subtilisins, E.C. 3.4.21.14) have gained the attention because of their wide applications in various industries, including detergent, food, feed, pharmaceutical, silk, leather, silk recovery from used X-ray films, contact lens cleaning, degumming of silk, isolation of nucleic acid and for preparation of high nutrition and easily digestible protein hydrolysates (Sharma *et al.*, 2019). Alkaline proteases show maximum activity in the neutral to alkaline pH range (pH 7-11).

Alkaline proteases are mainly produced using submerged fermentation. Media components and production conditions greatly influence the production of extracellular proteases from microbes (Wahab and Ahmed, 2018). The traditional method of optimization of media by "one-variable-at a time" is a time consuming and is expensive also when large number of variables need to be optimized. Moreover, the combined interactions between various variables cannot be established by this approach. Response surface methodology (RSM) is a statistical method employed for optimizing parameters, where a response of interest is changed by various variables to optimize the response. This method also helps in understanding interactions between several parameters without increasing the cost and with minimum number of experiments (Mishra and Varjani, 2019). Response surface methodology has been successfully applied for the production of microbial alkaline protease production (Jayakumar *et al.*, 2021; Shafique *et al.*, 2021). Central composite design (CCD) improves the information obtained by one

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variable at a time by minimizing the number of specific experiments required and also provides the information on interaction between various factors (Lakshmi and Hemalatha, 2016; Sreedevi *et al.*, 2017).

In the current work, the utilization of statistical factorial design (CCD) was done to optimize the production parameters of alkaline protease from the previously isolated strain of *Bacillus* viz., *Bacillus paramycoides*. The strain was isolated in our laboratory from the soil of slaughter house in Jalandhar. From the studies of production parameters of alkaline protease using one factor at a time, it was found that pH, temperature and production time have greatly affected the growth of alkaline protease. Therefore, the interactions of these three most affected parameters were found using central composite design (2^3 full factorial central composite designs). To the best of our knowledge, till now no literature is available on statistical optimization of media components and production parameters for *B. paramycoides*. Therefore, there is a potential of commercialization of the strain for the production of alkaline protease.

MATERIALS AND METHODS

In the present study, previously isolated strain of *Bacillus* identified as *B. paramycoides* was utilized for the production of alkaline protease. The strain was isolated from the slaughter house in Jalandhar. The stock culture of the strain was maintained as suspension in 30% glycerol (culture : suspension : 1 : 1) and was stored at -20°C for long term preservation. For routine work, these cultures were grown at 37°C for 24 h and maintained at 4°C and subcultured every month.

Inoculum was prepared by transferring a loopful of colonies from 24 h old slant into 250 ml Erlenmeyer flask containing 50 ml of nutrient broth. The inoculum was then kept at 37°C for 24 h at 180 RPM on a rotary shaker.

For the preparation of substrate, different agro waste viz., wheat bran, orange peel, papaya peel, potato peel, pineapple peel, beet root peel, chickpea husk, toor dal husk, wheat bran, rice bran, mustard oil cake, sunflower oil cake and soybean oil cake were washed with tap water followed by distilled water to remove adhered dust particles. The washed material was dried at 60°C for 6 h, grinded in a mixer and was passed through sieve of mesh size 450 µm. The powdered substrate was stored in desiccator for further analysis.

The alkaline protease was produced in the standard media using 3% potato peel as carbon source and 2% mustard oil cake as nitrogen source. The flasks were kept at 37 °C for 48 h at 120 rpm. At the end of the fermentation, media was centrifuged at 10000 rpm for 15 min, and cell free supernatant was used as a source of crude enzyme.

From the previous studies of effect of various production parameters viz., pH, temperature, inoculum age, inoculum size and production time on alkaline protease using one variable at a time, it was found that pH, temperature and production time greatly affected the production of alkaline protease. pH, temperature, inoculum age, inoculum size and production time were investigated in the range of pH 7-11, temperature 20-60°C, inoculum age 4-24 h, inoculum size 0.5-5% and production time, respectively. Therefore, to study the interaction between these components, the statistical analysis was done using response surface methodology in which central composite design (CCD) was developed using Design Expert version 6.0.10. The quadric design model was used to fit the data. Independent variables chosen were pH (X_1 : 7-11), temperature (X_2 : 20-60°C) and production time (X_3 : 24-120 h). The three significant variables studied at five coded levels ($-\alpha$, -1, 0, 1, α) are shown in Table 1. Actual levels of the variables were chosen based on the preliminary (one factor at a time) studies. The experimental design of CCD gave 20 trials including seven trials for factorial design, seven trials for axial points and six trials for replications of the central points (Table 2).

