Research Article

Optimization of Protease Extraction from *Billbergia pyramidalis* (Sims) Lindl. by Response Surface Methodology

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ABSTRACT

Proteases are involved in every aspect of physiology and development in both prokaryotes and eukaryotes and have been identified as therapeutic targets for many diseases and conditions. One such protease is Bromelain, a cysteine protease from *Ananas comosus* (Pineapple) of Bromeliaceae family. Bromelain is currently the thirteenth most widely used herbal medicine in Germany. In the present study, *Billbergia pyramidalis* of Bromeliaceae was chosen as a source of protease. The crude enzyme was extracted from the leaves of *B. pyramidalis* and extraction was optimized by a Response Surface Method (RSM) using a Central Composite Design (CCD). The most significant variables which enhance the protease extraction are screened by using the Plackett-Burman Design. The optimum levels of the variables such as sucrose (0.50M), DTT (3mM), $CaCl_2$ (5mM), pH (7.0), and temperature (30°C), established by the regression model, are validated experimentally and revealed an enhanced protease yield of 164.23 GDU/g. The optimal extraction conditions generated from RSM model were found to be significant.

Keywords: *B. pyramidalis*, Protease, Central Composite Design (CCD), Optimization, Plackett-Burman Design (PBD), Response Surface Methodology (RSM).

INTRODUCTION

Industrial and biotechnological demands for proteolytic enzymes are increasing as they execute a large variety of functions, extending from cellular level to the organ and organism level. Proteases are multifunctional enzymes that play a pivotal role in various complex physiological functions and have attracted worldwide attention in exploiting their biotechnological applications.[1,2] They have a long history of applications in both the food and non-food sectors. Widespread use of the proteases in industries such as detergent, leather, textile, pharmaceutical, food etc., has increased the demand for proteolytic enzymes in the global market.[3]

Although most of the commercial proteases are from animal and microbial sources the plant derived proteolytic enzymes have received special acquaintance because of their broad specificity, stability, reaction rate and sustainability to various pH and temperature conditions.[4-7] In the course of using animal proteases certain limitations has encountered due to various issues raised by food consumers such as food authenticity and adulteration by use of faithfully unlawful ingredients.[8-10] Therefore, it has become an essential motive of the industries to explore alternative sources of proteases, especially from plants.[11] Certain plant proteases have long been used both in food and the pharmaceutical industry. These include Bromelain extracted from leaves of *Ananas erectifolius*[12] from stem and fruit[13] of *Ananas comosus,* papain from (*Carica papaya*)[14,15], ficain from *Ficus glabrata* and *Ficuslaurifolia*[16,17] and many other are found to have strong proteolytic activity. Because of their selectivity, and potential for use in biological systems, plant proteases have found immense utility in various

diseased conditions and offer a new strategy for site-specific drug targeting.

In general, the extraction of protease from a plant source is complex when compared to other sources as the plants proteases are cellularly bound[18,19] and also contain many poly phenolic compounds and polyphenol oxidases which promote enzyme inactivation, degradation and reduced yield.[20,21] The performance or action of enzyme depends on the plant source, climatic conditions for growth and the methods used for its extraction and purification. In enzyme bioprocess, designing a suitable medium is highly significant as the components of the medium affect maximum protease production. Various reducing agents, activators, and detergents are thereby used by many researchers working on plant proteases in order to improve the extraction process and stability of the enzyme.[22,23]

One-Variable-at-a-time strategy is one of the conventional optimization strategies where an individual parameter is optimized by changing it while maintaining the other variables at constant values.[24] This strategy was performed for initial screening of the most significant factors required to enhance protease extraction. The traditional single variable optimization method unfortunately fails, as it requires more amount of work and time and is also expensive when more number of variables are used.[28,29] It mainly fails to identify the effect of interaction between the variables used.[25] To overcome these limitations, a statistical method, Response Surface Methodology (RSM) is applied.[26,27] RSM, is a collection of experimental strategies, statistical and mathematical methods which helps to explore the optimum concentrations of each of the variables and facilitates the

optimization process by evaluating the mutual interactions among the variables over a range of values in a statistically valid manner.[28,29] This method has a prominent role in vast areas of biotechnology including enzyme production and kinetics.[30,31]

