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Optimization of protease production by *Bacillus licheniformis* in Sugarcane bagasse using statistical experimental design

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Sugarcane bagasse, the residue obtained after extracting the sugar juice from sugarcane was tested for the production of protease under solid-state fermentation (SSF) using *Bacillus licheniformis.* The fermentation variables were selected in accordance with Plackett-Burman design and were further optimized via response surface methodological approach. Four significant variables (K2HPO4, Beef extract, NaNO3 and Glycine) were selected for the optimization studies. The optimum values for the selected variables were; K2HPO4 -0.3464g/gds, Beef extract- 0.1039g/gds, NaNO3- 0.0334g/gds and Glycine-0.1027g/gds.A second-order model equation was suggested and then validated experimentally.The model adequacy was very satisfactory as the coefficient of determination was 0.95.The maximum protease production was 146.28U/gds. Results from the study are promising for the economic utilisation of these agro residues, which are abundantly available in many countries.

Keywords: protease; sugarcane bagasse; *Bacillus licheniformis*; Optimization; RSM

The enzymes that hydrolyze peptide bonds are commonly called proteases. They have been classified into two types, the peptidases and proteases. Peptidases hydrolyse peptide bonds within the protein chain, previously called endopeptidases while proteases hydrolyze large polypeptides into smaller molecules. Proteolytic enzymes also regulate various metabolic processes such as blood coagulation, fibrinolysis (Imshenstskii et al., 1986), complement activation, phagocytosis and blood pressure control. Proteases constitute one of the commercially important groups of extra-cellular microbial enzymes and are widely used in several industrial sectors, particularly in the detergent, food, pharmaceutical, chemical, leather and silk, apart from waste treatment (Scheuer et al., 1990). A survey of literature has shown that a large proportion of commercially available proteases are derived from Bacillus strains (Gupta et al., 2002, Thangam et al., 2000). Proteases have been produced in submerged (SmF) and solid-state fermentations (SSF) (Sandhya et al. 2005). In the SSF process, microbes are grown on a porous solid substrate generally in the absence of free water. The water and nutrient absorbed on the substrate support the growth of cells. The growth and secretion of product occurs both on the surface of the solid support and within the support matrix (Pandey et al., 2001).

Media components were found to have great influence on extracellular protease

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production and are different for each microorganism. Therefore, the required constituents and their concentrations have to be optimized accordingly (Beg et al., 2003). A number of optimization techniques could be used for this purpose. Statistical approaches offer ideal ways for process optimization studies in biotechnology (Singh et al., 2004, Haaland et al., 1989, Krishna Prasad et al., 2005). Time consuming, requirement of more experimental data sets (Gupta et al., 2002, Ahuja et al., 2004) and missing the interactions among parameters are the obstacles in predicting the accurate results when the conventional optimization procedures like 'one-factor at a time' were applied (Bandaru et al., 2006). On the contrary, statistical procedures have advantages basically due to utilization of fundamental principles of statistics, randomization, replication and duplication (Sreenivas Rao et al., 2004).

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses (Myers et al., 2002).This method has been successfully applied in many areas of biotechnology such as enzyme production (Bocchini et al., 2002) With respect to protease production, it was utilized for example for Bacillus species (Puri et al., 2002, Beg et al., 2003). Extracellular protease production by microorganisms is greatly influenced by media composition (Varela et al., 1996).

At present, the overall cost of enzyme production is very high (due to high cost of substrates and mediums used) and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view. Furthermore, proteases produced by using commercial medium possess undesirable flavor, which are unsuitable for applications in food processing and pharmaceutical industries. Since the industrial use of proteases, particularly the alkaline proteases are expected to grow tremendously in the coming decade; therefore microbial proteases producing industries are always in search of new and cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme To achieve these goals, during the recent years, efforts have been directed to explore the means to reduce the protease production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate(s) for protease production (Sandhya et al., 2005, Prakasham et al., 2006).

In this study, protease production using agro-residue sugarcane bagasse from *Bacillus licheniformis* as a result of the interaction between four variables, such as K2HPO4, Beef extract, NaNO3 and Glycine which had played a significant role in enhancing the production of protease, was optimized with response surface methodology.