Comparison was done between predicted value, actual value and predicted error. The response that was checked for different experimental trials was alkaline protease activity (U/ml). The production medium (50 ml in 250 ml Erlenmeyer flask) was prepared and incubated at 37°C in rotary shaker at 120 rpm. The results were analyzed by using ANOVA i.e. analysis of variance suitable for the experimental design used. The P values were used as a tool to check the significance of each of the coefficients, which, in turn were necessary to understand the pattern of the mutual interactions between the test

Table 1. Coded levels for independent variables used in developing experimental data

Factor	Code	Levels				
		-α (1.68)	-1	0	1	α (1.68)
pH	X_1	7.32	8	9	10	10.68
Temperature (°C)	X_2	23.18	30	40	50	56.82
Production time (h)	X_3	31.64	48	72	96	112.36

Table 2. Variables and their levels employed in central composite design

Experiment	pH (X_1)		Temperature (X_2)		Production time (X_3)	
	Coded value	Uncoded value	Coded value	Uncoded value	Coded value	Uncoded value
1	-1	8.00	-1	30.00	-1	48.00
2	1	10.00	-1	30.00	-1	48.00
3	-1	8.00	1	50.00	-1	48.00
4	1	10.00	1	50.00	-1	48.00
5	-1	8.00	-1	30.00	1	96.00
6	1	10.00	-1	30.00	1	96.00
7	-1	8.00	1	50.00	1	96.00
8	1	10.00	1	50.00	1	96.00
9	-1.68	7.32	0	40.00	0	72.00
10	1.68	10.68	0	40.00	0	72.00
11	0	9.00	-1.68	23.18	0	72.00
12	0	9.00	1.68	56.82	0	72.00
13	0	9.00	0	40.00	-1.68	31.64
14	0	9.00	0	40.00	1.68	112.36
15-20	0	9.00	0	40.00	0	72.00

variables. Finally, the validation for the RSM model was also carried out. The productivity profile for alkaline protease production was also determined after validating the RSM model.

The enzyme assay was done using Hammerstein casein as a substrate. For the initiation of reaction, 100 µl of appropriately diluted solution was added to 500 µl Hammerstein casein (0.6% w/v casein prepared in 100 mM tris - HCl buffer, pH 8) and was incubated at 40°C for 15 min. At the end of the reaction, it was terminated by addition of 500 µl of 5% (w/v) trichloroacetic acid and was followed by centrifugation. The optical density of the supernatant was measured at 280 nm. One unit of alkaline protease activity was defined as the amount of enzyme required to liberate one µg of tyrosine/ml under experimental conditions (Mohan *et al.*, 2018).

RESULTS AND DISCUSSION

A perfect model was presented for optimization of production parameters in order to save, time, cost and labour. The optimization of production parameters was done to determine the optimum levels of these parameters and hence to maximize the production of alkaline protease.

At the end of one factor at a time, it was found that media components and physical factors especially pH, temperature and production time showed greater influence on the protease production. So, to examine the combined effect of these factors three factors (independent variables) viz., pH (X_1), temperature (X_2) and production time (X_3) were chosen to examine their effect on an alkaline protease production in RSM, they were further optimized by central composite design (CCD) of RSM. The independent as well as dependent variables were fitted to second order equation and were studied for goodness of fit (Chaudhary *et al.*, 2018). The experimental design and results of RSM for studying the effects of these independent variables are presented in Table 3.

Based on a regression analysis of the data from CCD, Table 4 shows the ANOVA for RSM. The effects of the independent variables on alkaline proteases production were predicted by a second-order polynomial equation for RSM as :

Alkaline protease activity (U/ml) = $2922.39 - 4.13\ X_1 + 242.95\ X_2 + 190.32\ X_3 - 468.74\ X_1^2 -$

Table 3. Effect of fermentation conditions (pH, temperature and production time) on alkaline protease activity (predicted vs. experimental)

Run	pH X_1	Temperature (°C) X_2	Production time (h) X_3	Alkaline protease activity (U/ml) predicted	Alkaline protease activity (U/ml) experimental
1	8.00	30.00	48.00	1208.77	1210.92±4.2[l]
2	10.00	30.00	48.00	1236.56	1230.10±15.3[j, k]
3	8.00	50.00	48.00	1382.67	1368.26±12.2[h]
4	10.00	50.00	48.00	1374.98	1385.17±35.4[g]
5	8.00	30.00	96.00	1285.66	1280.83±9.5[i]
6	10.00	30.00	96.00	1261.46	1265.85±4.5[i, j]
7	8.00	50.00	96.00	2111.37	2115.50±5.6[c]
8	10.00	50.00	96.00	2067.08	2062.60±24.2[d]
9	7.32	40.00	72.00	1603.55	1605.56±2.7[f]
10	10.68	40.00	72.00	1589.66	1590.95±12.6[f]
11	9.00	23.18	72.00	567.19	568.90±10.9[m]
12	9.00	56.82	72.00	1384.39	1385.98±8.4[g, h]
13	9.00	40.00	31.64	1826.30	1830.25±24.6[e]
14	9.00	40.00	112.36	2466.47	2465.83±20.21[c]
15	9.00	40.00	72.00	2922.39	2921.20±32.2[a, b]
16	9.00	40.00	72.00	2922.39	2918.50±27.4[b]
17	9.00	40.00	72.00	2922.39	2921.50±21.3[a, b]
18	9.00	40.00	72.00	2922.39	2925.70±20.23[a, b]
19	9.00	40.00	72.00	2922.39	2925.70±24.6[a, b]
20	9.00	40.00	72.00	2922.39	2921.00±19.3[a, b]

