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ORIGINAL ARTICLE

Enhanced Production of protease by Optimization of media constituents using response surface methodology

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ABSTRACT
Protease is an important industrial enzyme found in the food, chemical, beverage, and pharmaceutical industries. Bacillus licheniformis was used to optimise the growing conditions for optimum protease synthesis using agro industrial waste products such as groundnut shell as substrate in this study. The study used statistical methodology based on experimental designs. A Plackett-Burman design was used to screen twelve nutrients for their influence on protease synthesis. The beneficial effects of $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$, and glycine on protease synthesis led to their selection. Response Surface Methodology was used to optimise the specified components (RSM). The ideal conditions are (percentage weighted average): $MnSO_4.7H_2O$ - 0.03, beef extract - 0.10, $FeSO_4.7H_2O$ - 0.03 and glycine – 0.10. These circumstances were tested in the lab, and the result was a 152.5 U/gds increase in protease production.
Keywords*: protease, groundnut shell, Bacillus licheniformis, Optimization, RSM.*

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INTRODUCTION

Since antiquity, enzymes have been used in ancient fermentation processes. Their existence can be traced back to ancient Greece, where enzymes derived from microorganisms were used in baking, brewing, alcohol production, and cheese production, among other things. Today, better knowledge and advances in analytical techniques have demonstrated that they can perform a wide range of functions techniques as selective protein modification and lysis of fibroin clot etc [1]. Proteases are major industrial enzymes that account for 60% of total global enzyme sales and are one of the three most common types of industrial enzymes [2]. Proteases are naturally occurring enzymes that catalyse hydrolytic processes that break down protein molecules into peptides and amino acids [3].Proteases are found in all living things on the planet, including prokaryotes, fungi, plants, and animals. Proteases are widely used enzymes in a variety of industries, including food, leather, detergents, pharmaceuticals, diagnostics, waste management, and silver recovery [4]. Because of their unique nature of assisted digestion, these enzymes have the potential to contribute to the production of high-value-added goods[5].

Several microbiological strains have been documented, including fungus (*Aspergillus flavus, Aspergillus melleu, Aspergillus niger, Chrysosporium keratinophilum, Fusarium graminarum, Penicillium griseofulvin, Scedosporium apiosermum*) and bacteria (*Bacillus licheniformis, Bacillus firmus*) Proteases have been found in Bacillus alcalophilus, *Bacillus amyloliquefaciens, Bacillus proteolyticus*, *Bacillus licheniformis*, and *Bacillus thuringiensis*) [6]. The Bacillus genus has grown in importance on a large scale among these. Despite this, only a few investigations on proteolytic enzymes from Bacillus sp. have been conducted. Furthermore, research have shown that nutritional elements, such as carbon a source, have an impact on health[7].

In comparison to proteases obtained from plants and animals, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, simplifying downstream processing of the enzyme [8].

Solid - substrate fermentation (SSF) was used for this study because it has previously been demonstrated to have much higher productivity than submerged fermentation Ghildyal *et al*., [9] and Hesseltine, [10].

SSF has a number of advantages in terms of cost, including greater volumetric productivity, the use of smaller machinery, the use of low-cost substrates, and the ease of usage with submerged fermentation [11,12,13].Production of these biocatalysts using agro-biotech substrates under solid-state fermentation conditions offers several benefits in terms of productivity, cost-effectiveness in terms of labour, time, and medium components, as well as environmental benefits such as reduced effluent production and waste minimization [14].

Researchers are constantly researching diverse features of proteases due to their high demand in industry [15], [16], [17]. The purpose of this study was to determine the best medium components for producing proteases under solid state fermentation of Bacillus licheniformis using cassava waste as a substrate and to produce proteases under solid state fermentation of *Bacillus licheniformis* using cassava waste as a substrate.

MATERIAL AND METHODS

Bacterial strain

Bacterial strain used in this work is well preserved in the laboratory. Bacterial strain *Bacillus Licheniformis* was a stock of the Microbial Type Culture collection Centre (MTCC), Chandigarh, India. The strain was maintained on nutrient agar medium at 4◦C. The medium composition (g/L) was compressed off the following: beef extract- 1.0; yeast extract- 2.0; peptone- 5.0; NaCl- 5.0 and agar- 2. Cells were subcultures at monthly intervals.