Billbergia pyramidalis is a tropical and tender perennial plant originated at south Brazil. As with every plant of the Bromeliaceae family *B. pyramidalis* also contains bromelain like endopeptidase as a constituent.[32] Bromelain is one of the most promising multifunctional enzymes claimed for its wide range of applications in medicine, pharmacology and food industry.[33-35] Novel protease production schemes and procedures drive to expand the commercial value of proteases and have long been the subject of intensive research. Therefore, in response to the quest for search of novel plant proteases, present research works dealt with optimization of most significant parameters required to enhance protease extraction from leaves of *B. pyramidalis* using Plackett-Burman Design (PB) and Central Composite Design (CCD). PBD was exploited to determine the most significant variables required to enhance protease activity and subsequently a CCD was employed to study the interactions between the various variables. The optimized variables Sucrose, DTT, $CaCl_2$, pH, and temperature were used in the protease extraction from *B. pyramidalis*. The results were further analyzed by response surface analysis. This is the first report on optimization of various parameters for extraction of protease from *B. pyramidalis*, using experimental statistical designs.

MATERIALS AND METHODS

Plant material collection and authentication

The plant was collected from Hukumpeta area of Paderu, Andhra Pradesh, India (18.0833°N 82.6667°E) and taxonomically identified as *Billbergia pyramidalis* (Sims) Lindl. and deposited a voucher specimen(BSI/DRC/2013-2014/TECH/800) at Botanical Survey of India (BSI), Hyderabad.

Extraction of crude enzyme

Fresh leaves of *B. pyramidalis* were collected and washed thoroughly with Tween 20 (1%) and hydrogen peroxide (0.1%) solution. Leaf tissue was weighed (100 grams) and macerated in a mortar and pestle using liquid nitrogen to produce a fine powder and the enzyme was extracted using homogenization medium (250 ml), prepared with appropriate buffer solutions such as sodium acetate buffer (25mM) with pH 5.6 – 6.0, Tris buffer (25mM) with pH 7.0, phosphate buffer (25mM) with pH 8.0, glycine-sodium hydroxide buffer (25mM) with pH 9.0, along with sucrose, EDTA, 1, 4- dithiothreitol (DTT) and calcium chloride. The process of extraction was done by cold maceration and filtered using Whatman No.1 filter paper prior to centrifugation at 10,000 g at 4-6 °C for 10min. The supernatant thus obtained was stored at 4 °C for further analysis. Minor modifications were made to the existing enzyme extraction methods to enhance the protease activity, before further optimization of protease extraction using RSM.[36,37]

Protease Activity

Proteolytic activity of the enzyme was determined using gelatin digestion unit analytical method. 'One gelatin digestion unit is the amount of enzyme which will liberate one milligram (mg) of amino nitrogen from a standard gelatin solution after 20 min of digestion at 45℃ at pH 5.5'.[38] 1ml of crude extract was added to 25ml of gelatin substrate solution which had been previously equilibrated at 45 °C for 20 minutes in a water bath and was designated as test solution. After 20 minutes of incubation 0.1 ml of hydrogen peroxide (3%) solution was added and again incubated for 5 min. The test solution was adjusted to pH 6.0 and 10 ml of formaldehyde (37%) was added with constant stirring. The pH of the test solution was noted after 10 sec and 1 min respectively. Later three to four drops of phenopthalein indicator (0.1 %) was added and then titrated against 0.1 N sodium hydroxide till the test solution turns colorless to bright pink, which indicates the test solution is at pH 9.0 and the titration volume was recorded as test titer. The same digestion procedure was repeated to obtain blank titer B, by adding 0.1 ml of hydrogen peroxide (3%) prior to the addition of enzyme extract to the gelatin solution. Gelatin digestion unit (GDU) for the above method is defined as

$$GDU/g = \frac{(T-B)*14*N*1000}{mg\ of\ enzyme/RM}$$

Where 'T' is the test titer (ml), 'B' is the blank titer in ml, 'N' is the normality of standardized NaOH, 14 = mg of nitrogen per millmole nitrogen, mg of enzyme = amount per concentration of enzyme present in 1ml of crude extract and RM is the reaction mixture.

