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Protease production by newly isolated *Bacillus Sp.*: Statistical media optimization

A.V. N Gupta[1]*, S. Emmanuel[2] and M. Lakshminaras[3]

[1]*Department of Biotechnology, S S & N College, Narasaraopet, India*
[2]*Andhra Loyola College, Hyderabad, India*
[3]*Department of Biotechnology, Jawaharlal Nehru Technological University, Hyderabad, India*

Abstract

Impact of different carbon and nitrogen sources on protease production byBacillus sp. was investigated using Plackett Burman (PB) method. The inoculum concentration, incubation time, incubation temperature, medium pH, aeration and agitation were evaluated by conventional one factor at a time (OFAT) method. Fractional Factorial Central Composite Design (FFCCD) of Response Surface Methodology (RSM) was used to further optimize with respect to glucose (carbon source), soyabean meal (nitrogen source), inoculum concentration, medium volume, temperature and initial medium pH. High determination co-efficient (R^2 = 0.9165) and lower value of the coefficient of variation (CV = 8.19%) indicated a better precision and reliability of experimental data. ANOVA indicated linear effect by inoculum concentration whereas; incubation temperature vs volume, pH vs volume and volume vs inoculum size were more significant for protease production at interactive level. Response surface data revealed medium pH, glucose and soyabean meal concentrations were most significant with respect to inoculum concentration. Software predicted optimized conditions were validated at shake flask and improved protease production to the tune of 37 % was observed. Incubation temperature and medium volume depicted maximum interactive influence among all selected factors. Glucose, soyabean meal and medium pH play critical regulatory role in protease production by this isolate. Considerable improvement of enzyme production 37 % was noticed at optimized environment at shake flask. Carbon and nitrogen source ratio and medium pH were the major regulatory factors in protease production by Bacillus sp. Significant improvement 37% in enzyme production was achieved at optimized environment. Among all selected fermentation parameters, medium volume was the most interactive factor and showed significant interaction with inoculum concentration and medium pH while inoculum concentration had linear effect on enzyme production.

Key Words: Protease, *Bacillus* sp., Fermentation, Optimization, RSM.

INTRODUCTION

Proteases, especially alkaline, constitute 60–65% of the global industrial enzyme market [1]. In fact it is reported that the global proteolytic enzyme demand will increase dramatically to 1.0–1.2 billon dollars[2] because of their application potential in several industrial sectors especially food, meat tenderization, peptide synthesis, for infant formula preparations, baking and brewing, pharmaceuticals and medical diagnosis, detergent industry as additives, as well as in textile for process of dehairing [3]. Alkaline proteases belong to the group of proteases, which have either a serine center or metallo-type, exhibiting a wide pH range (6–13). Among these, the serine proteases have industrial importance [4] such as production of high nutritional value fish hydrolysate using *B. subtilis* protease [5], up-gradation of lean meat waste to edible products [6] and in the enzymatic modification of zein to produce a non-bitter peptide fraction with high Fischer ratio for patients with hepatic encephalopathy [7].

Among all protease producing microbial systems, microbes belonging to *Bacillus* genus gained importance due to their potential in production of these enzymes extracellularly under submerged fermentation conditions[8-11]. It was well documented that each microbial strain differs in its enzyme production character which mainly depend on fermentation, nutritional, physiological and genetic nature [4]. The composition of fermentation media plays an important role in the production of primary and secondary metabolites [9-12]. Moreover, the production characteristics would offer a competitive advantage over existing products. Discovering such species, producing proteases with novel characteristics will be of great value to the enzyme industry for different applications. Hence, designing of an appropriate fermentation medium was of critical importance as medium composition, product concentration, yield, and volumetric productivity [13] influences the product productivity. To achieve basic industrial objective of production capabilities of any new strain will be possible when the isolated strain was characterized for its growth and enzyme synthesis potential. This can be achieved by optimizing the different fermentation parameters.

Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiological and nutritional parameters [14-20]. Prior knowledge and understanding of fermentation parameters were necessary for achieving a more realistic model. In the present study, an isolated and identified bacterial strain, *Bacillus*, which was characterized for alkaline protease production under solid-state fermentation was used. Based on the results obtained by the classical approach, parameters found significantly affecting protease production from preliminary experimentation were taken into account. A 2[6-2] fractional-factorial central composite design (FFCCD) of RSM was used for optimization of medium components for the maximal production of protease under submerged fermentation. The regression analysis was performed to obtain the optimum medium concentration.

MATERIALS AND METHODS

Microorganism and culture conditions

A laboratory bacterial isolate identified as *Bacillus* sp. was used in this study. This microbial strain was grown on a medium consisting of (in g/l) yeast extract - 7.5, peptone - 7.5 and glucose - 10 at pH 9.0 by incubating at 33^{O}C and at 150 rpm in an orbital shaker (LabTech LSI – 3016

R). The organism was maintained on agar 20 (g/l) based above medium slants by sub-culturing at monthly intervals and stored at 4°C till further use.