Solid State Fermentation

Groundnut shell was obtained from a local market in Panruti, Cuddalore, Tamil Nadu, India, and employed as a protease production substrate. Table 1 shows the groundnut shell's chemical composition. Fermentation took place in 250 mL Erlenmeyer flasks containing 10g of groundnut shell and nutrients at concentrations determined by the experimental design. It was sterilized at 121°C for 15 min. After cooling the flasks to room temperature, the flasks were inoculated with 2 ml 24-h grown culture broth under sterile conditions. The contents of the flasks were agitated at 200 rpm and incubated at 33±1ºC for 120 hrs.

During the preliminary screening process, the experiments are carried out for 5 days and it was found that at the 28 hrs, the maximum production occurs. Hence experiments are carried out for 28 hrs.

Extraction of Protease

The enzyme was extracted according to the method described by Nagamine *et al*. [18]. Fermented medium was mixed thoroughly with 50 mM glycine–NaOH buffer, pH - 11 for 30 min and the extract was separated by squeezing through a cloth. This process was repeated three times and extracts were pooled together and then centrifuged at 200rpm The supernatant was used as enzyme source for protease assay.

Table 1.The Composition of Cassava Waste Table

Parameters	%
Cellulose	65.70
Carbohydrate	21.20
Protein	7.30
Mineral	4.50
Crude fibre	-
Moisture	-

Optimization of Protease production

RSM consist of a group of empirical techniques used for evaluation of relationship between cluster of controlled experimental factors and measured response. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach.

To determine which variables significantly affect protease production by *Bacillus licheniformis*, Plackett - Burman design was used. Twelve variables (Table 2) were screened in 20 experimental runs (Table 3) and insignificant ones were eliminated in order to obtain a smaller, manageable set of factors. The low level (-1) and high level (+1) of each factor are listed in (Table 3). The statistical software package 'Minitab 15', was used for analyzing the experimental data.

Table 2. Nutrients screening using a Plackett-Burman design

Variables		Levels	
Nutrient Code	**Components**	**Low Value (-1)**	**High Value (+1)**
A	Peptone	0.03	0.15
B	Glycine	0.03	0.15
C	$FeSO_4.7H_2O$	0.01	0.05
D	Fructose	0.15	0.35
E	$NaNO_3$	0.03	0.15
F	Casein	0.03	0.15
G	Mannose	0.15	0.35
H	Beef extract	0.03	0.15
J	K_2HPO_4	0.01	0.05
K	Lactose	0.15	0.35
L	$MnSO_4.7H_2O$	0.01	0.05
M	NH_4Cl	0.03	0.15

Table 3.Plackett–Burman experimental design matrix for screening of important Variables for protease production

Run Order	A	B	C	D	E	F	G	H	J	K	L	M	Activity (U/gds)
1	1	-1	1	-1	1	1	1	-1	-1	1	1	1	133.10
2	1	1	1	-1	-1	1	-1	1	-1	-1	1	-1	126.40
3	1	-1	-1	-1	-1	1	-1	1	1	1	-1	1	32.50
4	-1	-1	1	1	1	-1	-1	1	-1	-1	1	1	15.82
5	-1	1	-1	1	-1	1	-1	-1	1	1	1	-1	68.90
6	1	1	1	1	-1	-1	1	-1	1	-1	-1	1	28.70
7	-1	1	1	-1	1	-1	1	1	1	1	1	-1	15.22
8	1	1	-1	1	-1	-1	1	1	-1	1	1	-1	121.00
9	1	1	1	-1	1	-1	-1	-1	1	-1	-1	-1	21.40
10	1	1	-1	1	1	1	-1	-1	-1	-1	1	1	51.20
11	-1	-1	1	1	-1	-1	-1	-1	1	1	1	1	137.80
12	-1	-1	1	1	-1	1	1	1	-1	-1	-1	-1	112.00
13	-1	1	1	-1	-1	1	1	-1	-1	1	-1	1	18.60
14	-1	1	-1	-1	1	-1	-1	1	-1	1	-1	1	32.40
15	1	-1	-1	-1	-1	-1	1	1	1	-1	1	1	141.10
16	-1	-1	-1	-1	1	1	1	-1	1	-1	1	-1	100.78
17	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	16.11
18	-1	1	-1	1	1	1	1	1	1	-1	-1	1	74.20
19	1	-1	-1	1	1	-1	1	-1	-1	1	-1	-1	18.20
20	1	-1	1	1	1	1	-1	1	1	1	-1	-1	115.60

