International Journal of
Science Inventions Today
www.ijsit.com
ISSN 2319-5436
Research Article

Optimizing factors affecting protease production by a Bacillus cereus using groundnut shell under solid substrate fermentation

P. RATHAKRISHNAN, P. NAGARAJAN

Department of Chemical Engineering, Annamalai University, Annamalainagar-608002, Tamilnadu, India.

ABSTRACT

The physical factors affecting the production of protease from *bacillus cereus* was investigated. Agro industrial waste product groundnut shells were used as substrates in solid-state fermentation for protease enzyme production. The response surface methodology (RSM) was used to optimize protease production by implementing the central-composite design (CCD). The physiological fermentation factors such as pH (8.0), temperature (43°C), fermentation time (26 hrs), inoculum level (3.2 ml) and substrate concentration (9.6g) were optimized by statistical analysis using response surface methodology. The maximum yield of protease production was 76.75U/gds. This was evidenced by the higher value of coefficient of determination (R^2= 0.9994).

Keywords: RSM, groundnut shells, protease, CCD, *bacillus cereus,* physical factors optimization.

INTRODUCTION

Proteases (EC 3.4) form an important class of commercially important enzymes and find applications in detergent, leather, food and pharmaceutical industries, constituting approximately
40% of the total enzyme market [1].

In recent years, there has been an increasing trend towards efficient utilization and value-addition of agro-industrial residues such as coffee pulp and husk, cassava husk, cassava bagasse, sugarcane bagasse, sugar beet pulp, apple pomace, declassified potatoes, etc. [2–7].

Bacillus strains are known to produce and secrete large quantities of extracellular enzymes and constitute a major group of industrial enzyme producers due to the robust nature of the organism as well as its enzymes [8]. Bacterial systems are being increasingly investigated for the production of enzymes and metabolites by SSF.

Solid-state fermentation (SSF) has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must
Possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms. [9]

Statistical approaches offer ideal ways for process optimization studies in biotechnology [10, 11]. Response surface methodology (RSM) is now being routinely used for optimization studies in several biotechnological and industrial processes [12-14].Here, we report the use of RSM to study the effects various physicochemical factors on protease production from *B. cereus*

As the demand for proteases increases, it is expected that hyperactive strains will emerge and that the enzymes produced by new strains could be used as catalysts in different industries. In the present work, the strain employed was a strain of *Bacillus* sp. producing alkaline protease in SSF using agro-residue groundnut shell substrate. In this paper, we report on factors that influence the maximization of alkaline protease production by *Bacillus cereus* through SSF.

MATTERIALS AND METHODS

Microorganism and inoculums' preparation:

Bacterial strain used in this work is well preserved in the laboratory. Bacterial strain *Bacillus cereus* 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 comprised off the following: Beef extract 1.0; Yeast extract 2.0; Peptone 5.0; NaCl 5.0 and Agar 2.0. Cells were subcultered at monthly intervals.

Solid-state fermentation:

Groundnut shell was collected from local market in Panruti, Tamilnadu, India. The shells were washed thoroughly with tap water and then dried. The dried materials obtained were milled and sieved to powder for using as a carbon source for protease production. Fermentation was carried out in Erlenmeyer flasks (250 ml) with 10g of

Groundnut shell powder, supplemented with nutrients concentrations defined by the experimental design NaNO3 0.0386g/gds, Tryptone 0.0338g/gds, K2HPO4 0.2373g/gds and Malt extract 0.2090g/gds. Each flask was covered with hydrophobic cotton and autoclaved 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 well mixed 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. (2003) [15]. 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. The supernatant was used as enzyme source for protease assay.

Optimization of process parameters:

A full factorial design, which includes all possible factor combinations in each of the factors, is a powerful tool for understanding complex processes for describing factor interactions in multifactor systems. RSM is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations simultaneously. The experiments with different pH, temperature, fermentation time, innoculum size and substrate conc. were employed, simultaneously covering the spectrum of variables for the production of protease in the central composite design. In order to describe the effects of pH, temperature, fermentation time, inoculums size and substrate conc. on the protease production, batch experiments were conducted. The coded values of the process parameters were determined by the following equation.

$$x_i = \frac{X_i - X_0}{\Delta x} \qquad (1)$$

Where xi-coded value of the ith variable, Xi-uncoded value of the ith test variable and X0-uncoded value of the ith test variable at center point.

The range and levels of individual variables are given in Table 2. The experimental design is given in Table 3, along with experimental data and predicted responses. The regression analysis was performed to estimate the response function as a second order polynomial.