Experimental Design and Data Analysis

Screening of significant variables using Plackett-Burman Design (PB)

To determine the significant variables which affect the protease activity, a set of parameters were selected based on preliminary results. A total of eleven parameters were included for selection, with each variable represented at two levels and were designated as '+1' for high and '-1' for low values respectively (Table1). The variables are screened to remove the insignificant ones in order to obtain a smaller, manageable set of factors. Variables such as sucrose, maltose, Tris buffer, acetate buffer in molar concentration, EDTA, DTT, $CaCl_2$ in millimolar (mM) concentration, benzoic acid (%), pH, temperature (°C) and incubation time (hrs) were optimized with the PB design method. Eleven assigned variables were screened in PB design of 12 experiments (Table 2). The significant variables like the media components and environmental factors were analyzed using 'Analysis of variance' (ANOVA) listed in Table 3 and the variables were further analyzed by response surface methodology (RSM).

Optimization of significant nutrient factors by RSM using Central Composite Design (CCD) to enhance protease extraction

The levels of five significant factors and the interaction effects between various medium constituents which influence the protease activity significantly were analyzed and optimized by RSM, using a CCD design. Based on the results of PB design, five variables which show maximum effect on enzyme activity were selected (sucrose, DTT, $CaCl_2$, buffer, temperature and pH). All the five variables were analyzed at five coded levels, designated as -2, -1, 0, +1 and +2. The levels of the independent variables used for experimental design are shown in Table 3.

Thirty experimental runs with five duplicate runs at center point were designed, responses in terms of protease activity were determined for each experiment (Table 4). All parameters were taken at a central coded value considered as zero. The minimum and maximum ranges of parameters were investigated. The response values (Y) in each trial were the average of the triplicates. The above RSM method was executed using statistical software package 'Design Expert Ver.7.1.5'. The ranges of these variables were decided according to the ranges determined by the one-at-a-time method.

The significance of the model was analyzed by ANOVA and the protease activity from *B. pyramidalis* was expressed by the following regression equation:

Regression Equation (Y) = + intercept + A + B + D + E + A^2 + B^2 + C^2 + D^2 + E^2 + AB + AC + AD + AE + BC + BD + BE + CD + CE + DE   (i)

The above equation gives the empirical relationship between enzyme activity and tested variables. Where Y is the predicted response; A, B, C, D and E are linear terms , A^2, B^2, C^2, D^2, E^2 are quadratic terms and AB, AC, AD, AE, BC, BD, BE, CD, CE, DE represent the interaction terms of the variables tested. The optimal concentrations and interaction effects of variables were analyzed using the 3D response surface plots.

Statistical Analysis and Modelling

All analyses were performed in triplicates and the data presented as mean ± standard deviation. Upon the completion of experiments, the average maximum protease activities were taken as the response (Y), the data was subjected to ANOVA. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The significance of each coefficient was determined by Students t- test and p-values.

Validation of the Experimental Model

A verification test with respect to protease extraction was conducted at optimum levels of the variables predicted by the regression model. To validate the predicted optimized conditions obtained from RSM, a triplicate of experiments were performed and the protease activity was determined as described above and the results are compared. Thereby the accuracy and applicability of RSM in optimizing the protease extraction from *B. pyramidalis* can be determined.

RESULTS AND DISCUSSION

Identification of significant Variables by PB design

During the first step of optimization all the preferred nutrients were added to the homogenizing medium. The significant nutritional factors enhance the protease activity in *B. pyramidalis.* PB design showed a wide variation in protease activity which reflected the importance of optimization to enhance protease extraction in *B. pyramidalis.* Each independent factor which are set at two levels +1 for high and -1 for low (Table1). The variables were screened in PB design of 12 experiments (Table 2).

Evaluation of PB design on protease extraction using ANOVA

The PB results in Table 1 and 2 indicate that the media components and environmental factors have greater emphasis on protease extraction. From the ANOVA analysis of twelve PB experiments the variables sucrose, DTT, pH, $CaCl_2$, temperature showed a positive impact and greatly influenced the protease extraction. The variables in the model are said to be significant as the P<0.01. The coefficient of determination (R^2) was calculated as 0.98 which implies that the model is significant (Table 3).

CCD and Response Surface Methodology

According to PB design experiment, five significant variables X1-Sucrose, X6-DTT, X8-$CaCl_2$, X9-pH and X10-temperature were preferred for advanced optimization using CCD of Response Surface Method to evaluate the optimal values. The coded values of the variables at various levels for RSM analysis were listed in Table4.