Selection of production medium components

For selection of the best medium components and their concentrations, carbon (maltose, glucose, xylose, ribose, arabinose, mannose, sucrose, cellulose, starch fructose and galactose), nitrogen (potassium nitrate, ammonium sulphate, ammonium chloride, ammonium nitrate, yeast extact, beef extract, ammonium thiocynate, casein, corn steep liquor, typtone, ammonium sulphate, peptone, sodium nitrate, urea and soya bean meal) sources were selected based on Plackett-Burman approach. The selected carbon and nitrogen sources were replaced with glucose, yeast extract and peptone in growth medium. Whereas other factors such as pH, incubation temperature, inoculum concentration and medium volume were screened and selected based on classical approach. All these experiments were carried in Erlenmeyer flasks of 250 ml containing 100 ml of medium were inoculated with 1% of inoculum ($A_{600\ nm}$ at 1.00) and incubated at 33°C under shaking (150 rpm) unless otherwise stated. Protease yield was determined after 24 h of fermentation period.

Measurement of enzyme activity

Protease activity in the fermented sample was determined using modified Auson–Hagihara method [21]. In this method 1 ml of the diluted clear fermentation broth was added to 1 ml casein solution (1%, w/v casein solution prepared in 50 mM glycine–NaOH buffer, pH 11) and incubated at 70 °C for 20 min. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid and the contents were filtered through a Whatman No. 1 filter paper. The absorbance of the filtrate was read at 280 nm using UV–Visible spectrophotometer (Perklin-Emler λ25) and the protease activity was calculated using tyrosine standard curve. One unit of protease activity was defined as 1 μg of tyrosine liberated ml^{-1} under the assay conditions.

Experimental Design and Optimization

The optimum concentrations of production medium components for the protease production by *Bacillus* sp. were determined by means of RSM. The RSM consists of a group of empirical techniques devoted to the evaluation of relationships existing between a cluster of controlled experimental factors and measured responses according to one or more selected criteria[22]. According to this design, the total number of experimental combinations was

$$2\wedge k + 2*k + n0$$

- k is the number of independent variables
- $n0$ is the number of repetitions of the experiments at the center point

Based on the best results of above medium components selection approach, six critical components of the production medium were selected and further evaluated for their interactive behaviors by using a statistical approach. The levels of six medium variables *viz.* Glucose, 10 g/l ($x1$); soyabean meal, 10 g/l ($x2$); Temperature, 33 ^{o}C ($x3$); pH, 10 ($x4$ volume, 75 ml ($x5$) and inoculum size, 3% ($x6$) were selected and each of the variables were coded at five levels –2, –1, 0, 1, and 2 by using Eq. 1.

For statistical calculations, the variables *Xi* were coded as *xi* according to the following transformation.
The range and levels of the variables in coded units for RSM studies are given in Table 1.

$$x_i = X_i - X_0/\Delta X \text{-------- (1)}$$

- *xi* = dimensionless coded value of the variable X_i
- X_0 = value of the X_i at the center point
- ΔX = step change.

The behavior of the system was explained by the following quadratic model 2.

$$Y = \beta_0 + \sum \beta_i * x_i + \sum \beta_{ii} * x_i^2 + \sum \beta_{ij} * x_{ij} \text{ --------(2)}$$

- *Y* = predicted response
- β_0 = intercept term
- β_i = linear effect
- β_{ii} = squared effect
- βij = interaction effect

The full quadratic equation for four factors is given by model 3.

$$Y = \beta_0 + \beta_1 x1 + \beta_2 x2 + \beta_4 x3 + \beta_4 x4 + \beta_5 x5 + \beta_6 x6 + \beta_{11} x1*x1 + \beta_{12} x1*x2 + \beta_{13} x1*x3 + \beta_{14} x1*x\ 4 + \beta_{15} x1*x5 + \beta_{16} x1*x6 + \beta_{22} x2*x2 + \beta_{23} x2*x3 + \beta_{24} x2*x\ 4 + \beta_{25} x2*x5 + \beta_{26} x2*x6 + \beta_{33} x3*x3 + \beta_{34} x3*x4 + \beta_{35} x3*x5 + \beta_{36} x3*x6 + \beta_{44} x4*x4 + \beta_{45} x4*x5 + \beta_{46} x4*x6 + \beta_{55} x5*x5 + \beta_{56} x5*x6 + \beta_{66} x6*x6 \text{ ----------(3)}$$

Several experimental designs were considered for studying such models and FFCCD was selected based on its simplicity [23]. For this study, a 2[6-2] factorial design with twelve star points and six replicates at the central points was employed to fit the second order polynomial model, which indicated that 34 experiments were required for this procedure. STATISTICA 6.0 (Stat Soft, Inc, Tulsa, OK) software was used for regression and graphical analysis of the data obtained.