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

Where Y is the predicted response, βi, βj, βij are coefficients estimated from regression. They represent the linear, quadratic and cross products of X_1, X_2, and X3 on response

Variable	Code	Levels				
		-2.38	-1	0	+1	+2.38
pH	A	6	7	8	9	10
Temperature (^{o}C)	B	30	35	40	45	50
Fermentation Time (hrs)	C	8	16	24	32	40
Inoculums size (ml)	D	1	2	3	4	5
substrate concentration (g)	E	3	6	9	12	15

Table 2: Levels of different process variables in coded and un-coded form for protease production independent variable range and levels

Run. No	A-pH	B-Temperature	C-Fermentation time	D-Inoculums Size	E-substrate concentration	Protease activity(u/gds)	
						Experimental	Theoretical
1	0.00000	0.00000	0.00000	0.00000	0.00000	73.00	73.0603
2	2.37841	0.00000	0.00000	0.00000	0.00000	62.70	62.6081
3	1.00000	1.00000	-1.00000	-1.00000	1.00000	53.50	53.2997
4	-1.00000	-1.00000	1.00000	1.00000	-1.00000	50.00	49.9518
5	-2.37841	0.00000	0.00000	0.00000	0.00000	56.00	55.8666
6	0.00000	0.00000	-2.37841	0.00000	0.00000	60.00	59.8516
7	-1.00000	1.00000	-1.00000	-1.00000	1.00000	49.20	49.6277
8	1.00000	-1.00000	-1.00000	-1.00000	1.00000	62.30	62.2990
9	1.00000	1.00000	1.00000	-1.00000	1.00000	61.00	61.2235
10	1.00000	1.00000	1.00000	1.00000	1.00000	68.50	68.7547
11	1.00000	1.00000	-1.00000	-1.00000	-1.00000	53.00	53.1257
12	-1.00000	1.00000	-1.00000	1.00000	-1.00000	62.00	61.7850

13	-1.00000	1.00000	-1.00000	1.00000	1.00000	58.00	58.0715
14	-1.00000	1.00000	1.00000	1.00000	-1.00000	63.40	63.7275
15	1.00000	1.00000	1.00000	1.00000	-1.00000	60.00	59.9807
16	-1.00000	-1.00000	-1.00000	1.00000	1.00000	50.00	50.2833
17	0.00000	0.00000	0.00000	0.00000	0.00000	73.21	73.0603
18	0.00000	0.00000	0.00000	0.00000	2.37841	56.00	55.6909
19	0.00000	0.00000	0.00000	0.00000	0.00000	73.00	73.0603
20	-1.00000	-1.00000	-1.00000	-1.00000	-1.00000	61.10	61.1468
21	1.00000	-1.00000	-1.00000	1.00000	-1.00000	60.00	60.0563
22	0.00000	0.00000	0.00000	0.00000	0.00000	73.00	73.0603
23	1.00000	-1.00000	1.00000	1.00000	1.00000	58.90	58.7541
24	-1.00000	-1.00000	1.00000	1.00000	1.00000	50.00	50.2884
25	0.00000	0.00000	0.00000	0.00000	0.00000	73.00	73.0603
26	0.00000	0.00000	2.37841	0.00000	0.00000	62.20	62.1231
27	0.00000	0.00000	0.00000	0.00000	0.00000	73.00	73.0603
28	1.00000	1.00000	-1.00000	1.00000	-1.00000	60.00	60.1320
29	1.00000	-1.00000	1.00000	-1.00000	-1.00000	58.00	58.0425
30	1.00000	-1.00000	1.00000	1.00000	-1.00000	52.00	51.9488
31	-1.00000	-1.00000	1.00000	-1.00000	-1.00000	57.40	57.1893
32	0.00000	0.00000	0.00000	0.00000	0.00000	73.21	73.0603
33	-1.00000	-1.00000	-1.00000	-1.00000	1.00000	53.00	52.8833
34	-1.00000	-1.00000	1.00000	-1.00000	1.00000	55.00	54.9446

35	0.00000	0.00000	0.00000	0.00000	0.00000	73.21	73.0603
36	1.00000	1.00000	1.00000	-1.00000	-1.00000	55.35	55.0307
37	1.00000	-1.00000	1.00000	-1.00000	1.00000	62.00	62.2665
38	1.00000	-1.00000	-1.00000	-1.00000	-1.00000	64.00	64.0937
39	-1.00000	1.00000	1.00000	1.00000	1.00000	66.30	66.0328
40	0.00000	-2.37841	0.00000	0.00000	0.00000	59.00	59.0473
41	-1.00000	-1.00000	-1.00000	1.00000	-1.00000	56.00	55.9656
42	-1.00000	1.00000	1.00000	-1.00000	-1.00000	59.60	59.9212
43	-1.00000	1.00000	1.00000	-1.00000	1.00000	59.70	59.6453
44	0.00000	0.00000	0.00000	-2.37841	0.00000	42.00	41.9400
45	0.00000	0.00000	0.00000	2.37841	0.00000	44.90	44.7347
46	1.00000	1.00000	-1.00000	1.00000	1.00000	62.60	62.8872
47	0.00000	2.37841	0.00000	0.00000	0.00000	65.00	64.7274
48	-1.00000	1.00000	-1.00000	-1.00000	-1.00000	56.00	55.9225
49	0.00000	0.00000	0.00000	0.00000	-2.37841	55.00	55.0838
50	1.00000	-1.00000	-1.00000	1.00000	1.00000	61.00	60.8428

