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Optimization of Process Parameters Using a Statistical Approach for Protease Production by *Bacillus Subtilis* using Cassava Waste

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Abstract: In the present study, the effect of pH, temperature, fermentation time, inoculums Size and substrate concentration on protease production by *Bacillus Subtilis* using cassava waste has been investigated. The central composite experimental design (CCD) in response surface methodology (RSM) was used for designing the experiments as well as for full response surface estimation. The optimum conditions for maximum protease production (212.56U/gds) were as follows: pH (9), temperature (42°C), fermentation time (32 hrs), Inoculums size (3ml) and substrate concentration (10g). This was evidenced by the higher value of coefficient of determination (R^2= 0.982).

Keywords: Response surface methodology, *cassava waste*, protease, central composite Design, *Bacillus Subtilis,* optimization.

INTRODUCTION

Proteases are proteolytic (protein-digesting) enzymes that are mainly classified on the basis of their pH optimum as acidic, neutral, and alkaline proteases[1]. Proteases, also known as Peptidyl-peptide hydrolyses, which is the most important category of enzymes from industrial point of view. Protease are responsible for approximately 60% of all enzyme sales and are utilized extensively in a variety of industries, including detergents, meat tenderization, cheese making, dehairing , baking, brewery, and the recovery of silver from photographic film. The uses of these enzymes are detergent additives stimulated their commercial development and resulted in a considerable expansion of fundamental research into these enzymes. These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion [2].

Microbial proteases are gaining more importance than conventional chemicals that cleave peptides because of the cheaper production cost and use of renewable resources. Microbial proteases can be produced from bacteria, fungi and yeast using many processes like solid-state fermentation, submerged fermentation [3 4 5]. Several microbial strains including fungi (*Aspergillus flavus, Aspergillus melleu, Aspergillus Niger, Chrysosporium keratinophilum, Fusarium graminarum*, *Penicillium griseofulvin, Scedosporium apiosermum*) and bacterial (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus proteolyticus, Bacillus subtilis, Bacillus thuringiensis)* are reported to produce proteases. Among these, *Bacillus* genus has gained importance at industrial scale [6] .

Proteases are generally produced using submerged fermentation due to its apparent advantages in consistent enzyme production characteristics with defined medium and process conditions and advantages in downstream in spite of the cost –intensiveness for medium components. In this context, solid-state fermentation has gained renewed interest and fresh attention from researchers owing to its importance in recent developments in biomass energy conservation, in solid waste treatment and in its application to produce secondary metabolites. Production of these biocatalysts using agro-biotech substrates under solid-state fermentation conditions provide several advantages in productivity, cost-effectiveness in labour, time and medium components in addition to environmental advantages like less effluents production, waste minimization [7].

Industrial Fermentation is moving away from traditional and largely empirical operation towards knowledge based and better controlled process [8]. There are several reports describing use of agro-industrial residues for the production of alkaline protease, e.g. nug meal and *bacillus* sp. AR009 [9], pigeon pea and *bacillus* sp. JB-99 [10], wheat bran and *Rhizopus oryzae* [11]. However, these production characteristics would have to offer a competitive advantage over existing products. In general, each microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties.

Process optimization is a topic of central importance in industrial production processes. With particular regard to biotechnological production processes, in which even small improvements can be decisive for commercial success, process optimization is presently an undisputed component of the agenda of any commercial concern. In fermentation technology, improvements in the productivity of the microbial metabolite are achieved, in general, via the manipulation of nutritional and physical parameters and by strain improvements as the result of mutation selection [12]. These measures can alter the product yield significantly. Statistical methodologies are also generally preferred, due to the variety of recognized advantages to their use [13 14 15 16].

The present investigation aimed to exploit the locally available, inexpensive agro-substrate, cassava waste, for protease production using *Bacillus sp*. under solid-state fermentation and to investigate the combined effect of pH, temperature, fermentation time, inoculums size and substrate concentration using CCD in RSM.