In order to search for the optimum combination of major components of the production medium, experiments were performed according to the FFCCD experimental plan (Table 2). The results of FFCCD experiments for studying the effect of six independent variables were presented along with the mean predicted and observed responses in Table 3. The regression equations obtained after the analysis of variance (ANOVA) gave the level of protease production as a function of the initial values of glucose, soyabean meal, incubation temperature, pH, volume and inoculum concentration. Validation experiments were performed at shake flask level and two-liter bioreactor (B-Braun) at software predicted optimized medium conditions.

RESULTS

The influence of different fermentation parameters such as carbon source, nitrogen source, medium pH, incubation temperature, inoculum size and medium volume were optimized by conventional method changing one factor at a time using growth medium. Maximum enzyme production (3245 U/ml) was observed after 24 h of incubation. Whereas, selected carbon and nitrogen sources influence on protease production by this *Bacillus* sp. was investigated using Plackett-Berman design (Fig 1 and 2) under submerged fermentation conditions. The data indicated that the enzyme production affected the type of carbon and nitrogen and their concentration in the fermentation medium as reported in literature in different microbial systems [4,8,24]. Maximum protease production by this strain was observed with galactose followed by glucose as carbon source (Fig 1). While in case of nitrogen sources, soya bean meal and peptone supplementation showed maximum enzyme production (Fig 2). The observed difference in enzyme production was very less i.e., <5 and <2% in case of carbon (between galactose and glucose) and nitrogen (between soya bean meal and peptone) sources, respectively. Considering the cost and availability of galactose and glucose, glucose was considered as carbon source for further optimization. In case of nitrogen sources, soya bean meal as nitrogen source was selected as one of the optimization parameters using RSM. The six independent variables, glucose, soya bean meal, incubation temperature, medium pH, medium volume in 250 ml conical flask (volume) and inoculum concentration were chosen to optimize the protease production by *Bacillus* sp. Selected ranges of all these fermentation parameters are depicted in Table 1. Table 2 depicts the FFCCD experimental design layout and experimental results. A predicted value for each performed experiment was calculated and the correlation between experimental and predicted values is shown in Fig 3.

The above results were analyzed using statistical programme and the coefficient of determination (R^2) was calculated as 0.9165 for protease production by this *Bacillus* sp. (Model summary, Table 3), indicating that the statistical model can explain 91.65% of variability in the response and only 8.35% of the total variations were not explained by the model. The R^2 value should always be between 0 and 1. If the R^2 is closer to 1.0, the stronger the model and the better it predicts the response [25]. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (R^2 = 0.8032) was also very high suggesting a higher significance of the model used for analyzing the data [22,26]. In this enzyme production study the adjusted R^2 value (0.8032) was lesser than the R^2 value (0.9165). This is because, if there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. At the same time, a relatively lower value of the coefficient of variation (CV=8.19%) indicated a better precision and reliability of the experiments carried out.

The protease experimental data was analyzed by applying multiple regression and the results of the FFCCD design were fitted with a second order full polynomial equation. The empirical relationship between protease production (Y) and the four test variables in coded units obtained by the application of RSM is given by equation 4.

Y = 6688.395 + 200.74 * x1 + 20.49 * x2 - 76.50 * x3 - 102.71 * x4 - 122.15 * x5 + 411.15 * x6 - 194.56 * x1x1 - 296.55 * x2x2 - 252.50 * x2x5 - 565.43 * x3x3 - 263.82 * x3x4 - 480.04 * x3x5 - 489.22 * x3x6 - 499.22 * x4x4 - 408.70 * x4x5 - 282.63 * x4x6 - 203.12 * x5x5 - 463.34 * x5x6 - 243.98 * x6x6 ---------- (4)

Where *Y*, protease production in U/ml, was response and glucose, 10 g/l (*x*1); soyabean meal, 10 g/l (*x2*); Temperature, 33 ^{o}C (*x3*); pH, 10 (*x*4) volume, 75 ml in 250 ml conical flask (*x*5) and inoculum size, 3.0% (*x*6) were the coded values of the test variables.

The ANOVA was conducted for the second order response surface model. The significance of each coefficient was determined by Student's *t*-test and *p*-values, which were listed in Table 3 and 4. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient [22,25,26]. This implied that the linear effects of inoculum size ($p < 0.00218$) and interactive effects of temperature and volume ($p < 0.003083$), pH and volume and ($p < 0.008849$) volume and inoculum size ($p < 0.003945$) were more significant than the other factors, i.e., ($p < 0.05$). The model F-value of 8.019, and values of probability > F (<0.05) indicated that the model terms were significant.