Once the critical factor was identified through the screening, the central composite design (CCD) was used to obtain a quadratic model, consisting of factorial trials and star points to estimate quadratic effects and central points to estimate the pure process variability with protease production as response. Response surface methodology (RSM) was employed to optimize the four significant factors viz., $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine which enhances the protease production. The four independent variables were studied at four different levels (Table 4) and a set of 20 experiments were carried out (Table 5). The statistical software package 'Design Expert 7.1.5 was used to analyze the experimental data. All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table 4. Upon the completion of experiments, the average maximum proteases were taken as the response (Y). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second order polynomial equation is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i Z_i + \sum_{i=1}^{k} \beta_{ii} Z_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} Z_i Z_j \quad (1)$$

Where Y is the measured response, β_0 is the intercept term, $\beta_i$are linear coefficients, β_{ii} are quadratic coefficient, β_{ij} are interaction coefficient and Z_i and Z_j are coded independent variables. The optimal

concentrations of the critical variables were obtained by analyzing contour plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

Table 4. Ranges of nutrients used in RSM for protease production by *B. licheniformis* using groundnut shell

Variables		Levels, g/gds				
	Code	-2	-1	0	1	2
$MnSO_4.7H_2O$	x_1	0.01	0.02	0.03	0.04	0.05
Beef extract	x_2	0.03	0.06	0.09	0.12	0.15
$FeSO_4.7H_2O$	x_3	0.01	0.02	0.03	0.04	0.05
Glycine	x_4	0.03	0.06	0.09	0.12	0.15

Table 5.Central composite design (CCD) of factors in coded levels with enzyme activity as response for protease production by *B. licheniformis* using groundnut shell

Run	x_1	x_2	x_3	x_4	Protease Production, U/gds	
					Experimental	Predicted
1	-2	0	0	0	50.00	49.352
2	0	0	-2	0	44.98	43.869
3	0	0	2	0	89.25	88.804
4	0	0	0	0	152.41	153.564
5	0	2	0	0	100.10	99.560
6	1	1	-1	1	77.19	77.921
7	0	0	0	0	152.87	153.564
8	-1	1	-1	-1	57.80	58.724
9	1	-1	1	1	103.00	104.041
10	-1	1	1	1	114.00	115.113
11	1	1	1	1	140.00	140.206
12	-1	1	-1	1	77.75	77.579
13	1	-1	-1	1	66.50	66.770
14	-1	-1	1	1	63.78	63.205
15	1	1	-1	-1	60.78	60.947
16	0	-2	0	0	58.34	57.323
17	0	0	0	-2	65.00	64.782
18	0	0	0	2	111.00	109.661
19	0	0	0	0	152.89	153.564
20	2	0	0	0	93.32	92.411
21	-1	-1	1	-1	30.00	31.235
22	0	0	0	0	150.10	149.499
23	1	1	1	-1	88.95	89.296
24	1	-1	-1	-1	65.70	66.552
25	-1	-1	-1	1	45.00	46.620
26	-1	-1	-1	-1	49.20	48.586
27	1	-1	1	-1	74.19	73.953
28	-1	1	1	-1	63.00	62.322
29	0	0	0	0	150.80	149.499
30	0	0	0	0	150.12	149.499

Z_1-$MnSO_4.7H_2O$ Z_2-Beef extract Z_3-$FeSO_4.7H_2O$ Z_4 - Glycine

Assay of enzyme activities

Protease activity was determined using modified Auson–Hagihara method [19]. In this 1 ml of the enzyme solution was added to 1 ml casein solution (1%, w/v casein solution prepared in 50 mM glycine–NaOH buffer, pH 11) and incubated at 70ºC for 20 min. The reaction was terminated by adding 4 ml of 10% trichloroacetic acid and the contents were filtered through a Whatman No. 1 filter paper. The filtrate absorbance was read at 280 nm using UV–Visible spectrophotometer and the protease activity was calculated using tyrosine standard curve. One unit of alkaline protease activity was defined as 1 µg/ml tyrosine liberated per min under the assay conditions.