Table 3: Experimental conditions and observed response values of 2^5 Central Composite Design

A statistical program package Design Expert 7.1.5, was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation. The equations were validated by the statistical tests called the ANOVA analysis. The significance of each term in the Equation is to estimate the goodness of fit in each case. Response surfaces were drawn to determine the individual and interactive effects of the test variable on the protease production. The optimal values of the test variables were first obtained in coded units and then converted to the uncoded units.

Protease assay:

Protease activity was determined using modified Auson–Hagihara method [16]. 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 of tyrosine liberated ml^{-1} under the assay conditions.

RESULT AND DICUSSION

To examine the combined effect of five different process parameters (independent variables), on the protease production, a central composite design of 2^5 = 32 plus 8 centre points and (2x5 = 10) star points leading to a total of 50 experiments were performed. Equation (3) represents the mathematical model relating the protease production and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert 7.1.5.The coded values of the independent variables are given in Table 2. The experimental and predicted values of protease production are also given in table 3.

The results were analyzed by using ANOVA i.e., analysis of variance suitable for the experimental design used and cited in Table 4. The ANOVA of the quadratic regression model indicates the model to be significant. The Model F-value of 2602.26 implied the model to be significant. Model F-value was calculated as a ratio of mean square regression and mean square residual. Model P value (Prob>F) is very low [0.0500]. This reiterates that the model is significant. The P values are used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The F value and the corresponding P values, along with the coefficient estimate are given in Table 4. The smaller the magnitude of the P, the more significant is the corresponding coefficient. Values of P less than 0.0500 indicates the model terms to be significant. The coefficient estimates and the corresponding P values along with the coefficient estimate are given in table 4.The coefficients estimate and the corresponding P values suggests that, among the test variables used in the study, A, B, C, D, E, AB, AC, AD, AE, BC, BD, BE, CD, CE, DE, A^2, B^2, C^2, D^2, E^2 are significant [where A-pH, B-temperature, C-fermentation time, D-inoculums size and E-substrate conc.] model terms. Values greater than 0.1000 indicate the model terms are not significant.

Source	Coefficient factor	Sum of square	DF	Mean square	F	P value P>F
Model	73.06	2891.74	20	144.59	2602.26	< 0.0001
A	1.42	87.00	1	87.00	1565.76	< 0.0001
B	1.19	61.76	1	61.76	1111.53	<0.0100
C	0.48	9.88	1	9.88	177.75	< 0.0001
D	0.59	14.95	1	14.95	269.08	<0.0001
E	0.13	0.71	1	0.71	12.70	0.0013
A*A	-2.44	331.80	1	331.80	5971.73	<0.0001
B*B	-1.98	216.78	1	216.78	3901.51	<0.0001
C*C	-2.13	253.11	1	253.11	4555.38	<0.0001
D*D	-5.25	1534.14	1	1534.14	2761.18	<0.0001
E*E	-3.12	542.37	1	542.37	9761.52	< 0.0001
A*B	-1.44	65.98	1	65.98	1187.52	<0.0001
A*C	-0.52	8.77	1	8.74	157.80	<0.0001
A*D	0.29	2.62	1	2.62	47.09	<0.0001
A*E	1.62	83.69	1	83.69	1506.23	<0.0001
B*C	1.99	126.60	1	126.60	2278.60	<0.0001
B*D	2.76	243.93	1	243.93	4390.20	< 0.0001
B*E	0.49	7.75	1	7.75	139.52	< 0.0001
C*D	-0.51	8.46	1	8.46	152.20	<0.0001
C*E	1.50	72.45	1	72.45	1303.96	<0.0001
D*E	0.65	13.33	1	13.33	239.83	< 0.0001
Residua		1.61	29	0.056		
Lack of fit		1.53	22	0.069	5.88	0.0112
Pure Error		0.083	7	0.012		
Cor Total		2893.36	49			

Table 4: Analysis of Variance (ANOVA) for Response Surface Quadratic Model

Std. Dev. 0.24; R^2 = 99.94%; R^2(pred) 99.80%; R^2(adj) 99.91%; C.V. % 0.39

Protease activity = +73.06+1.42* A+1.19 * B+0.48 * C+0.59 * D+0.13* E -1.44 * A * B-5.2 * A * C+0.29 * A * D+1.62 * A * E+1.99 * B * C+2.76 * B * D +0.49 * B * E-0.52 * C * D+1.50 * C * E+0.65 * D * E -2.44 *A^2 -1.98 * B^2-2.13 * C^2-5.25 * D^2-3.12 * E^2