The regression model developed was represented in the form of 2D and 3D surface and contour plots. The yields of protease for different concentrations of variables could also be predicted from the respective contour plots as shown in Figures 1–8. Each contour curve represents an infinite number of combinations of tow test variables with the other two maintained at their respective 0 level. It was evident from the contour curves that the protease production was highly and interactively influenced by all selected fermentation parameters (Fig 3 and 4). An interesting enzyme productivity status was noticed with the interactive state of carbon and nitrogen source supplemented environment. The enzyme production was inhibited by higher concentrations of these two nutrients (in the selected range) and more enzyme yield was observed when one of these nutrient concentrations was below critical level (0 level at selected concentrations) and other at above critical level (below '0' level as per present experiment) (Fig 3D). Moreover, the figure also suggested that for achieving maximum enzyme production, glucose concentration should be more than 2.5% while soya bean meal concentration should be low (below 1.0%) (Fig 3D). Moreover, higher enzyme production was also observed in controlling the ratio of soyabean meal and glucose. This can be evidenced from the figure 3D that protease production improved by supplementation of glucose in the range of 1.0% while increasing the soyabean meal concentration ranging from 0.5 to above 2.0%. Similarly, the interaction influence of soyabean meal with inoculum concentration was observed to be interesting (Fig 3C). Maximum enzyme production could be achieved with soyabean meal supplementation in the range of 0.2–1.0% with 3 – 8% inoculum concentration. Though influence of increased inoculum on enhanced enzyme production was well reported in the literature [8], however, soya bean meal concentration regulated productivity as observed in this study was not documented. Similar trend was also noticed with incubation temperature and volume of the medium versus inoculum level (Fig 3A and 3B).

The interactive influence of glucose as carbon source with respect to medium pH and inoculum level in this microbial strain was depicted in Fig 4 (A and B). Glucose showed positive effect on protease production at lower level of medium pH (7 – 10 pH range) than optimized pH value

(Fig 4A) i.e., at individual level maximum enzyme production was observed at 10 pH. However, improved enzyme production at higher glucose and inoculum levels was expected (Fig 4B) as glucose as carbon source provides more carbon skeletons and energy for any metabolite production. Similar trend was noticed with medium pH and inoculum level (Fig 4D) in the investigated range. Optimum enzyme production was depicted with selected range of medium pH and incubation temperature at central points of optimization process (Fig 4C). The above data indicated that interaction among fermentation parameters was one of the important aspects in achieving optimum productivity of any metabolite and further improvement in enzyme production is possible with regulation of interactive influences between selected fermentation parameters.

A numerical method given by Myers and Montgomery [23] was used to solve the regression equation 4. The optimal values of the six test variables in coded units were observed to be x1=0.24, x2=0.46, x3=0.53, x4=-0.35, x5=0.32 and x6=2.2. The predicted value of Y (U/ml) at these values of x was 7886 U/ml. The real values of the six test variables were obtained by substituting the respective coded values in equation 1.

The optimum conditions of selected fermentation parameters were predicted using RSM and the maximum predicted protease production (7886 U/ml) could be achieved with the medium consisting of (g/l) glucose -1.12 and soyabean meal – 1.23 at 34.6^{O}C at pH 9.65 with medium volume of 83 ml in 250 ml flask and 5.2 ml of initial inoculum concentration of 18 hours culture having absorbance of 0.8 at 600 nm. The validation experimental protease production data revealed 7717 and 9685 U/ml under optimized conditions indicating an overall improvement of 137 and 198% after optimization at shake flask and bioreactor conditions, respectively. The experimental value of the protease production was almost equal if we consider 95% of the confidence limits for the prediction of Y value at optimized conditions with shake flask results. Further validation was performed for optimized fermentation parameters. Incubation temperature and fermentation medium pH was maintained at 34.6^{O}C and 9.65, respectively throughout the fermentation period. Maximum enzyme production under validation conditions was found to be 9685 U/ml at 24 h. The optimization of medium components and process conditions will be efficient and cost effective at industrial level production.

DISCUSSION

Increasing emphasis on the environmental protection, the use of biocatalysts gained considerable attention in this biotechnological era. Extremozymes are now-a-day replacing chemical catalyst in manufacturing of chemicals, textiles, pharmaceuticals, paper, food and agricultural chemicals. Microbial protease constitutes one of the most important groups of industrial enzymes, accounting for at least 25% of the total enzyme market. Among different proteases, emphasis was given to on protease and several microbes have been tested for their ability to secrete these enzymes [8-11,24,29]. Industrial potential of any new microbial strain will be known when it is characterized for production capabilities. The classical method of experimental optimization involves changing one variable at a time keeping the others constant, which is tedious, laborious, time-taking, cost intensive and is not practical to carry out experiments with every possible factorial combination of the test variables. In addition, this does not consider the interactions effect of various parameters. An alternative and more efficient approach is the use of statistical

method, which would be crucial to develop a process for the maximum production of protease and standardization of media components[11].

The RSM is an effective and sequential and stepwise procedure. The lead objective of the RSM was to run rapidly and efficiently along the path of improvement towards the general vicinity of the optimum. It is appropriate when the optimal region for running the process was identified before performing RSM experiments. It was reported that effects of a specific carbon and nitrogen supplement on protease production differ from organism to organism although complex nitrogen sources are usually used for protease production [8,24].