Table 6.Analysis of Variance (ANOVA) for response surface quadratic model for the production of protease by *B. licheniformis* using groundnut shell

Source	Coefficient factor	Sum of squares	DF	F	P
Model	151.53	44004.35	14	590.327	< 0.0001
x_1	10.934	2869.344	1	538.9	< 0.0001
x_2	11.06	2939.749	1	552.12	< 0.0001
x_3	11.064	2937.979	1	551.790	< 0.0001
x_4	12.06	3494.507	1	656.313	< 0.0001
x_1*x_1	-20.459	11480.75	1	2156.23	< 0.0001
x_2*x_2	-18.569	9457.542	1	1776.24	< 0.0001
x_3*x_3	-21.5952	12791.4	1	2402.39	< 0.0001
x_4*x_4	-16.374	7353.779	1	1381.135	< 0.0001
x_1*x_2	-4.19	280.8976	1	52.756	< 0.0001
x_1*x_3	5.93	563.3502	1	105.80	< 0.0001
x_1*x_4	-0.21	0.748225	1	0.1405	0.7130
x_2*x_3	5.49	482.4612	1	90.612	< 0.0001
x_2*x_4	4.95	392.238	1	73.667	< 0.0001
x_3*x_4	8.23	1083.726	1	203.53	< 0.0001
Residual		79.86669	15		
Lack of fit		70.88121	10		
Pure Error		8.985483	5		
Total		44084.22	29		

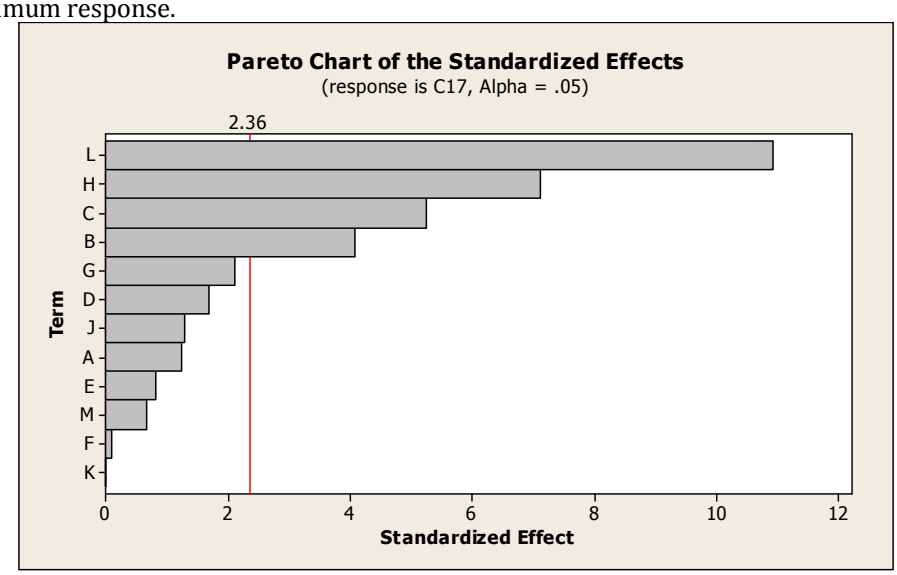
Std. Dev -2.31; R^2 - 99.82%; Mean -89.93; Adj R^2 - 99.65%; C.V. % - 2.57; Pred R^2-99.04%; Adeq Precision -73.00.

Validation of the experimental model

The statistical model was validated with respect to protease production under the conditions predicted by the model in shake-flasks level. Samples were drawn at the desired intervals and protease activity was determined as described above

RESULTS AND DISCUSSION

Plackett–Burman experiments (Table 3) showed a wide variation in protease activity. This variation reflected the importance of optimization to attain higher productivity. From the Pareto chart (Fig.1) the variables viz., $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine were selected for further optimization to attain a maximum response.