The predicted R^2 of 0.9980 is in reasonable agreement with the adjusted R^2 of 0.9991. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The fit of the model was also expressed by the coefficient of regression R^2, which was found to be 0.9994 indicating from properly that 99.94% the variability in the response could be explained by the model. This implies that the prediction of experimental data is quite satisfactory. The Coefficient of Variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of the CV is, the lower the reliability of the experiment. Here a lower value of CV (0.39) indicates greater reliability of the experiments performed. The Response surface estimation for protease production as discussed in the previous section, the response surface methodology was used with five process variables to evaluate their effect on the protease production. The response Eq. (3) was obtained for the protease production. To investigate the interactive effect of two factors on the protease production, the response surface methodology was used and three-dimensional plot was drawn. The inferences so obtained are discussed below. The interaction effects and optimal levels of the variables were determined by plotting the response surface curves.

The 3D response surface curves are shown in Figs. 1 to 10. Figure 1 represents the interactive effect of pH and temperature on protease production. From Fig. 1 it was inferred that with the increase in pH, the protease production increases with the temperature. The optimum value of both the factors, viz, pH and temperature can be analyzed by saddle point or by checking the maxima formed by the X and Y coordinates. The combined effect of pH and fermentation time on protease production shown in Fig 2.

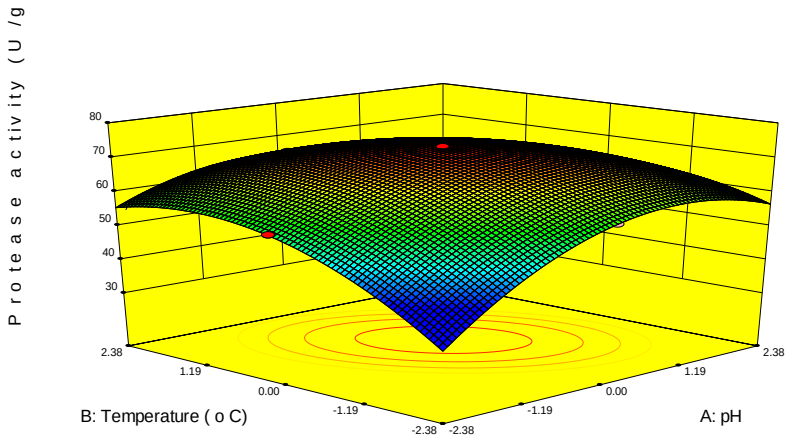

Figure 1: Response surface Plot for protease production from groundnut shell by Bacillus *cereuss* in solid state fermentation as a function of pH and Temperature

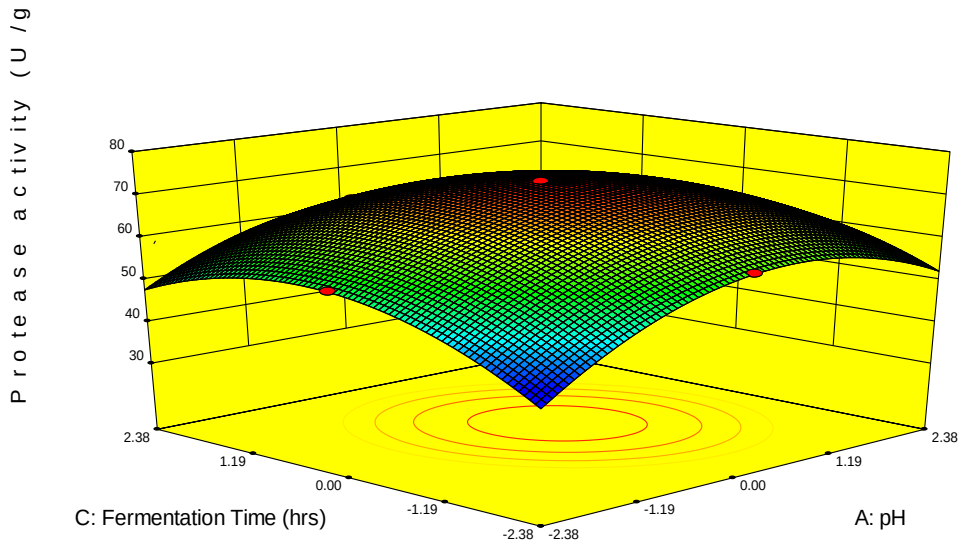

Figure 2: Response surface Plot for protease production from groundnut shell by Bacillus *cereus* in solid state fermentation as a function of pH and fermentation time