CCD was used to explore conditions that increase protease extraction. Thirty experimental runs were performed at different combinations of the variables depicted in Table 5.

A high degree of similarity was observed between the predicted and experimental optimum protease activity values and the correlation between predicted and experimental values was showed in Figure 1. The results were further analyzed by ANOVA.

The significance of the model was analyzed by ANOVA and the protease activity from *B. pyramidalis* was expressed by the following regression equation:

Regression Equation (Y) = +172.55+18.45A+8.02B-0.92C+14.64D+10.08E+32.24A^2-18.66B^2+21.24C^2-42.98D^2+39.57E^2+48.21AB+10.81AC-69.48AD-3.71AE+24.32BC + 65.47BD+13.05BE+1.836CD + 7.081CE - 0.692DE.

The five significant variables Sucrose, DTT, CaCl₂, pH and temperature which are selected for further optimization were divided into three groups (Linear, Quadratic, Interaction effects). From the study the t-value of the linear term Sucrose (A) was found to be higher which indicates that the Sucrose level on protease activity has the strongest effect. Among all the 10 interaction terms sucrose. pH (AD) interaction had strongest effect on the protease activity. All the interaction terms were also found to be significant as $p<0.0500$ whereas, values greater than 0.100 indicate that the model terms are not significant.

Based on the t-values listed in Table 6, main, quadratic and interaction group variables for protease activity were arranged in the following ascending order.

The ascending order of the main group was, CaCl₂(C) < Temperature (E) < DTT (B) < pH (D) < Sucrose (A), the quadratic group was $D^2 < B^2 < C^2 < A^2 < E^2$ and for the interaction group was DE <CE < CD < AE < AC < BE < BC < BD < AB < AD.

Statistical Analysis and Modelling

The significance of each coefficient was determined by Students t- test and p- values listed in the Table 6 and 7.

The smaller the 'p' value the larger is the magnitude of t-value, and the more significant is the corresponding coefficient.
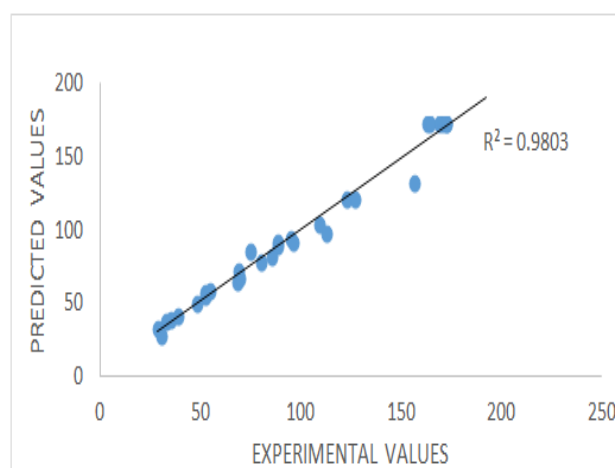

Figure 1: Correlation between predicted and experimental values

Table 1: Screening of Independent Variables for PB design

Variable code	Name of the Variable	Coded levels	
		-1	1
X1	Sucrose (Molar conc.)	0.10	1.00
X2	Maltose (Molar conc.)	0.10	1.00
X3	Tris buffer (Molar conc.)	0.01	0.10
X4	Acetate buffer (Molar conc.)	0.01	0.10
X5	EDTA (mM conc.)	1.00	5.00
X6	DTT (mM conc.)	1.00	5.00
X7	Benzoic acid (%)	0.20	1.00
X8	CaCl₂(mM)	2.50	10.0
X9	pH	6.00	9.00
X10	Temperature (°C)	20.0	40.0
X11	Incubation time (hrs)	24.0	48.0

Table 2: PB experimental design matrix for screening of variables affecting protease extraction