Where, A - peptone, B - glycine, C – $FeSO_4.7H_2O$, D - fructose, E – NaNO3, F -casein, G - mannose, H - beef extract, J - K_2HPO_4, K -lactose, L -$MnSO_4.7H_2O$, M - NH_4Cl

Figure1. Pareto chart for screening of nutrients for the production of Protease by *B. licheniformis* using groundnut shell

The levels of factors ($MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine) and the effect of their interactions on protease production were determined by central composite design of RSM. Twenty experiments were performed at different combinations of the factors shown in Table 4. The predicted and

observed responses along with design matrix are presented in Table 5 and the results were analyzed by ANOVA. The second-order regression equation provided the levels of protease activity as the function of $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine, which can be presented in terms of coded factors as in the following equation (2):

$$Y = 151.53 + 10.93x_1 + 11.07x_2 + 11.06x_3 + 12.07x_4 - 20.46x_1^2 - 18.57x_2^2 - 21.60x_3^2 - 16.37x_4^2 - 4.19x_1x_2 + 5.93x_1x_3 - 0.22x_1x_4 + 5.49x_2x_3 + 4.95x_2x_4 + 8.23x_3x_4 \quad (2)$$

Where Y is the protease activity (U/gds), Z_1, Z_2,Z_3 and $Z_4$are $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine respectively. ANOVA for the response surface is shown in Table 6. The Model F-value of 590.33implies 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 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant. In the present work, linear effects of x_1, x_2, x_3 and x_4, interactive effects of x_1 x_2, x_1x_3, x_2x_3 x_2x_4 and x_3x_4, square effects of x_1, x_2, x_3 and x_4 are significant for protease production. The coefficient of determination (R^2) for protease activity was found to be 0.9982, which was very close to 1 and can explain up to 99.82% variability of the response. The predicted R^2 value of 0.9904 was in reasonable agreement with the adjusted R^2 value of 0.9965. An adequate precision value greater than 4 was desirable. The adequate precision value of 73.00 indicates an adequate signal and suggests that the model can be used to navigate the design space. The above model can be used to predict the protease production within the limits of the experimental factors. Figure 2 shows that the actual response values agree well with the predicted response values.

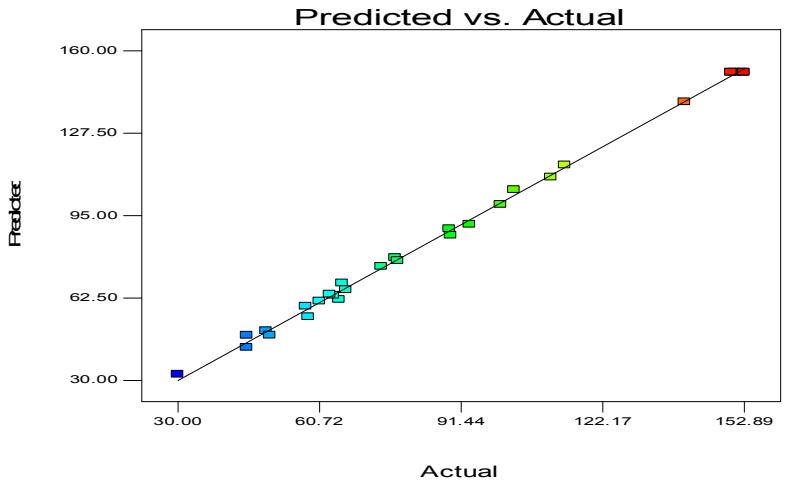


Figure2. Predicted response versus actual value of protease production by *B. licheniformis* using groundnut shell

The interaction effects of variables on protease production were studied by plotting 3D surface curves against any two independent variables, while keeping another variable at its central (0) level. The 3D curves of the calculated response (protease production) and contour plots from the interactions between the variables are shown in Figs. 3-8.

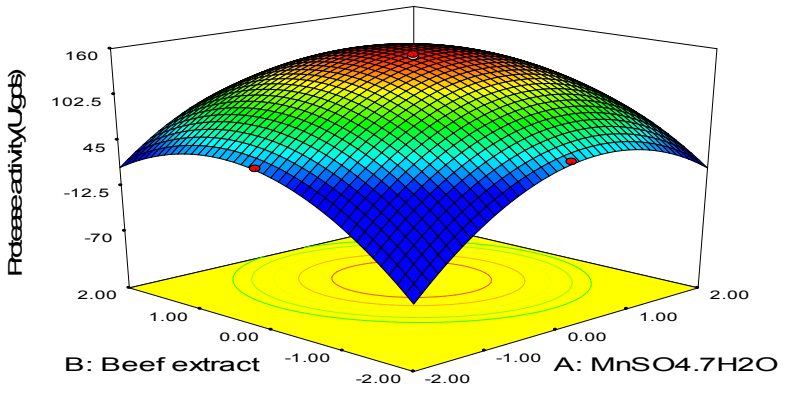


Figure 3.3D Plot showing the effect of Beef extract and $MnSO_4.7H_2O$ on protease activity.