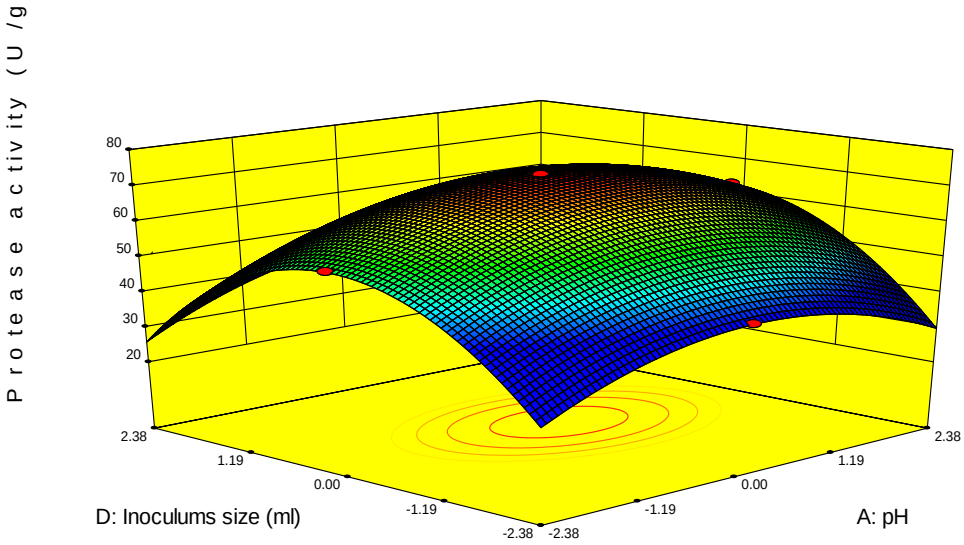

Figure 3: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of pH and inoculums size

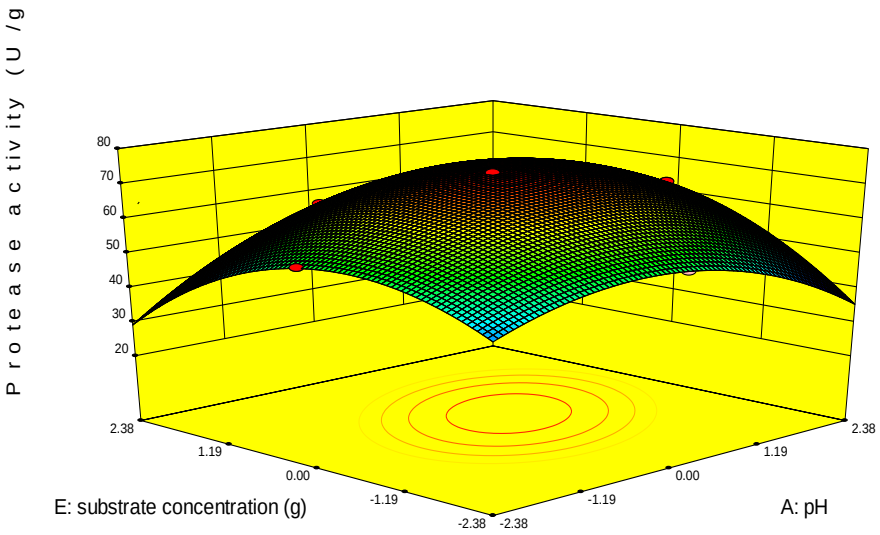

Figure 4: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of pH and substrate concentration

Figure 3 depicts the interaction of pH and Inoculums size, Figure 4 shows the effect of pH and substrate concentration on the protease production. The shape of the contour show good interaction between the pH and substrate concentration, which is clearly illustrated in Fig. 4. The combined effect of temperature and fermentation time was shown in the form of 3D plot in Fig. 5 Combined effect of temperature and innoculum size has been analyzed from the CCD three-dimensional plot shown in Fig. 6.

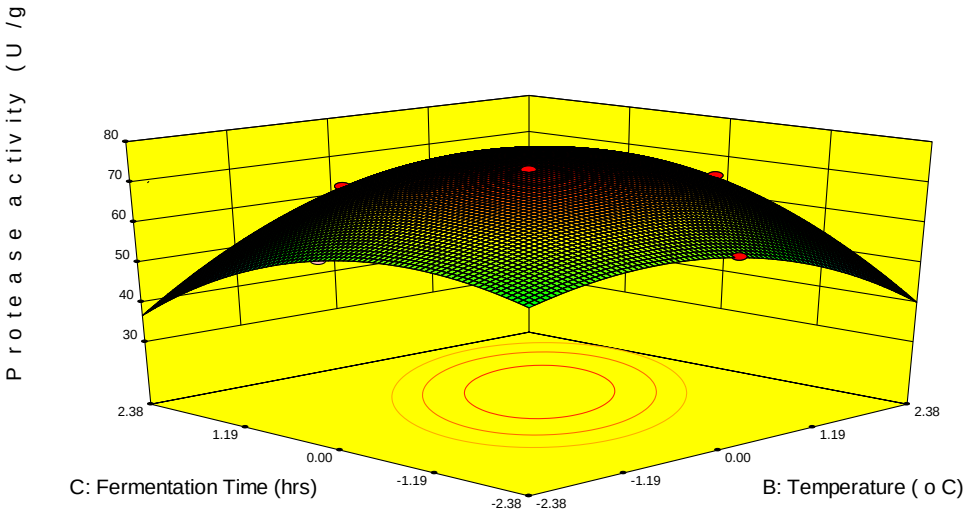

Figure 5: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of temperature and fermentation time