MATTERIALS AND METHODS

MICROORGANISM AND INOCULUMS' PREPARATION

Bacterial strain used in this work is well preserved in the laboratory. Bacterial strain *Bacillus Subtilis* 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

Cassava waste was procured from the Balamurugan chaggo factory-Vada Chennimalai, Kallakuruchi – TamilNadu, India and used as substrate for protease production. The composition of the Cassava waste is given in Table 1. Fermentation was carried out in Erlenmeyer flasks (250 ml) with 10g of Cassava waste, supplemented with nutrients concentrations (% w/w): $MgSO_4.7H_2O$- 0.14, casein- 1.4 and glucose- 2.64. 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 3ml 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 32 hrs, the maximum production occurs. Hence experiments are carried out for 32 hrs.

Table 1. The Composition of Cassava Waste

Parameters	% (w/w)
Moisture	79.50
Protein	2.03
Crude Fat	0.20
Crude Fiber	14.35
Ash	2.38
Starch	61.84

EXTRACTION OF PROTEASE

The enzyme was extracted according to the method described by Nagamine et al. (2003) [17] . 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.

OPTIMISATION 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.

$$x_i = \frac{X_i - X_o}{\Delta x} \quad (1)$$

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.

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

Variable	Code	Levels				
		-2.38	-1	0	+1	+2.38
pH	A	6	7	8	9	10
Temperature (^oC)	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 3. Experimental conditions and observed response values of 2^5 Central Composite Design

Run. No	A-pH	B-Temperature	C-Fermentation time	D-Inoculums Size	E-substrate concentration	Protease activity	
						Experimental (u/gds)	Theoretical (u/gds)
1	-1	-1	1	-1	1	178.400	182.000
2	-1	1	1	1	-1	209.400	205.060
3	-1	1	-1	1	1	148.100	154.200
4	0	0	0	0	0	202.800	205.050
5	1	-1	-1	1	1	165.600	169.900
6	1	1	1	1	1	171.800	174.800
7	0	0	0	-2.37841	0	138.800	143.800
8	1	1	1	-1	1	195.000	191.000
9	1	1	-1	-1	1	168.000	164.000
10	0	0	0	0	0	205.300	205.050
11	0	0	0	0	0	205.300	205.050
12	1	1	-1	1	1	142.200	137.200
13	-1	1	-1	1	-1	155.200	160.400
14	0	0	0	0	0	205.300	205.050
15	1	1	-1	-1	-1	108.200	113.700

16	2.37841	0	0	0	0	105.500	110.070
17	0	0	0	0	0	205.300	205.050
18	1	1	-1	1	-1	102.200	103.700
19	1	-1	1	-1	1	180.800	175.700
20	0	2.37841	0	0	0	152.400	148.070
21	1	1	1	-1	-1	125.000	126.200
22	-1	-1	-1	-1	1	180.500	174.080
23	1	-1	1	1	1	165.000	166.700
24	1	-1	-1	-1	-1	154.800	147.600
25	-1	1	1	-1	-1	191.300	189.300
26	-1	1	1	1	1	207.800	213.300
27	-1	-1	-1	1	-1	184.700	185.300
28	-1	-1	-1	-1	-1	168.200	172.09
29	-1	-1	1	1	-1	184.700	189.200
30	1	-1	1	1	-1	125.300	127.900
31	-1	-1	1	-1	-1	165.200	165.483
32	0	0	0	0	-2.37841	132.000	134.919
33	1	-1	1	-1	-1	125.300	119.485
34	0	0	0	0	0	205.300	205.055
35	0	0	0	0	0	205.300	205.055
36	-1	1	-1	-1	-1	160.600	155.169
37	0	0	0	0	0	205.300	205.055
38	0	-2.37841	0	0	0	155.000	158.663
39	1	-1	-1	1	-1	152.600	145.733
40	-2.37841	0	0	0	0	190.200	184.961
41	0	0	0	0	2.37841	198.000	194.417
42	-1	-1	1	1	1	194.600	188.307
43	-1	1	1	-1	1	210.170	215.122
44	0	0	0	0	0	205.300	205.055
45	0	0	-2.37841	0	0	171.500	170.836
46	-1	-1	-1	1	1	168.200	169.891
47	-1	1	-1	-1	1	165.000	166.414
48	0	0	0	2.37841	0	145.200	139.462
49	1	-1	-1	-1	1	183.080	189.415
50	0	0	0	0	0	205.300	205.055

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 [18] . 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 81.85 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, 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. The model terms D, [P<0.100] were found to be insignificant.