Figure 3 shows the dependency of protease on $MgSO_4.7H_2O$, and Beef extract. The protease activity increased with increase in $MgSO_4.7H_2O$ to about 1.4 (% w/w) and thereafter protease activity decreased with further increase in $MgSO_4.7H_2O$.

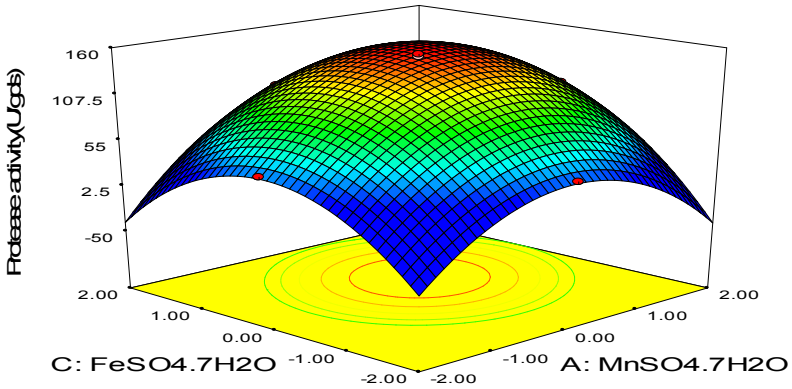


Figure 4.3D Plot showing the effect of $FeSO_4.7H_2O$ and $MnSO_4.7H_2O$ on protease activity.

The same trend was observed in figs. 4 and 5. Increase in $MnSO_4.7H_2O$ resulted increase in protease activity upto 11.4 (% w/w).This is evident from Figs.3, 4. Figure 4, 5 shows the dependency of protease activity on $MnSO_4.7H_2O$,a similar type of trend was observed for heavy metal removalusing *Phanerochaete Chrysosporium* biosorbent [20].

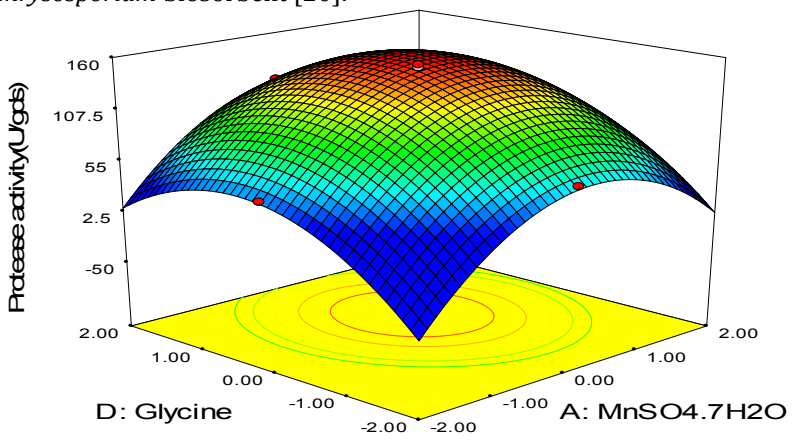


Figure 5.3D Plot showing the effect of Glysine and $MnSO_4.7H_2O$ on protease activity.

The optimal operation conditions of $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine for maximum protease activity were determined by response surface analysis and also estimated by regression equation. The predicted results are shown in Table 5. The predicted values from the regression equation closely agreed with that obtained from experimental values.

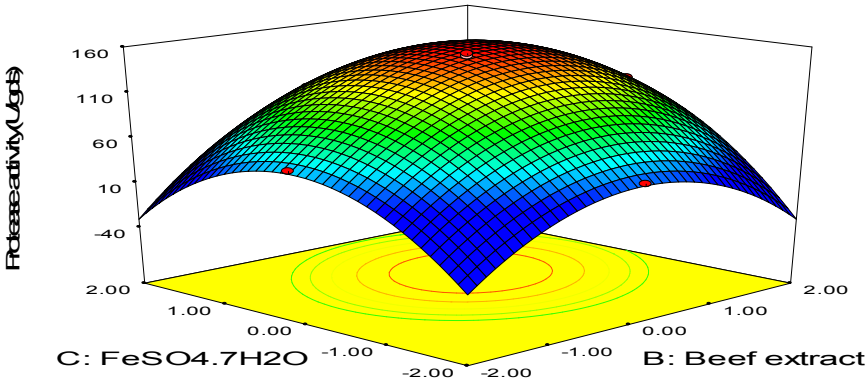


Figure 6. 3D Plot showing the effect of $FeSO_4.7H_2O$ and Beef extract on protease activity.