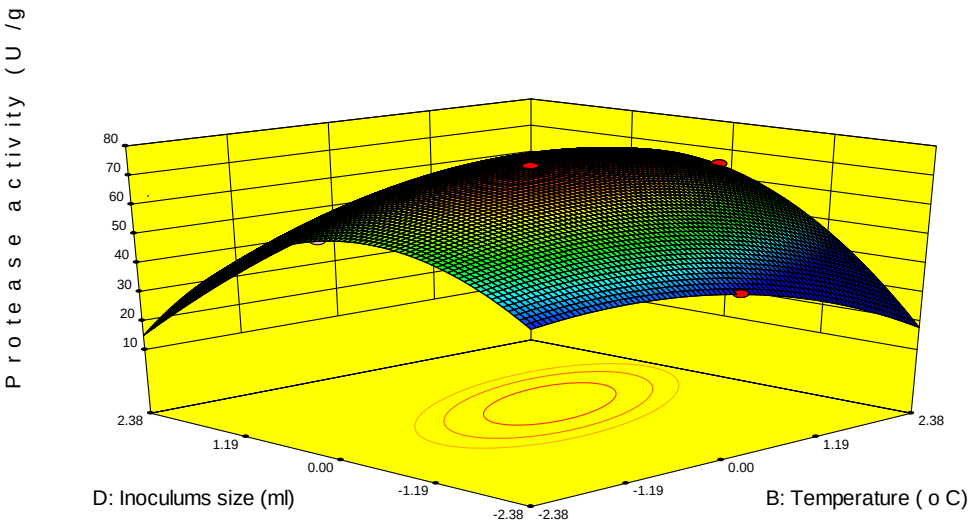

Figure 6: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of temperature and inoculum size

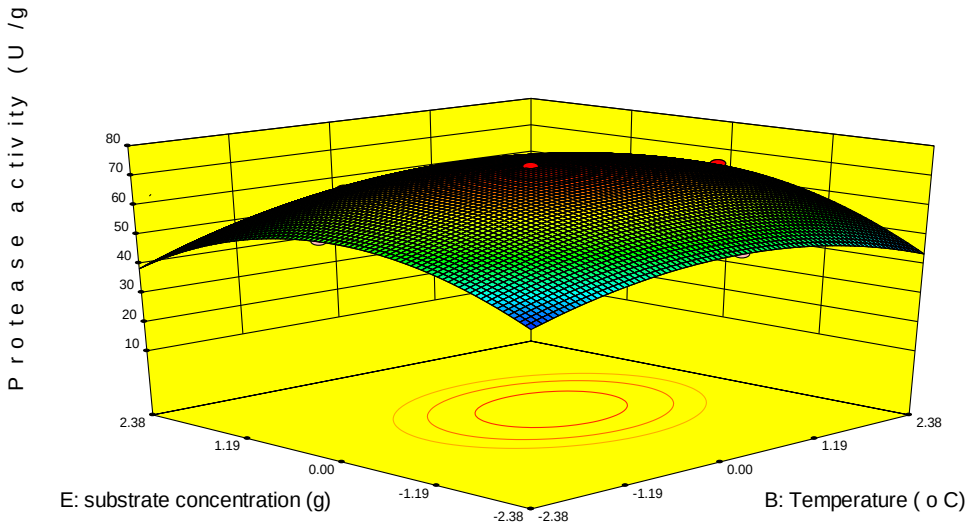

Figure 7: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of temperature and substrate concentration

Figure 7 shows the effect of temperature and substrate concentration on the protease production. Figure 8 shows the response surface curves of protease production as a function of inoculums size and fermentation time. Figure 9 shows the response surface curves of protease production as a function of substrate concentration and fermentation time. Substrate concentration and fermentation time are the most important environmental parameters influencing the protease production. Figure 10 depicts the interaction of inoculums size and substrate concentration, where the maximum protease production of 61 U/gds was found to occur with inoculums size of 3ml and substrate concentration of 8.5 g.