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 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

Sugarcane bagasse was collected from E.I.D PARRY (INDIA) Ltd in Nellikuppam -

Tamilnadu, India and washed thoroughly with tap water and then dried. The dried material were milled and sieved to powder then sterilized at 121°C, 15 lbs pressure for 15 min and stored at 4°C before further use. Fermentation was carried out in Erlenmeyer flasks (250 ml) with 10g of Sugarcane bagasse, supplemented with nutrients concentrations defined by the experimental design. 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±1°C 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). 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 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 1) were screened in 20 experimental runs (Table 2) 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 1. Nutrient screening using a Plackett-Burman design

Variables		Levels (g/g dry substrate)	
Nutrient Code	Nutrient	Low (-1)	High (+1)
A	Peptone	0.03	0.15
B	Glycine	0.03	0.15
C	$FeSO_4 .7H_2O$	0.004	0.02
D	NaCl	0.03	0.15
E	$NaNO_3$	0.01	0.05
F	Casein	0.01	0.05
G	Mannose	0.03	0.15
H	Beef extract	0.03	0.15
J	$K_2 HPO_4$	0.1	0.5
K	$MgSO_4 .7H_2O$	0.1	0.5
L	$MnSO_4.7H_2O$	0.1	0.5
M	NH_4Cl	0.03	0.15

Once the critical factor were 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., K_2HPO_4, Beef extract, $NaNO_3$ and Glycine which enhances the protease production. The four independent variables were studied at five different levels (Table 3) and a set of 30 experiments were carried out (Table 4). 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 3. 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:

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

Where Y is the measured response, $\beta 0$ is the intercept term, βi are linear coefficients, βii are quadratic coefficient, βij are interaction coefficient and Zi and Zj 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 2. Plackett-Burman experimental design matrix for screening of important variables

Run Order	A	B	C	D	E	F	G	H	J	K	L	M	Protease activity U/gds
1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	143.64
2	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	134.21
3	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	43.40
4	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	168.29
5	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	78.34
6	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	38.34
7	-1	1	1	1	1	-1	-1	1	1	-1	1	1	162.00
8	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	130.00
9	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	28.00
10	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	60.32
11	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	146.00
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	24.00
14	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	41.20
15	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	150.21
16	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	110.21
17	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	16.00
18	1	1	1	1	-1	-1	1	1	-1	1	1	-1	82.27
19	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	24.00
20	1	-1	1	-1	1	1	1	1	-1	-1	1	1	125.40

Table 3. Ranges of the independent variables used in RSM

Variables	Code	Levels (g/g dry substrate)				
		-2	-1	0	+1	+2
K2HPO4	*X1*	0.1	0.2	0.3	0.4	0.5
Beef extract	*X2*	0.03	0.06	0.09	0.12	0.15
NaNO3	*X3*	0.01	0.02	0.03	0.04	0.05
Glycine	*X4*	0.03	0.06	0.09	0.12	0.15

Assay of enzyme activities

Protease activity was determined using modified Auson–Hagihara method (Hagihara et al., 1958). 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.

Table 4. Central composite design (CCD) of factors in coded levels with enzyme activity as response

Run Order	X1	X2	X3	X4	Protease activity (U/gds)	
					Experimental	Predicted
1	1	1	1	1	110.00	115.348
2	-1	1	-1	1	63.00	64.455
3	-1	-1	1	1	58.00	58.771
4	1	-1	-1	1	28.00	29.420
5	-1	1	1	-1	42.00	44.168
6	-1	-1	-1	-1	28.00	26.240
7	1	-1	1	-1	56.00	58.133
8	1	1	-1	-1	70.00	72.816
9	0	0	0	0	130.00	122.825
10	0	0	0	0	130.00	122.825
11	0	0	-2	0	50.00	46.201
12	-2	0	0	0	48.00	52.923
13	2	0	0	0	100.20	93.965
14	0	0	0	0	130.60	132.925
15	0	2	0	0	90.11	90.655
16	0	-2	0	0	30.40	28.543
17	0	0	0	-2	55.00	58.300
18	0	0	0	2	96.23	91.618
19	0	0	0	0	130.00	132.925
20	0	0	2	0	85.46	87.946
21	1	-1	-1	-1	38.40	41.529
22	0	0	0	0	130.40	137.250
23	1	1	1	-1	100.60	95.457
24	-1	-1	1	-1	62.60	57.656
25	-1	-1	-1	1	36.80	39.668
26	-1	1	1	1	95.00	89.596
27	0	0	0	0	135.00	137.250
28	-1	1	-1	-1	68.10	64.414
29	1	-1	1	1	90.00	91.411
30	1	1	-1	1	102.35	105.019