Table 4. Analysis of variance for product response

Response	Source	d. f.	Sum of squares	Mean squares	*F*-value	*P*-value
Alkaline protease activity (U/ml)	Regression	9	1.110E+007	1.234E+006	50312.90	<0.0001
	Lack-of-fit	5	204.58	40.92	5.04	0.0503
	Pure error	5	40.62	8.12		
	Residual	10	245.20	24.52		
	Total	19	1.110E+007			
	R^2 (0.96)					

$688.23\ X_2^{\ 2} - 274.36\ X_3^{\ 2} - 5.02\ X_1X_2 - 13.00\ X_1X_3 + 164.88\ X_2X_3$

The coefficient of determination (R^2) for alkaline protease production was found to be 0.96. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1.00, the stronger the model is and better it predicts the response. When expressed as a percentage, R^2 is interpreted as the per cent variability in the response explained by statistical model. It implied that the sample variation of 96% for alkaline protease production was attributed to the independent variables and only 4% of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The "Predicted R^2" of 0.96 was in reasonable agreement with the "Adjusted R^2" of 1.0000. This indicated a good agreement between the experimental and predicted values for alkaline proteases production. The model F-value for alkaline proteases 50312.90 implied that the model was significant. Values of "Prob > F" less than 0.05 indicated that the model terms were significant. According to present model factors X_1, X_2, X_3, $X_1^{\ 2}$, $X_2^{\ 2}$, $X_3^{\ 2}$, X_1X_2, X_1X_3 and X_2X_3 were significant model terms. The 3D surface plots described by the regression model were drawn to illustrate the effects of the independent variables, and interactive effects of each independent variable on the response variable. The shape of the corresponding contour plots indicated whether the mutual interactions between the independent variables were significant or not. Each contour curve represented an infinite number of combinations of two test variables with the other two maintained at their respective zero levels. Elliptical nature of the contour in 3D response surface graphs (Figs. 1, 2 and 3)

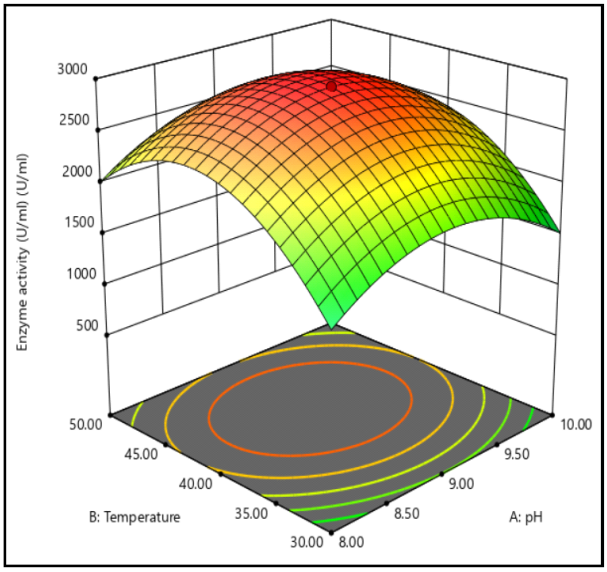

Fig. 1. Surface response plot for alkaline protease production (Effect of pH and temperature when other variables were held at zero level).

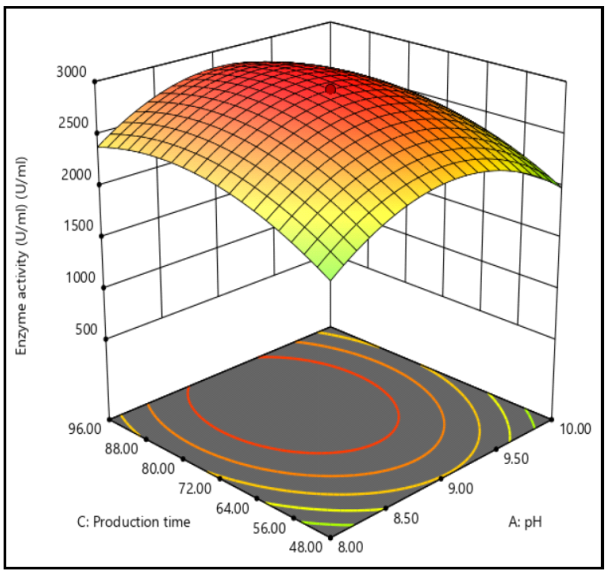

Fig. 2. Surface response plot for alkaline protease production (Effect of pH and production time when other variables were held at zero level).