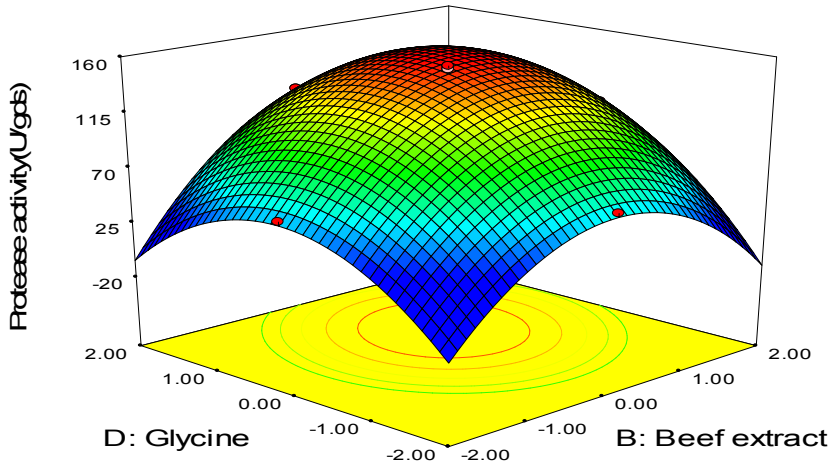


Figure 7. 3D Plot showing the effect of Glycine and Beef extract on protease activity.

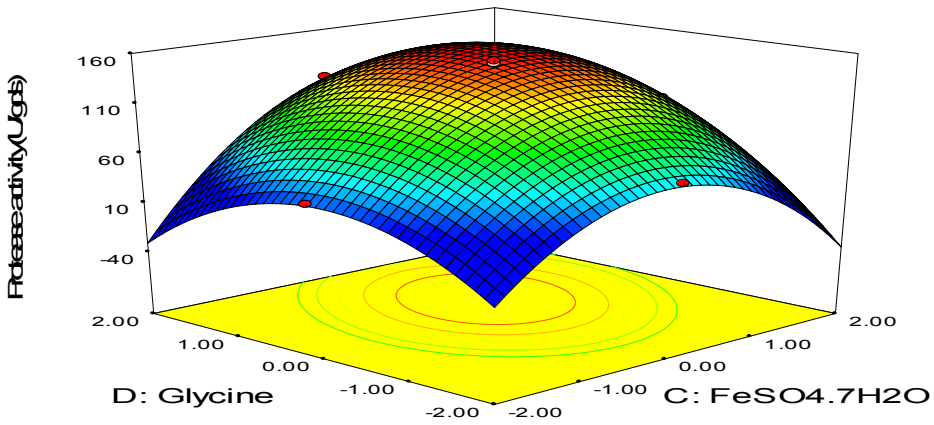


Figure 8.3D Plot showing the effect of Glycine and $FeSO_4.7H_2O$ on protease activity.

Validation of the experimental model

Validation of the experimental model was tested by carrying out the batch experiment under optimal operation condition are (% w/w):$MnSO_4.7H_2O$ – 0.03, beef extract – 0.10, $FeSO_4.7H_2O$ – 0.03 and glycine – 0.10 established by the regression model. Three repeated experiments were performed and the results are compared. The protease activity (152.87U/gds) obtained from experiments was very close to the actual response (153.564 U/gds) predicted by the regression model, which proved the validity of the model.

CONCLUSION

In this work, Plackett Burman design was used to test the relative importance of medium components on protease production. Among the variables, $MnSO_4.7H_2O$, beef extract, $FeSO_4.7H_2O$ and glycine were found to be the most significant variables. From further optimization studies the optimized values of the variables for protease production were as follows (% w/w):$MnSO_4.7H_2O$ – 0.03, beef extract – 0.10, $FeSO_4.7H_2O$ – 0.03 and glycine – 0.10.This study showed that the groundnut shell constitutes a good substrate for the production of protease. Using the optimized conditions, the produced activity reaches 152.5 U/gds. The results show a close concordance between the expected and obtained activity level.

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