Protease production in most of the microbial strains was regulated by several fermentation factors [8,11]. In general it was noticed that higher concentration of any carbon source results in reduction in protease production in variety of protease producing microbial systems due to catabolic repression of glucose. In alkalophillic *actinomyces* sp. supplementation of more than 0.5% of carbon source resulted in decreased protease production [28] (> 60%). Such carbon source mediated repression also observed in *Arthrobacter* sp.[29] and alkaliphilic *Bacillus* sp.[30]. However, it was evident from figure 3D that glucose mediated regulation of enzyme production was fine tuned with other nutrient sources. This is the first report of its kind where soya bean meal concentration mediated regulation of protease production in presence of glucose as carbon source or vice versa. It was evident from the surface response figure 3D that, available carbon and nitrogen source ratio was the critical factor in enzyme production in this microbial strain and more production was noticed in low concentrations of soyabean meal (Fig 3D). However, enhanced protease production was reported with soya bean meal [31] and corn steep liquor[32] as nitrogen sources. Comparatively, in this isolate higher concentration of nitrogen source (> 1.0%) in presence of higher glucose concentration (1-2.5%) reduced protease production. This might be due to the fact that, nitrogen sources known to affect the enzyme secretion [33]. Similar results were observed in *B. firmus [34]* when present in higher concentrations. However, the influence of glucose concentration in reduction of medium pH was not ruled out as Glucose dependent protease production was observed in several microbial strains [4,8,10]. These types of nutritional regulatory key aspects are very important in large-scale production where enzyme yield will be continuous even in limited availability of one of the nutrients.

Table 1: Range and Levels of the fermentation variables in coded units for alkaline protease production by isolated *Bacillus* sp.

Parameter/level	**-2**	**-1**	**0**	**1**	**2**	**Δ x**
Glucose, X 1, (gm/100 ml)	0.25	0.5	1.0	1.5	2.5	0.5
Soyabean meal X 2, (gm/100) ml	0.25	0.5	1.0	1.5	2.5	0.5
Temp(^{o}C) X 3	27	30	33	36	39	3
pH, X 4	8	9	10	11	12	1
Volume, X 5, (ml)	25	50	75	100	125	25
Inoculum size, X 6, (ml)	1.0	2.0	3.0	4.0	5.0	1.0

Validation experimental data suggested that, protease production by this isolated bacterial strain could be achieved 37% more under optimized conditions compared to initial fermentation environment at shake flask.

Table 2: Protease production Design of Experiments by Fractional Factorial Central Composite Design (CCD) for RSM Studies

Run number	x1	x2	x3	x4	x5	x6	Co-efficient assessed by FFCCD	Protease production (U/ml)	
								Measured	Predicted
1	-1	-1	-1	-1	-1	-1	2^{6-2} fractional factorial points (16 experiments)	1560	1714.196
2	1	-1	-1	-1	1	-1		5236	5933.415
3	-1	1	-1	-1	1	1		5500	5918.822
4	1	1	-1	-1	-1	1		5586	7409.165
5	-1	-1	1	-1	1	1		4948	6459.677
6	1	-1	1	-1	-1	1		5750	7467.352
7	-1	1	1	-1	-1	-1		4600	6496.108
8	1	1	1	-1	1	-1		5318	5908.304
9	-1	-1	-1	1	-1	1		5500	6480.003
10	1	-1	-1	1	1	1		5660	7382.189
11	-1	1	-1	1	1	-1		4527	5987.810
12	1	1	-1	1	-1	-1		4200	6271.739
13	-1	-1	1	1	1	-1		4682	5541.641
14	1	-1	1	1	-1	-1		5460	5342.902
15	-1	1	1	1	-1	1		5500	6281.083
16	1	1	1	1	1	1		2757	2376.245
17	-2	0	0	0	0	0	2x6 star points (12 experiments)	5265	5508.635
18	2	0	0	0	0	0		6100	6311.628
19	0	-2	0	0	0	0		4950	4727.243
20	0	2	0	0	0	0		5600	4809.219
21	0	0	-2	0	0	0		4970	6028.908
22	0	0	2	0	0	0		3428	5722.901
23	0	0	0	-2	0	0		5027	6893.824
24	0	0	0	2	0	0		3900	6482.967
25	0	0	0	0	-2	0		6500	6932.701
26	0	0	0	0	2	0		4797	6444.090
27	0	0	0	0	0	-2		4423	5866.093
28	0	0	0	0	0	2		6547	7510.698
29	0	0	0	0	0	0	6 central points	6760	6688.395
30	0	0	0	0	0	0		6901	6688.395
31	0	0	0	0	0	0		6890	6688.395
32	0	0	0	0	0	0		6802	6688.395
33	0	0	0	0	0	0		6790	6688.395
34	0	0	0	0	0	0		6896	6688.395

Table 3: Summary data of protease production by isolated *Bacillus* sp.