RUN	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Protease activity GDU/g
1	1	-1	-1	1	-1	1	1	-1	1	1	-1	70.2
2	1	1	1	-1	1	1	1	-1	-1	-1	-1	75.5
3	-1	1	-1	1	1	-1	1	1	1	-1	-1	8.4
4	-1	-1	1	-1	1	1	-1	1	1	1	-1	21.6
5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	62.7
6	-1	-1	1	1	-1	1	1	1	1	-1	1	64.8
7	1	1	-1	-1	-1	1	-1	1	1	-1	1	69.6
8	-1	1	1	-1	-1	-1	1	-1	1	1	1	78.2
9	1	-1	-1	-1	1	-1	1	1	-1	1	1	58.1
10	1	-1	1	1	1	-1	-1	-1	1	-1	1	75.4
11	-1	1	-1	1	1	1	-1	-1	-1	1	1	16.2
12	1	1	1	1	-1	-1	-1	1	-1	1	-1	68.2

(X1- Sucrose, X2-maltose, X3-Tris buffer, X4-Acetate buffer, X5-EDTA, X6-DTT, X7-Benzoic acid, X8-Cacl2, X9-pH, X10-Temperature, X11-incubation time).

Table 3: ANOVA for PB experimental design for protease extraction

Variable	Sum of Squares (SS)	Degree of Freedom (DF)	Mean Square (MS)	F- value	P-value
X1	24.82	1	24.82	1464.2	0.0006
X2	1.83	1	1.83	54.68	0.0224
X3	4.58	1	4.58	188.26	0.0088
X4	0.13	1	0.13	4.48	0.1462
X5	0.04	1	0.04	0.26	0.5854
X6	9.82	1	9.82	466.45	0.0034
X7	3.40	1	3.40	112.2	0.0059
X8	8.64	1	8.64	344.26	0.0046
X9	6.10	1	6.10	284.64	0.0041
X10	4.88	1	4.88	220.45	0.0072
X11	0.13	1	0.84	5.26	0.2589

$R^2 = 0.98$; Adj $R^2 = 0.97$; SD = 0.18

Table 4: Range of the independent variables for RSM analysis

Coded Variables	-2	-1	0	1	2
A-Sucrose	0.1M	0.25M	0.50M	0.75M	1M
B-DTT	1mM	2mM	3mM	4mM	5mM
C-$CaCl_2$	0.00mM	2.5mM	5mM	7.5mM	10mM
D-pH	5.0	6.0	7.0	8.0	9.0
E-Temperature ($^\circ$C)	10	20	30	40	50

Table 5: CCD of factors in coded levels with enzyme activity as response

	Variables					Protease Activity GDU/g	
S.No.	A-Sucrose(M)	B- DTT(mM)	C- $CaCl_2$(mM)	D- pH	E- Temperature (°C)	Actual	Predicted
1	0.25	2.0	2.5	6.0	20	108.94	98.24
2	0.25	4.0	2.5	6.0	20	122.76	110.28
3	0.75	2.0	2.5	6.0	20	95.78	84.27
4	0.75	4.0	2.5	6.0	20	68.77	78.25
5	0.25	2.0	7.5	6.0	20	88.14	90.12
6	0.25	4.0	7.5	6.0	20	94.89	94.99
7	0.75	2.0	7.5	6.0	20	88.64	92.05
8	0.75	4.0	7.5	6.0	20	80.26	74.24
9	0.25	2.0	7.5	6.0	40	32.6	58.24
10	0.25	4.0	2.5	8.0	40	74.85	86.26
11	0.25	2.0	2.5	8.0	40	28.27	36.57
12	0.75	4.0	2.5	8.0	40	54.42	62.78
13	0.25	2.0	7.5	8.0	40	38.88	46.58
14	0.25	4.0	7.5	8.0	40	52.28	72.84
15	0.75	2.0	7.5	8.0	40	52.46	63.21
16	0.75	4.0	7.5	8.0	40	48.12	52.67
17	0.50	1.0	5.0	7.0	30	69.58	57.55
18	0.50	5.0	5.0	7.0	30	112.54	94.62
19	0.10	3.0	5.0	7.0	30	68.52	58.42
20	1.00	3.0	5.0	7.0	30	126.82	118.45
21	0.50	3.0	0.0	7.0	30	30.28	27.81
22	0.50	3.0	10.0	7.0	30	85.24	79.25
23	0.50	3.0	5.0	5.0	20	156.14	112.12
24	0.50	3.0	5.0	9.0	50	24.86	34.58
25	0.50	3.0	5.0	7.0	30	172.35	172.55
26	0.50	3.0	5.0	7.0	30	168.59	172.55
27	0.50	3.0	5.0	7.0	30	170.75	172.55
28	0.50	3.0	5.0	7.0	30	164.23	172.55
29	0.50	3.0	5.0	7.0	30	172.48	172.55
30	0.50	3.0	5.0	7.0	30	156.28	172.55