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 2) 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., K2HPO4, Beef extract, NaNO3 and Glycine were selected for further otimization to attain a maximum response.

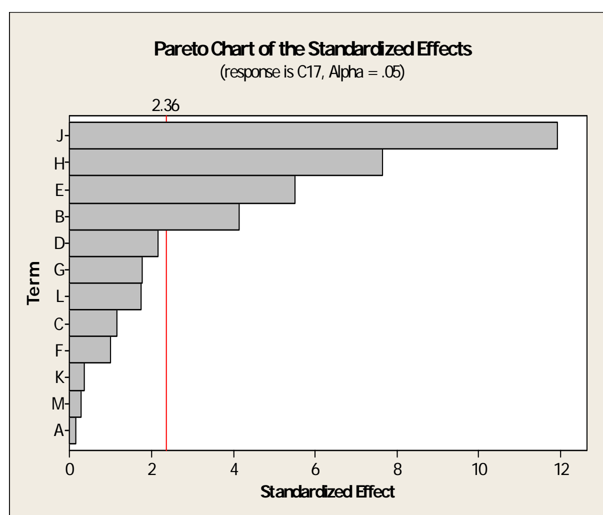

Fig 1: Pareto chart showing the effect of media components on protease activity (J- K2HPO4, H- Beef extract, E- NaNO3 and B- Glycine)

The levels of factors (K2HPO4, Beef extract, NaNO3 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 3. The predicted and observed responses along with design matrix are presented in Table 4 and the results were analyzed by ANOVA. The second-order regression equation provided the levels of protease activity as the function of K2HPO4, Beef extract, NaNO3 and Glycine, which can

be presented in terms of coded factors as in the following equation:

$$Y = 131.00 + 10.26X1 + 15.53\ X2 + 10.44X3 + 8.33X4 + 5.49X1X2 + 3.51X1X3 + 0.83X1X4 - 5.70X2X3 + 3.87X2X4 + 4.13X3X4 - 14.87\ Z1^2 - 18.33\ Z2^2 - 16.46\ Z3^2 - 14.49\ Z4^2 \quad \ldots\ldots(2)$$

Where Y is the protease activity (U/gds), Z1, Z2, Z3 and Z4 are K2HPO4, Beef extract, NaNO3 and Glycine respectively. ANOVA for the response surface is shown in Table 5. The Model F-value of 23.62 implies 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, all the linear, interactive effects of Z1Z2,Z2Z3 and all the square effects of Z1, Z2 Z3and Z4 were significant for Protease production. The coefficient of determination (R^2) for protease activity was calculated as 0.9566, which is very close to 1 and can explain up to 95.66% variability of the response. The predicted R^2 value of 0.7524 was in reasonable agreement with the adjusted R^2 value of 0.9161. An adequate precision value greater than 4 is desirable. The adequate precision value of 14.480 indicates an adequate signal and suggests that the model can be used to navigate the design space.