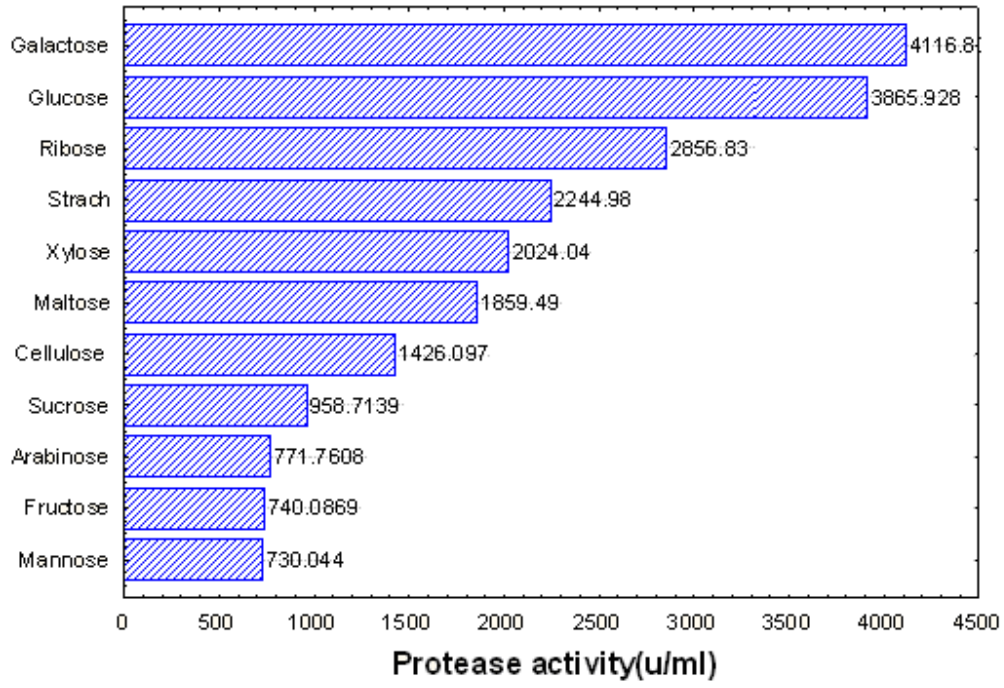
Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	6688.395	203.3553		32.89019	1.17E-14
	x1	200.7481	109.8244	0.141135	1.827900	0.088950
	x2	20.4939	109.8244	0.014408	0.186606	0.854647
	x3	-76.50168	109.8244	-0.053784	-0.696582	0.497468
	x4	-102.7142	109.8244	-0.072213	-0.935259	0.365508
	x5	-122.1527	109.8244	-0.085879	-1.112255	0.284764
	x6	411.1514	109.8244	0.289058	3.743715	**0.002180***
	x1x1	-194.566	96.79426	-0.155610	-2.010098	0.064096
	x2x2	-296.5597	96.79426	-0.237182	-3.063815	**0.008416***
	x2x5	-252.5092	134.5069	-0.144949	-1.877295	0.081472
	x3x3	-565.4308	96.79426	-0.452220	-5.841574	4.28E-05
	x3x4	-263.8274	134.5069	-0.151446	-1.961441	0.070027
	x3x5	-480.0411	134.5069	-0.275560	-3.568895	**0.003083***
	x3x6	-489.2228	134.5069	-0.280831	-3.637157	**0.002693***
	x4x4	-499.226	96.79426	-0.399271	-5.157599	**0.000145***
	x4x5	-408.7024	134.5069	-0.234609	-3.038523	**0.008849***
	x4x6	-282.6342	134.5069	-0.162242	-2.101261	0.054207
	x5x5	-203.1227	96.79426	-0.162453	-2.098500	0.054484
	x5x6	-463.3478	134.5069	-0.265977	-3.444788	**0.003945***
	x6x6	-243.9848	96.79426	-0.195134	-2.520654	0.024470

** Values are significant at 95% confidence limits (p < 0.05)*

Table 4: ANOVA table for data obtained for protease production by isolated *Bacillus* sp.

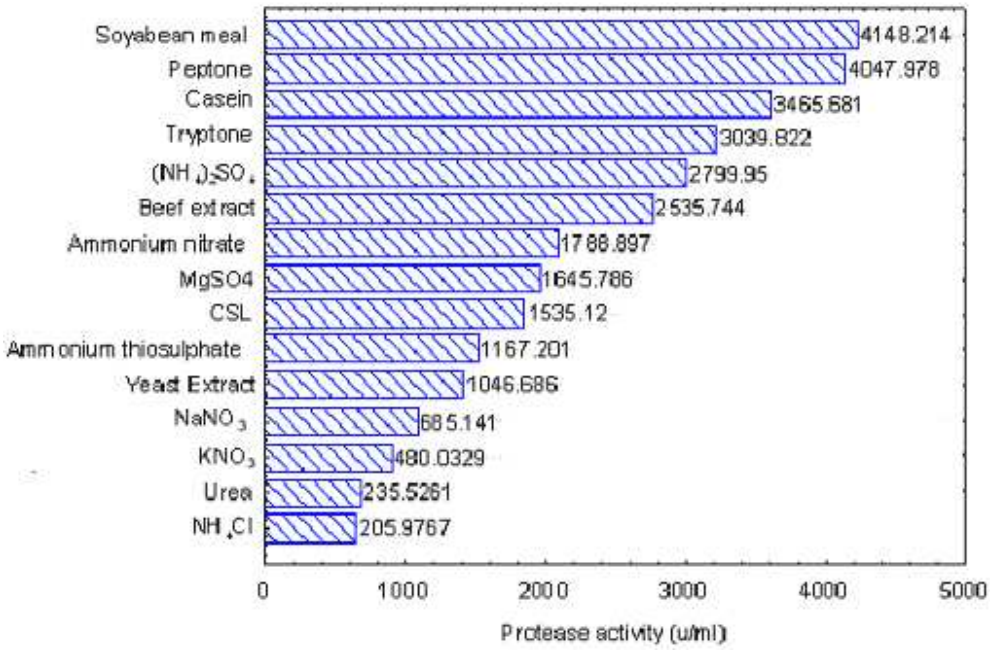
Model	Sum of Squares	Degree of Freedom (DF)	Mean Square	F	Significance
Regression	44503576	19	2342293	8.091557	0.000128
Residual	4052633	14	289473.8		
Total	48556209	33			
R= 0.95736; CV=8.19%; R Square=0.916537; Adjusted R Square= 0.803266; Std. Error of the Estimate= 538.0277					