Table 6: ANOVA for response surface regression model

Terms	Coefficient estimate	Sum of Squares	DF	Mean Square	F- value	Probability >F	t-value
A	18.45	24.88	1	24.88	1464.2	<0.0001	9.67
B	8.02	8.64	1	8.64	344.26	0.0016	4.19
C	-0.92	4.58	1	4.58	188.26	0.0104	-1.88
D	14.64	9.82	1	9.82	466.45	<0.0001	7.44
E	10.08	4.88	1	4.88	220.45	0.0021	3.15
A*A	32.24	8.21	1	8.21	318.5	0.0014	4.32
B*B	-18.66	1.642	1	1.642	48.34	0.6851	0.62
C*C	21.24	3.25	1	3.25	102.24	0.0035	-3.38
D*D	-42.98	0.46	1	0.21	8.66	0.5622	0.31
E*E	39.57	11.24	1	11.24	542.33	<0.0001	8.74
AB	48.21	12.63	1	12.63	606.27	<0.0001	7.89
AC	10.81	6.87	1	6.87	312.55	0.0011	4.66
AD	-69.48	14.59	1	14.59	666.64	<0.0001	8.62
AE	-3.71	3.22	1	3.22	99.48	0.0069	3.85
BC	-24.32	8.2	1	8.2	318.02	0.0011	-0.52
BD	65.47	10.84	1	11.84	568.06	0.0003	6.99
BE	13.05	7.55	1	7.55	282.12	0.0439	3.59
CD	1.836	2.69	1	2.69	87.65	0.0082	3.97
CE	7.081	4.21	1	4.21	165.56	0.0002	5.25
DE	-0.692	1.4	1	1.4	46.02	0.0032	-2.99

Table 7: ANOVA for the variables of response surface quadratic model

Source of Variation	Sum of Squares	Degree of Freedom	Mean Square	F-Value	P-Value
Model	58248.04	16	4647.29	18.28	<0.0001
Residual	5504.94	14	336.24	--	--
Lack of Fit	5545.26	10	545.29	68.94	<0.0003
Pure error	54.62	5	11.15	--	--
Total	63752.98	30	--	--	--

R^2=0.9124, Adjusted (Adj) R^2=0.8962 Predicted (Pred) R^2=0.7585;

Coefficient of Variation (CV) = 2.64%, Predicted Errors Sum Square (PRESS) = 28.24.

The model F-Value of 18.28, and values of probability >F (<0.05) indicated that the model terms are significant. The R^2 value (multiple correlation coefficient) was 0.9124, and is said to be satisfactory as the value of 0.75 indicates aptness of the model. The smaller sample size and more number of terms in the model results in a smaller Adj R^2 value, compared to R^2. The present study, also, reported a smaller Adj R^2 value of 0.8962 than the R^2 value 0.9124. Furthermore, the Adj R^2 value should be in commensurate agreement with Pred R^2 value 0.7585, thereby indicating the model as highly significant and can be used to analyze the data. At the same time a relatively lower value of CV = 2.64% and PRESS value of 28.24 implied a better fidelity and reliability of the experiments carried out.

3D response surface plots

The interaction effects of variables were studied using the 3D response surface plots.[39] The response surface plots reveal the interaction between the response and experimental levels of test variables on protease activity (Figure 2).

From the 3D surface response plots it can be concluded that the ranges of temperature, pH , CaCl$_2$, DTT and sucrose in optimizing the protease production are 30 – 40°C, pH 6.5 – 7.0, CaCl$_2$ 5mM – 10mM, DTT 3mM – 5mM and sucrose 0.25M – 0.75M, respectively. For these variables further increase in temperature, pH or concentrations would decrease the protease activity.