Table 5. Analysis of Variance (ANOVA) for Response Surface Quadratic Model

Source	Coefficient factor	Sum of square	DF	F	P value P>F
Model	131.00	34367.10	14	23.62	< 0.0001
A	-10.26	2526.63	1	24.31	0.0002
B	-15.53	5786.79	1	55.68	<0.0001
C	-10.44	2613.97	1	25.15	0.0002
D	-8.33	1665.17	1	16.02	0.0012
A*A	-14.87	6065.18	1	58.36	< 0.0001
B*B	18.33	9217.27	1	88.69	<0.0001
C*C	16.46	7433.81	1	71.53	<0.0001
D*D	14.49	5760.15	1	55.42	<,0.0001
A*B	5.49	482.35	1		0.0479
A*C	3.51	197.05	1	4.64	0.1887
A*D	0.83	10.97	1	1.90	0.7497
B*C	-5.70	520.41	1	0.11	0.0408
B*D	3.87	239.09	1	5.01	0.1501
C*D	4.13	273.49	1	2.30	0.1256
Residual		1558.94	15	2.63	
Lack of fit		1539.42	10	39.43	0.0004
Pure Error		19.52	5		
Cor Total		35926.04	29		

Std. Dev. 10.19; R^2 = 95.66%; R^2(pred) 75.24%; R^2(adj) 91.61%; C.V. % 12.80

The above model can be used to predict the protease production within the limits of the experimental factors. Figure2 shows that the actual response values agree well with the predicted response values.

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.

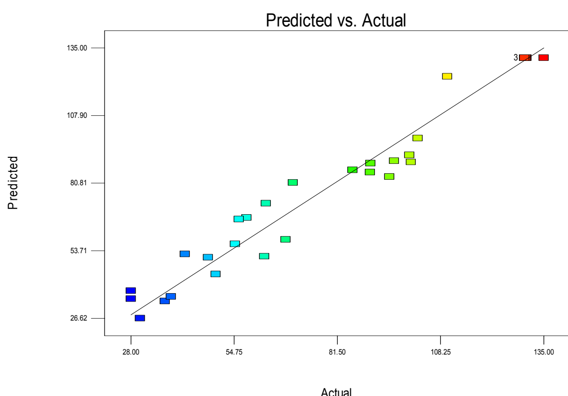

Fig 2: Predicted response versus actual value

Figure 3 shows the dependency of protease on K2HPO4 and Beef extract. The protease activity increased with increase in K2HPO4 and Beef extract up to 0.3464 and 0.1039g/gds respectively, and thereafter protease activity decreased with further increase in K2HPO4 and Beef extract. The same trend was observed in figures. 4&5. Increase in NaNO3 results increase in protease activity up to 0.0334g/gds. This is evident from Figures 4, 7 and 8. Figure 5, 6 and 8 shows the dependency of protease activity on Glycine. The effect of Glycine on protease activity was similar to other variables. The optimal operation conditions of K2HPO4, Beef extract, NaNO3 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 4. The predicted values from the regression equation closely agreed with that obtained from experimental values.

Validation of the experimental model

Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions (K2HPO4 -0.3464g/gds, Beef extract- 0.1039g/gds, NaNO3- 0.0334g/gds and Glycine- 0.1027g/gds) established by the regression model. Three repeated experiments were performed and the results are compared. The protease activity (135.00U/gds) obtained from experiments was very close to the actual response (137.250 U/gds) predicted by the regression model, which proved the validity of the model.

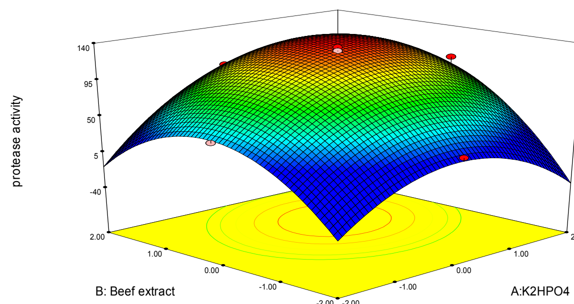

Fig 3: 3D Plot showing the effect of K2HPO4 and Beef extract on protease activity