Fig 1: Influence of different carbon sources on protease production by Isolated *Bacillus* sp.



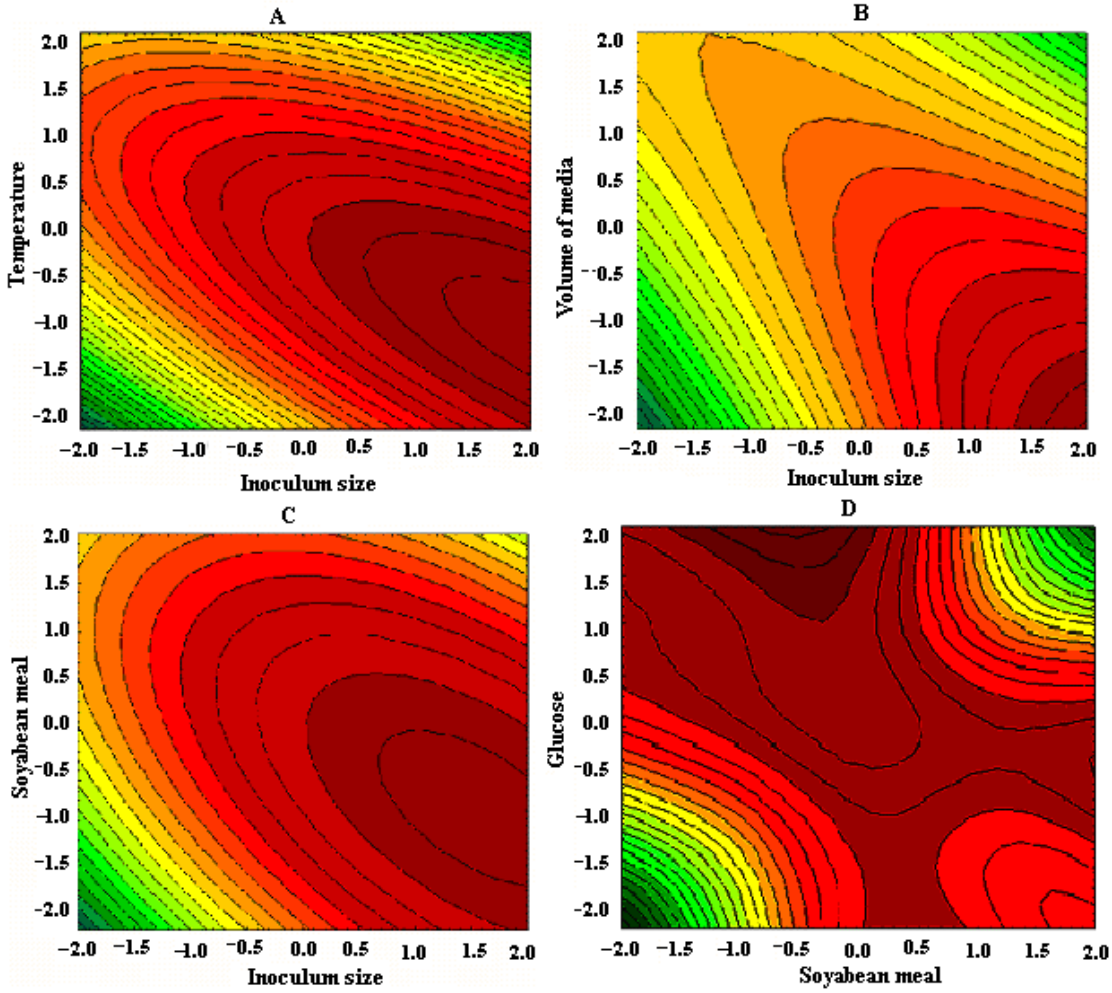
"X" axis represents protease activity (U/ml) and "Y" axis represents different carbon sources.