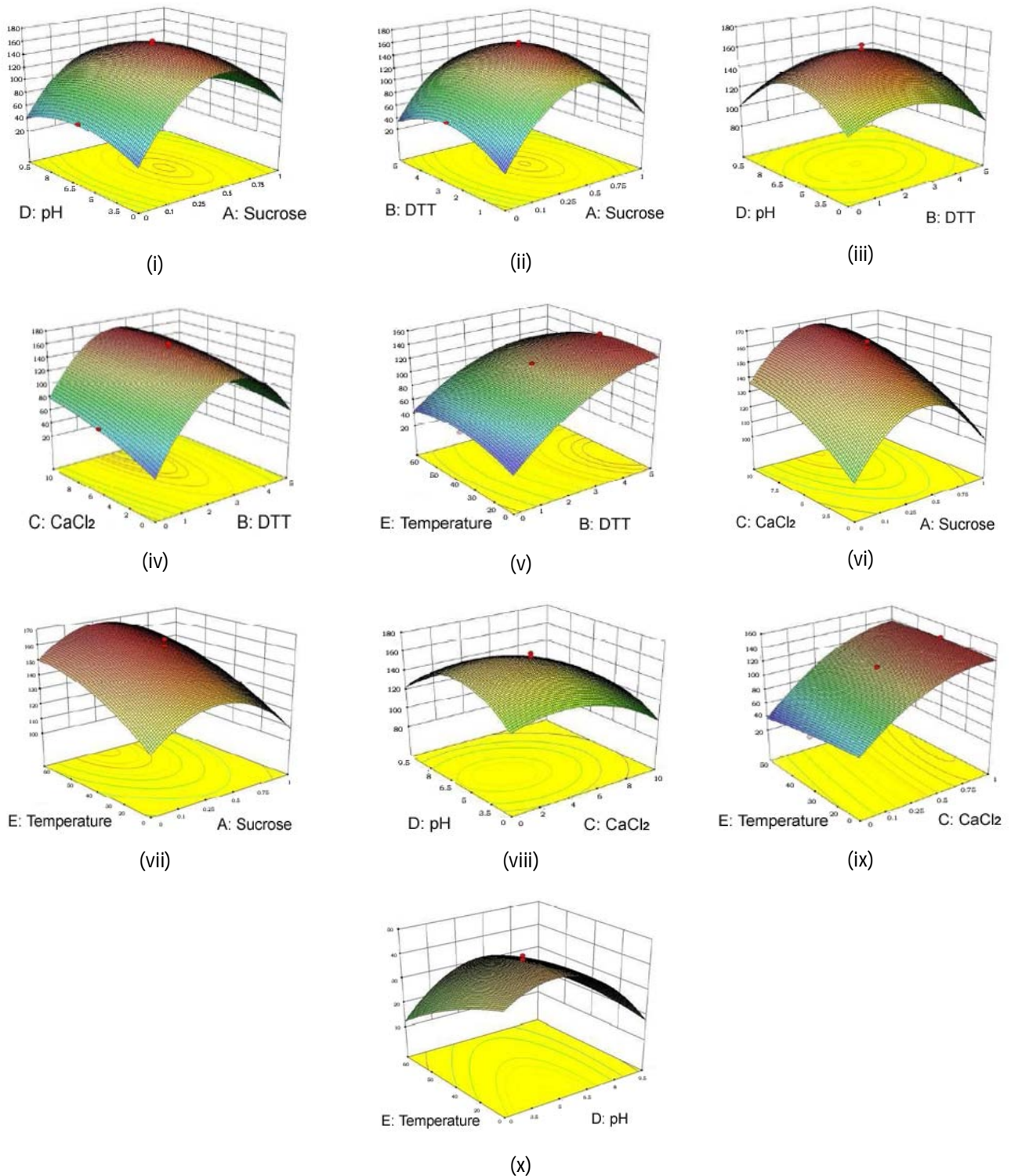**Figure 2:** Interaction between the variables on protease extraction

Figure 2: 3- Dimensional surface plots for the interaction effect of variables- (i) sucrose, pH (ii) sucrose, DTT (iii) DTT, pH (iv) DTT, CaCl$_2$ (v) DTT, temperature (vi) sucrose, CaCl$_2$ (vii) sucrose, temperature (viii) CaCl$_2$, pH (ix) CaCl$_2$, temperature (x) pH, temperature on protease activity.