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

Source	Coefficient factor	Sum of square	DF	Mean square	F	P value P>F
Model	205.25	44291.06	20	2214.55	81.85	< 0.0001
A	-15.74	10290.63	1	10290.63	380.34	< 0.0001
B	-2.23	205.80	1	205.80	7.61	0.0100
C	7.74	1873.47	1	1873.47	69.24	< 0.0001
D	-0.93	35.70	1	35.70	1.32	0.2601
E	12.51	6495.98	1	6495.98	240.09	< 0.0001
A*A	-10.21	6004.01	1	6004.01	20.30	0.0001
B*B	-9.18	4849.26	1	4849.26	32.58	<0.0001
C*C	-2.84	256.37	1	256.37	16.25	0.0004
D*D	-11.24	7281.97	1	7281.97	110.25	<0.0001
E*E	-7.18	2967.57	1	2967.57	116.06	< 0.0001
A*B	-4.26	549.20	1	549.20	4.50	0.0425
A*C	-5.40	881.48	1	881.48	5.98	0.0208
A*D	-3.81	439.78	1	439.78	7.66	0.0097
A*E	9.93	2982.85	1	2982.85	14.79	0.0006
B*C	10.19	3140.11	1	3140.11	21.36	<0.0001
B*D	-2.01	121.79	1	121.79	221.91	< 0.0001
B*E	2.31	161.79	1	161.79	179.23	< 0.0001
C*D	2.62	207.22	1	207.22	9.48	0.0045
C*E	3.64	400.21	1	400.21	269.14	<0.0001
D*E		578.01	1	578.01	109.68	< 0.0001
		784.64	1	27.06		
		784.64	29	39.23		
		0.000	20	0.000		
		45075.70	9			

Residua Lack of fit Pure Error Cor Total	-4.37		49			

Std. Dev. 5.20; R^2 = 98.26%; R^2(pred) 93.04%; R^2(adj) 97.06%; C.V. % 3.03

Protease activity = +205.25-15.74* A-2.23 * B+7.74 * C-0.93 * D+12.51* E -4.26 * A * B-5.40 * A * C-3.81 * A * D+ 9.93 * A * E+ 10.19 * B * C-2.01 * B * D +2.31 * B * E+2.62 * C * D+3.64 * C * E-4.37 * D * E -10.21 *A^2 -9.18 * B^2-2.84 * C^2-11.24 * D^2-7.18 * E^2 .....(3)

The predicted R^2 of 0.9304 is in reasonable agreement with the adjusted R^2 of 0.9706. 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 0.9826 indicating from properly that 98.26% 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 (3.03) 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 maximum protease production is 180.8U/gds at a particular range of pH 9 and temperature in the range 43°C .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. It was inferred that the maximum production was observed at a pH 9 and a fermentation time of 32 hrs.

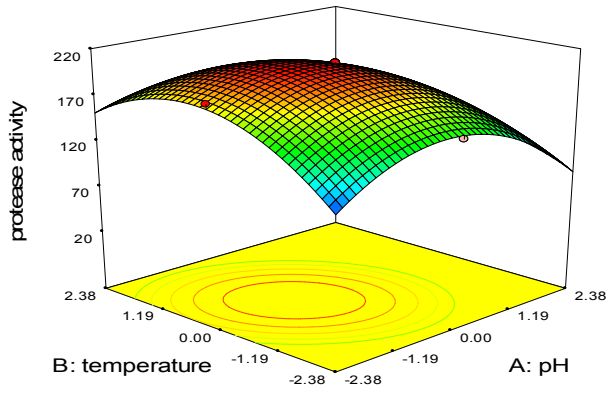

Fig. No.1.Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of pH and Temperature