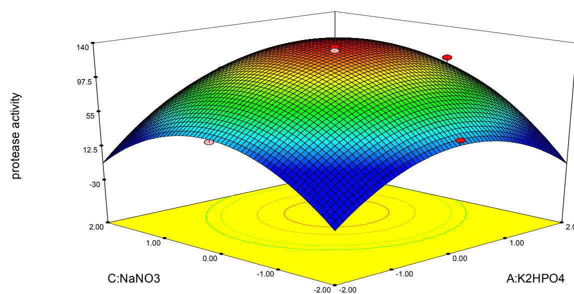

Fig 4: 3D Plot showing the effect of K2HPO4and NaNO3 on protease activity

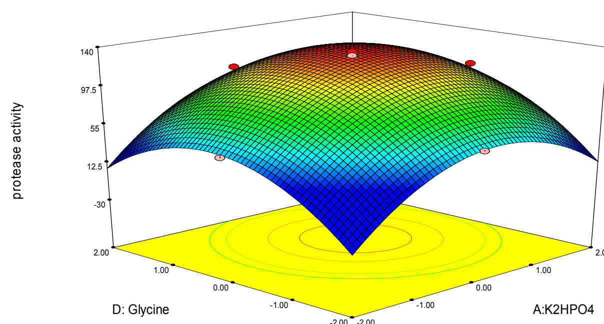

Fig 5: 3D Plot showing the effect of K2HPO4 and Glycine on protease activity

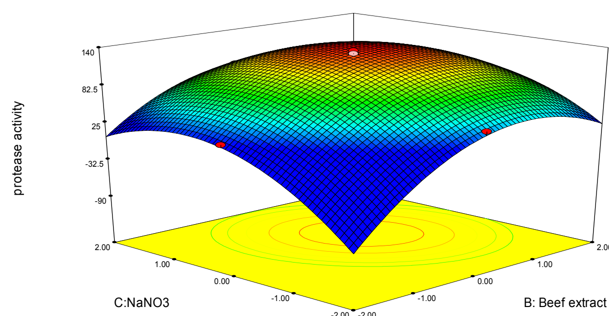

Fig 6: 3D Plot showing the effect of Beef extract and NaNO3 on protease activity

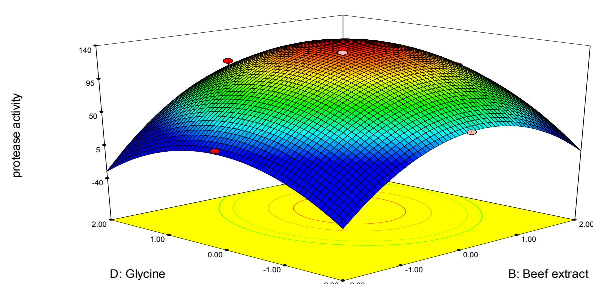

Fig 7: 3D Plot showing the effect of Beef extract and Glycine on protease activity

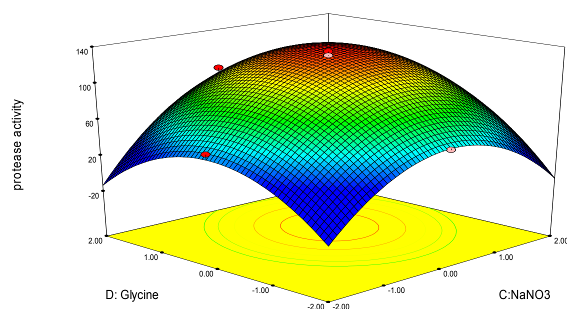

Fig 8: 3D Plot showing the effect of NaNO3 and Glycine on protease activity

Conclusion

B. licheniformis used in the present study could utilize sugarcane bagasse as low-cost residue for protease production under SSF condition. In this study Plackett-Burman design was employed to optimize the medium for protease production. The final composition of the optimized medium was as follows: K2HPO4 -0.3464g/gds, Beef extract-0.1039g/gds, NaNO3- 0.0334g/gds and Glycine- 0.1027g/gds. This study showed that the sugarcane bagasse constitutes a source for the production of protease. Using the optimized conditions, the produced activity reaches 146.28U/gds U/gds. The results show a close concordance between the expected and obtained activity level.

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References

Ahuja, S.K., Ferreira, G.M. and Moreira, A.R. 2004. Application of Plackett–Burman design and response surface methodology to achieve exponential growth for aggregated shipworm bacterium. *Biotechnol Bioeng*., **85**: 666–675.

Beg, Q.K., Sahai, V. and Gupta, R. 2003. Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. *Process Biochem*., **39**: 203–209.