Validation of the experimental model

A verification test was conducted at optimum levels of the variables like sucrose 0.50M, DTT 3mM, CaCl$_2$ 5mM, pH 7.0, and Temperature 30 °C established by the regression model (Table 8). To validate the predicted

optimized conditions obtained from RSM, a triplicate of experiments were performed by using those values of variables and the results are compared. The validity of the model was there by conformed satisfactory, as the obtained experimental value (164.23 GDU/g) is close to

the actual response (172.55 GDU/g) predicted by regression model. This reflects the accuracy and applicability of RSM in optimizing the protease extraction from *B. pyramidalis.*

The optimized variables sucrose, DTT, CaCl$_2$, pH, and temperature evaluated from the model played a significant role in enhancing the protease extraction from leaves of *B. pyramidalis.* From the RSM model sucrose is found to be a significant variable enhancing the protease extraction based on the t-values. The function of sucrose is to reduce "non-specific" interactions between proteins and acts as an osmolyte.[8] Sucrose helps stabilizes proteins and helps prevent aggregation similar to glycerol. High concentrations of sucrose will prevent bacterial growth and protect proteins during freezing by preventing ice crystal formation causing protein degradation. Optimum protease activity was obtained at 0.50M concentration of sucrose. The protease extraction from *B. pyramidalis* is thereby facilitated by using sucrose as one of the variable as it improves the stability of the target protein.

DTT also called Cleland's reagent acts as protein modulator. The protease extracted from *B. pyramidalis* is a highly active thiol proteinase, hence, DTT is commonly used as a thiol group protectant which confers protective effects against oxidation and it also maintain protease activity for a longer time.[40-43] Because of the high tendency of the thiol group to oxidation, the environment of the enzyme should contain a reducing component therefore addition of mercaptoethanol or dithiothreitol is required for *in vitro* experiments. The optimum DTT level evaluated from our RSM model was 3mM. This shows that DTT enhance the total protease availability and eventually prevented the protease from denaturation. Further, the addition of DTT enhanced protease activity and thermo stability and these results are in accordance with the earlier reports.[44-,45] As per the data obtained, the optimum pH and temperature of the extraction medium are significant at pH 7.0 and temperature 37 °C, respectively. As reported earlier, pH optima for bromelain enzyme lies in a broad pH range, around pH 7.0.[46]

Denaturation of the enzyme and its activity was controlled by maintaining the pH at optimum levels using the appropriate buffer. The pH of plant cells usually ranges between pH 6.0 and 7.0.[47] The ability of the buffer to protect enzyme against pH denaturation enhanced the extraction process.[48] Temperature is one of the major factor along with pH which maintains the enzyme activity. Further, increase of pH and temperature conditions has shown adverse effects on protease extraction. In the study pH was more effective in increasing protease extraction than DTT. Ions maintain or increase the strength of the medium and facilitate the release of soluble proteins and other cellular components into the solution.[49,50] In the present study, 5 mMCaCl$_2$ was found to enhance the protease activity due to the ability of CaCl$_2$ to maintain and stabilize the confirmation of

enzyme molecule. It also acts a cofactor as few enzymes require cofactor to function well.

All the five significant variables had significant effects on protease activity and the interaction terms are also found to be significant as $p<0.0500$. The optimal extraction conditions generated from RSM therefore enhanced the protease activity. Further advancement in biotechnological techniques promotes the development of proteases and will continue to facilitate their applications in various pharmaceutical and industrial sectors.

CONCLUSION

The optimum levels of the variables such as sucrose 0.50M, DTT 3mM, CaCl$_2$ 5mM, pH 7.0, and temperature 30 °C, established by the regression model, enhanced the protease activity. The optimal extraction conditions generated from CCD of RSM model were found to be significant with good precision. The validity of the model was there by conformed satisfactory, as the obtained experimental value (164.23 GDU/g) is close to the actual response (172.55 GDU/g) predicted by regression model. This reflects the accuracy and applicability of RSM in optimizing the protease extraction from *B. pyramidalis.* Further, it provides a way for enhanced protease extraction from various valuable plant sources as the proteases are identified as therapeutic targets for numerous diseases and industrial applications. Therefore, the existing knowledge about the proteases when coupled with protein engineering techniques may lead to the development in identification of novel potential proteases.

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International Journal of Pharmaceutical Sciences Review and Research
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275

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