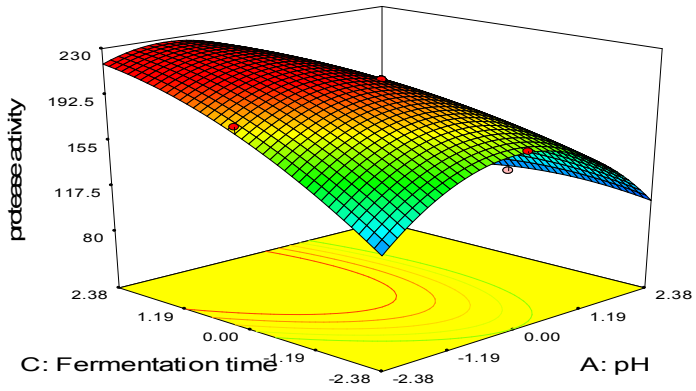

Fig. No.2. Response surface Plot for protease production from cassava waste by Bacillus *Subtilis* in solid state fermentation as a function of pH and fermentation time

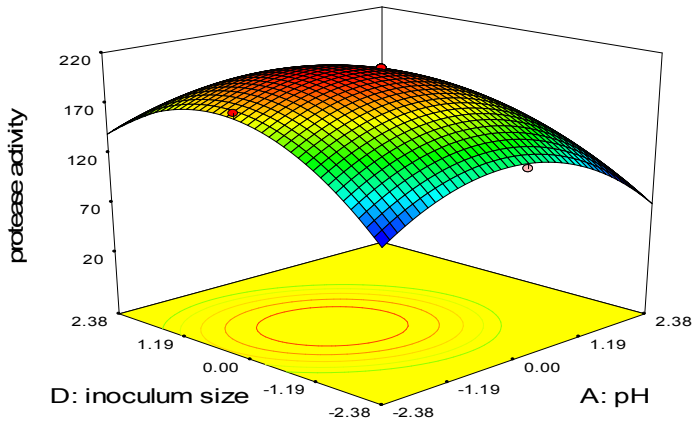

Fig. No.3. Response surface Plot for protease production from cassava waste by Bacillus *Subtilis* in solid state fermentation as a function of pH and inoculums size

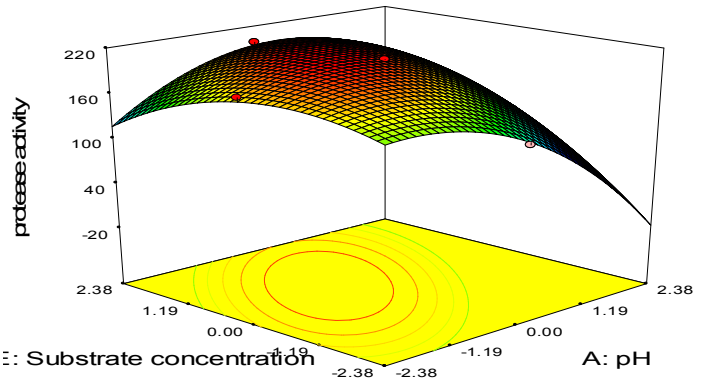

Fig. No.4. Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of pH and substrate concentration

Figure 3 depicts the interaction of pH and Inoculums size, where the maximum protease production 182 U/gds was found to occur with a pH 9 and Inoculums size 3 ml. Figure 4 shows the effect of pH and substrate concentration on the protease production. From the figure it was observed that the maximum protease production occurs at the pH 9.0 when the substrate concentration is 10 gms, which is in accordance with the model. 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 It is inferred from figure that the increase in temperature up to 43°C leads to increase in protease production after that the production decreases. The maximum protease production occurs at the temperature of 42°C and fermentation time of 32 hrs. Combined effect of temperature and innoculum size has been analyzed from the CCD three-dimensional plot representing the maximum protease production 184 U/gds was obtained in the temperature range of 43°C and at an Inoculums size of 3 ml is shown in Fig. 6.