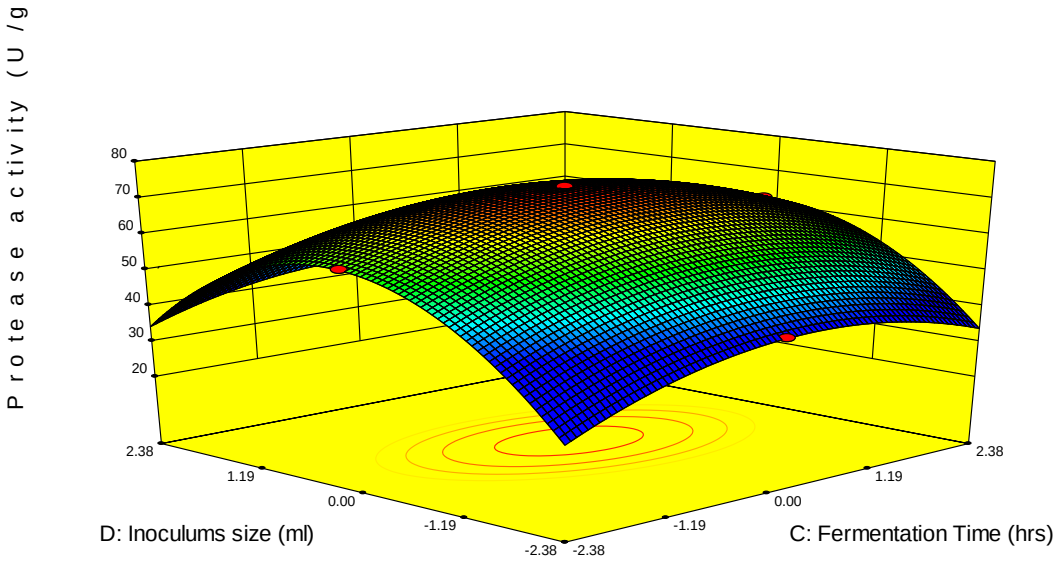

Figure 8: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of fermentation time and inoculum size

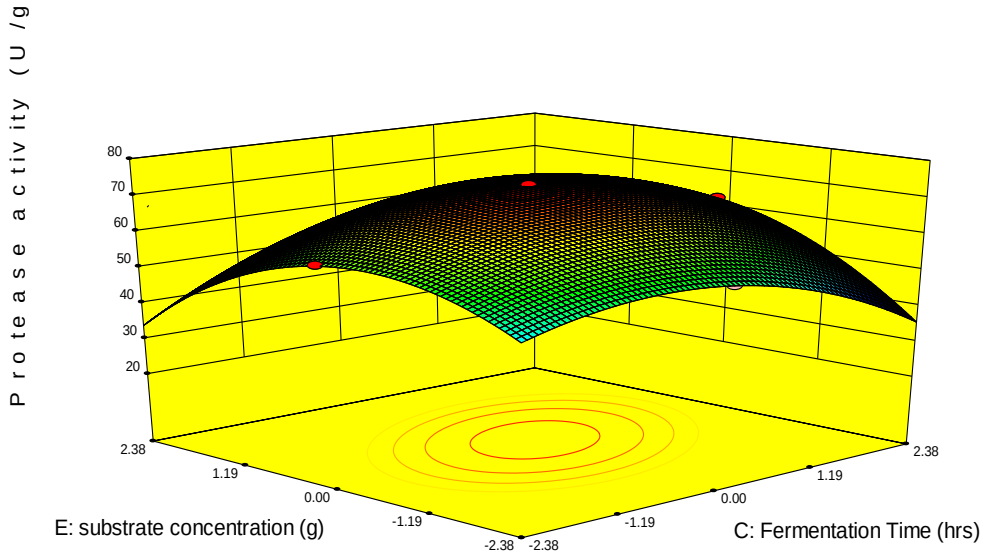

Figure 9: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of fermentation time and substrate concentration

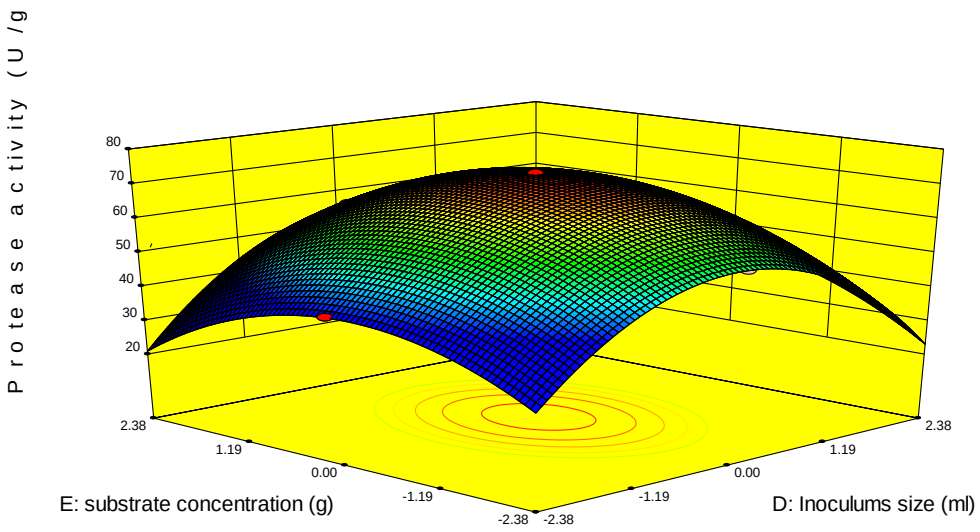

Figure 10: Response surface Plot for protease production from groundnut shell by *Bacillus cereus* in solid state fermentation as a function of inoculum size and substrate concentration