Fig 2: Influence of different nitrogen sources on protease production by isolated *Bacillus* sp. under submerged fermentation



"X" axis represents protease activity (U/ml) and "Y" axis represents different nitrogen sources.

Fig 3: Contour graphs for protease production by *Bacillus* sp.



"X" and "Y" axis represents assigned levels of selected factor. The plot area color represents the protease activity (U/ml).

A) X- Inoculum size, Y-Temperature

1000 2000 3000 4000 5000 6000

B) X-Inoculum size, Y- Volume of media

2000 3000 4000 5000 6000 7000

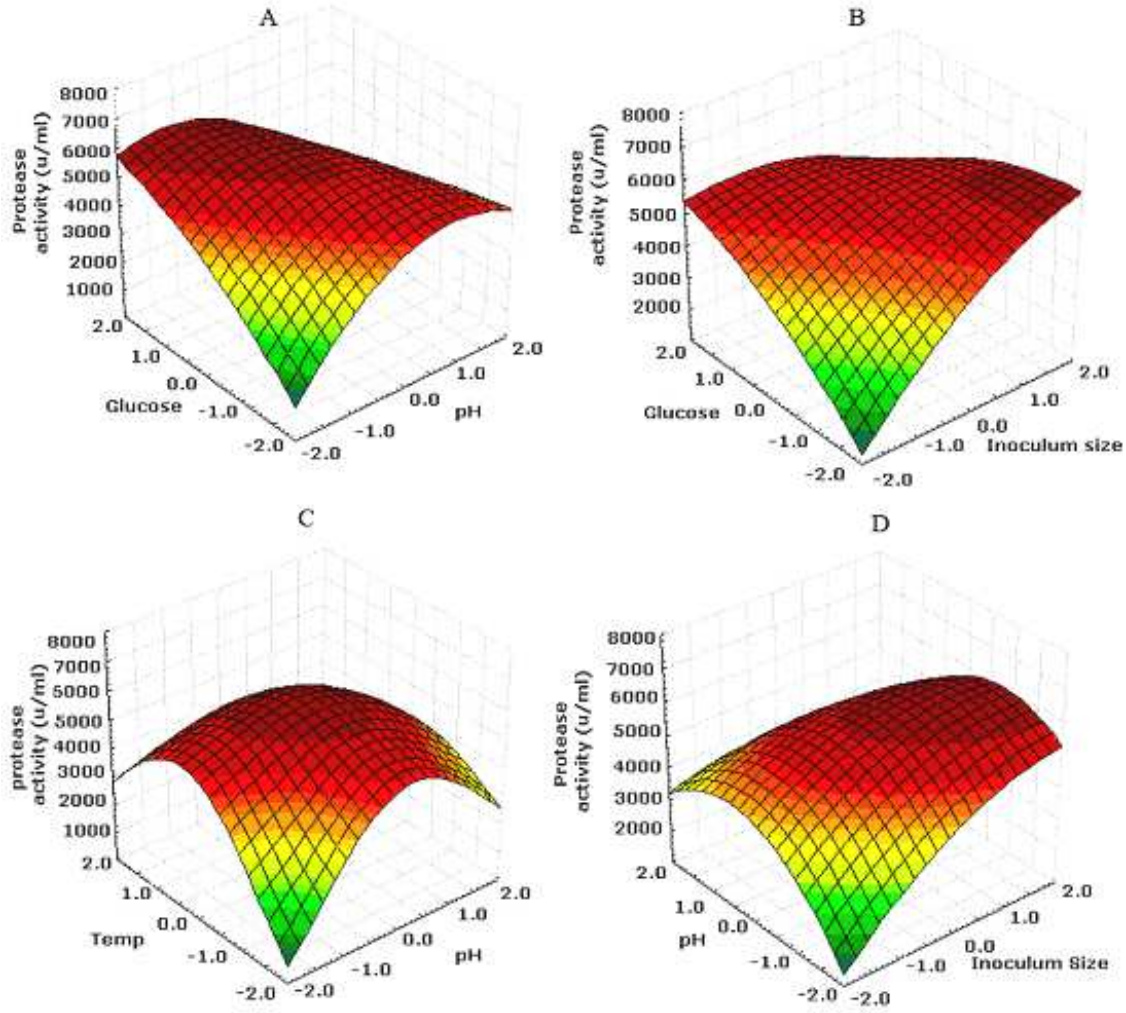
C) X-Inoculum size, Y-Soya bean meal

1000 2000 3000 4000 5000 6000

D) X-Soya bean meal, Y-Glucose

1000 2000 3000 4000 5000 6000

Fig 4: **Response surface graphs for protease production by *Bacillus* sp.**



"X", "Y" axis represents assigned levels of selected factor and Z-axis represents the protease activity (U/ml).

A) X- pH, Y-Glucose, B) X- Inoculum size, Y-Glucose, C) X-pH, Y-Temp,

D) X-Inoculum size, Y- pH.

1000 2000 3000 4000 5000 6000

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