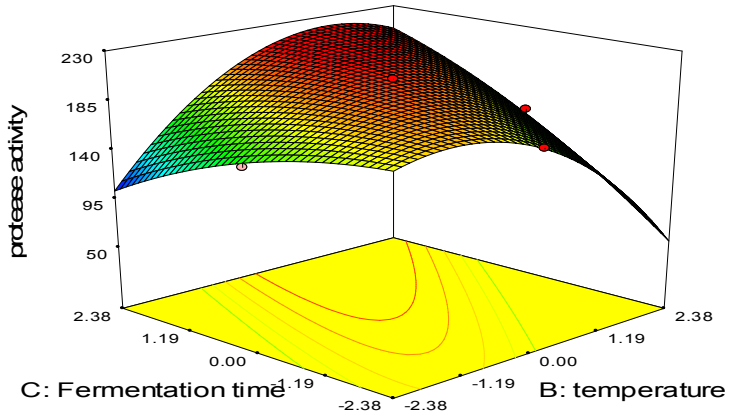

Fig . No.5. Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of temperature and fermentation time

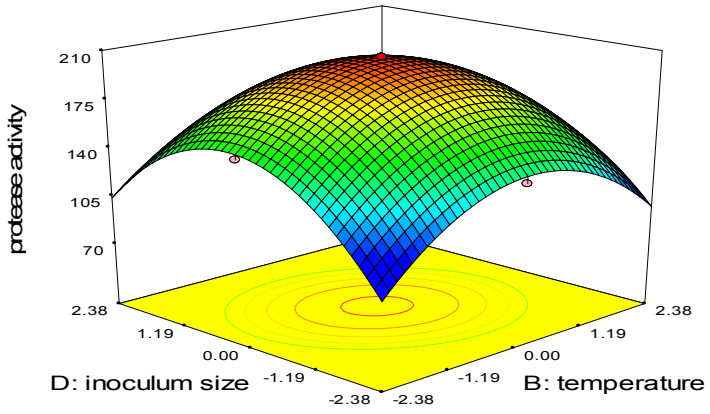

Fig . No.6.Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of temperature and inoculum size

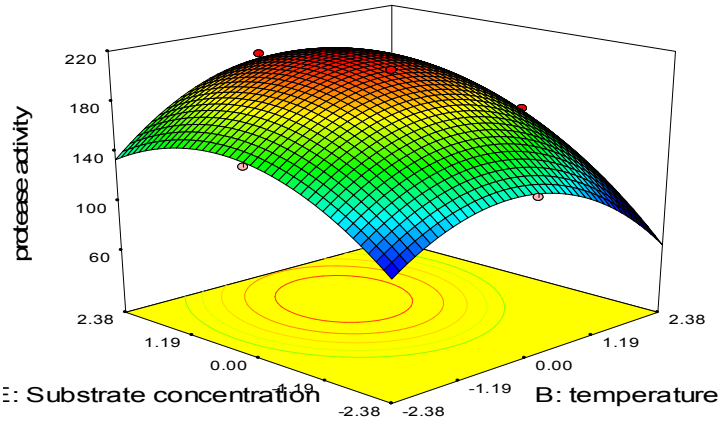

Fig . No.7. Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of temperature and substrate concentration

Figure 7 shows the effect of temperature and substrate concentration on the protease production. The optimum values for the maximum protease production were temperature 43°C and substrate concentration 10 gms.

Figure 8 shows the response surface curves of protease production as a function of inoculums size and fermentation time. The maximum protease production occurs at the inoculums size 3 ml and at fermentation time 32 hrs. 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. The maximum protease production 205 U/gds is obtained when the substrate concentration is 10 gms at a fermentation time of 32 hrs. Figure 10 depicts the interaction of inoculums size and substrate concentration, where the maximum protease production of 210 U/gds was found to occur with inoculums size of 3ml and substrate concentration of 10 gms.