Bandaru,V.V.R., Somalanka, S.R., Menduc, D.R., Madicherla, N.R. and Chityala, A. 2006. Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas mobilis* using response surface methodology. *Enzyme Microb Technol*.. **38**:209–214.

Bocchini, D.A., Alves-Prado, H.F., Baida, L.C., Roberto. I.C., Gomes, E. and Da Silva, R. 2002. Optimization of xylanase production by Bacillus circulans D1in submerged fermentation using response

surface methodology. *Process Biochem.,* **38** :727–31.

Gupta, R., Beg, Q.K., Khan, S. and Chahuan, B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol.,* **60**: 381–95.

Gupta, R., Beg, Q.K. and Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microb Biotechnol*., **59**: 15–32.

Haaland, P.D. 1989. Statistical problem solving, in: P.D. Haaland (Ed.), Experimental Design in Biotechnology. Marcel Dekker., New York. 1–18.

Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, K. 1958. Crystalline bacterial proteinase. I. Preparation of crystalline proteinase of *Bacillus subtilis. J Biochem.,* **45** :185–194.

Imshenstskii, A.A., Kasatkina, I.D., Cherkesova, G.V. and Lebedeva, I.M. 1986. The fibrinolytic activity of *Bacillus mesenteroides* strains. *Microbiologiya.,* **55**: 217–22.

Krishna Prasad, K., Venkata Mohan, S., Sreenivas Rao, R., Ranjan Pati, B. and Sarma, P.N. 2005. Laccase production by *Pleurotus ostreatus* 1804: optimization of submerged culture conditions by Taguchi DOE methodology. *Biochem Eng J.,* **24**:17–26.

Myers, R.H. and Montgomery, D.C. 2002. Response surface methodology: process and product optimization using designed experiments. New York: Wiley.

Nagamine, K., Murashima, K., Kato, T., Shimoi, H. and Ito, K. 2003. Mode of alpha-amylase production by the Shochu Koji Mold *Aspergillu skawachii*. *Biosci Biotechno. Biochem.,* **67**: 2194–2202.

Pandey, A., Soccol, C.R., Leo, G.R. and Nigam, P. 2001. Solid-State Fermentation in biotechnology. *Asiatech Publ Inc.* New Delhi. p. 221.

Puri, S., Beg, Q.K. and Gupta, R. 2002. Optimization of alkaline protease production from *Bacillus sp.* by response surface methodology. *Curr Microbiol* .,**44**: 286–90.

Prakasham, R.S., Subba Rao, C.H. and Sarma, P.N. 2006. Green gram husk—an inexpensive substrate for alkaline protease production by *Bacillus* sp. In solid-state fermentation. *Bioresour Technol.,* **97**: 1449–1454.

Scheuer, P.J .1990. Some marine ecological phenomena: chemical basis and biomedical potential. *Science*., **248**:173–177.

Sandhya, C., Madhavan Nampoothiri, K. and Pandey, A. 2005. Microbial proteases. In: Barredo JL, editor. Microbial enzymes and biotransformations. New Jersey, USA. *The Human Press Inc*. **17**:165–80.

Singh, J., Vohra, R.M. and Sahoo, D.K. 2004. Enhanced production of alkaline proteases by *Bacillus sphaericus* using fed-batch culture. *Process Biochem.,* **39** :1093–1101.

Sreenivas Rao, R., Prakasham, R.S., Krishna Prasad, K., Rajesham, S., Sarma, P.N. and Venkateswar Rao, L. 2004. Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach. *Process Biochem*., **39**: 951–956.

Sandhya, C., Sumantha, A., Szakacs, G. and Pandey, A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. *Process Biochem*., **40**: 2689–2694.

Thangam, E.B. and Rajkumar, G.S. 2000. Studies on production of extracellular protease by *Alkaligenes faecalis*. *World J Microbiol Biotechnol.,* **16**: 663–6.

Varela, H., Ferrari, M.D., Belobradjic, L., Weyrauch, R. and Loperena, M.L. 1996. Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhearing activity. *World J Microbiol Biotechnol.,* **12** :643–5.