depicted the mutual interactions of all the variables. There was a relative significant interaction between every two variables, and there was a maximum predicted yield as indicated by the surface confined in the smallest ellipse in the contour diagrams. From the central point of the contour plot or from

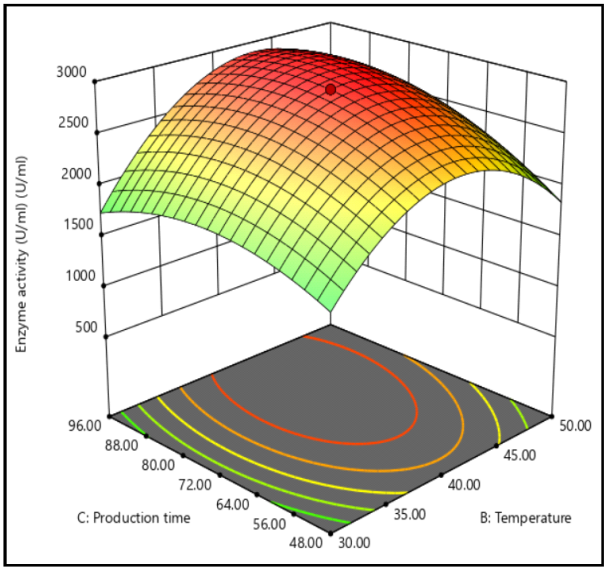

Fig. 3. Surface response plot for alkaline protease production (Effect of temperature and production time when other variables were held at zero level).

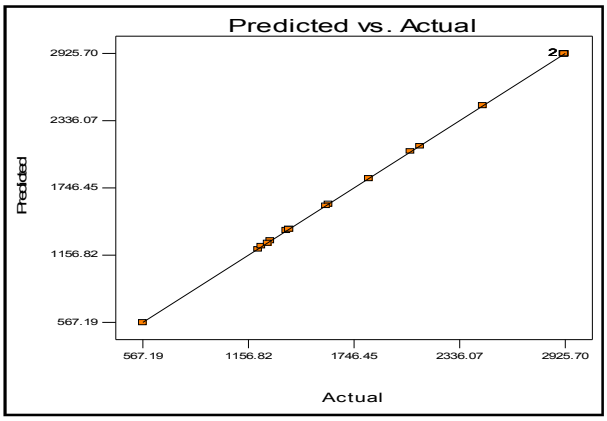

Fig. 4. Parity plot showing actual vs. predicted values.

the bump of the 3D plot the optimal composition of medium components was identified.

In the parity plot, alkaline protease yields were close to diagonal i. e. zero error line (Fig. 4). This implied that the prediction of experimental data was quite satisfactory. Accordingly, three-dimensional graphs were generated for the pair-wise combination of the three factors, while keeping the other two at their center point levels. The model predicted that maximum alkaline protease production of 2927.31 U/ml could be achieved using the

Table 5. Validation model

pH	Temperature (°C)	Production time (h)	Protease activity (U/ml) (Predicted)	Protease activity (U/ml) (Experimental)
9.29	40.63	78.96	2927.31	2958.32±38.2

media having pH 9.29, temperature 40.63 and production time of 78.96 h. The results for model validation of model are given in Table 5.

CONCLUSION

The statistical approach enabled the combination of experiments to explicate the substantial parameters of the production conditions for maximum production of alkaline protease from *Bacillus paramycoides*. The statistical tools enabled hustle free identification of the significant parameters for production of alkaline protease and the interactions between them. Selecting the suitable range of the significant parameters is one of the essential steps in the initial experiments. The ultimate objective of RSM was to determine the optimum conditions which maximized the production of alkaline protease. The central composite design enabled to explore the production conditions in just 20 experimental runs with an overall 1.1-fold increase in protease production in the optimized culture condition over the final optimized media by one variable at a time (2958.32 U/ml : 2752.5 U/ml) and an overall 5.3-fold increase (2958.32 U/ml : 557.2 U/ ml) over the basal medium. The alkaline protease activity predicted by model fitted with the experimental data and hence confirmed the model validity. Because of the high activity of alkaline protease in a semi-defined media, the enzyme is having the potent to be used as industrial enzyme.

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