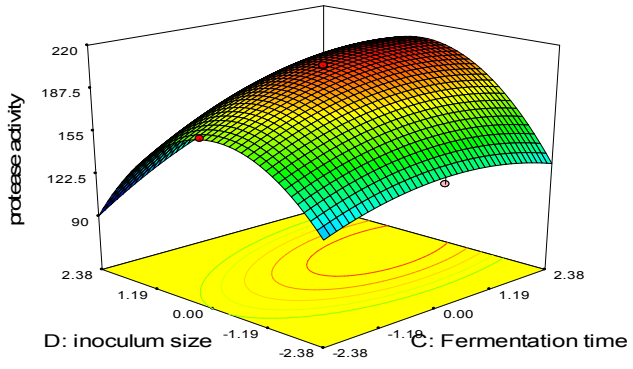

Fig. No.8. Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of fermentation time and inoculum size

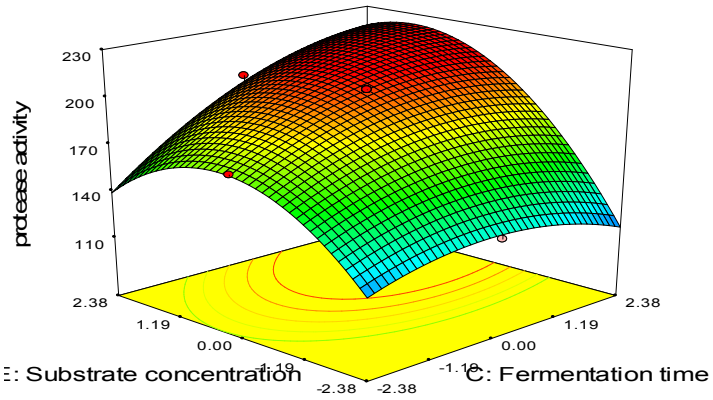

Fig.No.9.Response surface Plot for protease production from cassava waste by *Bacillus Subtilis* in solid state fermentation as a function of fermentation time and substrate concentration

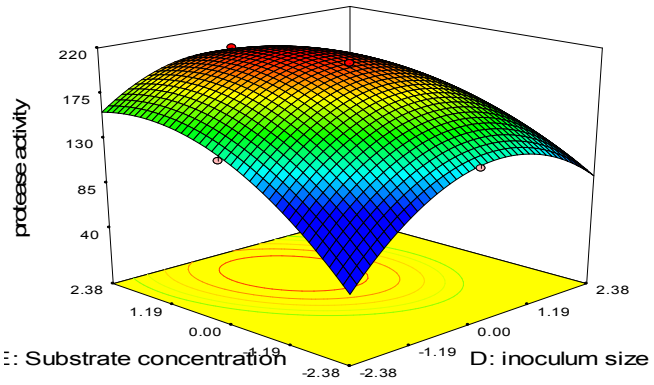

Fig.No.10. Response surface Plot for protease production from cassava waste *Bacillus Subtilis* in solid state fermentation as a function of inoculum size substrate concentration

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 9, temperature 43°C, fermentation time32hrs, inoculums size 3 ml and substrate concentration 10g. 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-9), (temperature – 43°C), (fermentation time-32hrs), (Inoculums size-3ml) and (substrate concentration-10g of medium) established by the regression model. Three repeated experiments were performed and the results are compared. The protease activity (210.170U/gds) obtained from experiments was close to the actual response

(215.122U/gds) predicted by the regression model, which proved the validity of the model.

CONCLUSION

The feasibility of using an Agro-residue (Cassava waste) as possible substrate for the protease production was studied using the response surface methodological approach. The optimum conditions for the maximum protease production 212.56 U/gds using cassava waste are as follows: pH 9, temperature 43°C, fermentation time 32hrs, inoculums size 3ml and substrate concentration 10g. The enzyme production in this range from this vastly available by-product is significant. Other commonly employed agro-wastes have been reported to produce protease activity in the similar range, viz. 266 U/g [19] , 1210 U/g [20], 10,772 U/g [21] from Pseudomonas sp., Bacillus sp. P-2, Penicillium sp., . As Cassava waste is readily available, it could represent a cheap source of substrate for protease production.

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