The response surfaces of mutual interactions between the variables were found to be elliptical for most cases. The stationary point or central point is the point at which the slope of the contour is zero in all directions. The coordinates of the central point within the highest contour levels in each of these figures will correspond to the optimum values of the respective constituents. The optimum values drawn from these figures are in close agreement with those obtained by optimizing the regression model Eq. (3). The sequential quadratic programming in MATLAB 7 is used to solve the second-degree polynomial regression Eq. (3). The optimum values for maximum protease production were: pH (8.0), temperature (43°C), fermentation time (26 hrs), inoculum level (3.2 ml) and substrate concentration (9.6g). The optimal values for the variables as predicted by MATLAB were found to be within the design region. This shows that the model correctly explains the influence of the chosen variables on the protease production.

Validation of the experimental model:

Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions pH (8.0), temperature (43°C), fermentation time (26 hrs), inoculum level (3.2 ml) and substrate concentration (9.6g) established by the regression model. Three repeated experiments were performed and the results are compared. The protease activity (73.00U/gds) obtained from experiments was close to the actual response (73.06U/gds) predicted by the regression model, which proved the validity of the model.

CONCLUSION

The feasibility of using an Agro-residue (groundnut shell) as possible substrate for the protease production was studied using the response surface methodological approach. The optimum conditions for the maximum protease production 76.75 U/gds using cassava waste are as follows: pH 8, temperature 43°C, fermentation time 26hrs, inoculums size 3.2ml and substrate concentration 9.6g. The enzyme production in this range from this vastly available by-product is significant.

REFERENCES

1. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 1998; 62:597–635.
2. A. Pandey, C.R. Soccol, Bioconversion of biomass: a case study of ligno-cellulosics bioconversions in solid-state fermentation, Braz. Arch. Biol. Technol. 1998; 41: 379–390.
3. A. Pandey, C.R. Soccol, P. Nigam, V.T. Soccol, Biotechnological potential of agro-industrial residues. I. Sugarcane bagasse, Biores. Technol. 2000; 74: 69–80.
4. A. Pandey, C.R. Soccol, P. Nigam, V.T. Soccol, L.P.S. Vandenberghe, R. Mohan, Biotechnological potential of agro-industrial residues. II. Cassava bagasse, Biores. Technol. 2000; 74: 81– 87.
5. A. Pandey, P. Selvakumar, C.R. Soccol, P. Nigam, Solid-state fermentation for the production of industrial enzymes, Curr. Sci. 1999; 77: 149–162.
6. A. Pandey, C.R. Soccol, Economic utilization of crop residues for the value addition: a futuristic approach, J. Sci. Ind. Res. 2000; 59: 12–22.
7. A. Pandey, C.R. Soccol, D. Mitchell, New developments in solid-state fermentation. I. Bioprocesses and products, Process Biochem. 2000; 35: 1153–1169.
8. Lin X, Lee CG, Casale ES, Shih JCH. Purification and characterization of a keratinase from a feather-degrading Bacillus licheniformis strain. Appl Environ Microbiol 1992;58: 3271–5.
9. A. Pandey, Solid-state fermentation, Biochem. Eng. J. 2003 ; 13:81–84.
10. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002; 59:15- 32.
11. Haaland PD. Statistical problem solving. In: Haaland PD, editor. Experimental Design in Biotechnology. New York: Marcel Dekker, Inc, 1989;1- 18.
12. Beg QK, Saxena RK, Gupta R. Kinetic constants determination for an alkaline protease from Bacillus mojavensis using response surface methodology. Biotechnol Bioeng 2002;78:289- 95.
13. De Coninck J, Bouquelet S, Dumortier V, Duyme F, Denantes VI. Industrial media and fermentation process for improved growth and protease production by Tetrahymena thermophila BIII. J Ind Microbiol Biotechnol 2000; 24:285- 90.
14. Puri S, Beg QK, Gupta R. Optimization of alkaline protease production from Bacillus sp. using

response surface methodology. Curr Microbiol 2002; 44:286- 90.

15. Nagamine K., Murashima K., Kato T., Shimoi H., Ito K., Mode of alpha-amylase production by the Shochu Koji Mold Aspergillu skawachii, *Biosci. Biotechnol. and Biochem.* 2003; 67: 2194–2202.
16. Hagihara B., Matsubara H., Nakai M., Okunuki K., Crystalline bacterial proteinase of *Bacillus subtilis*, *The Journal of Biochem.* 1958; 45: 185–194.