International Journal of Molecular and Clinical Microbiology 2 (2013) 325-333

International Journal of
Molecular and Clinical Microbiology

Response of surface optimization for the enhanced production of alkaline protease isolated from *Bacillus* sp. with bean husk as a new substrate

Masoumeh Anvari [1*] (Ph.D), Mehdi Shahriari Nor [2] (Ph.D) and Mohammad Reza Safari [1](Ph.D)

1 Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, P.O. Box 41335-3516, Rasht, Iran*
2) Department of Microbiology, Sciences and research branch, Islamic Azad University, Rasht Iran

ARTICLE INFO

Article history:
Received 2 June 2013
Accepted 17 November 2013
Available online 1 December 2013

Keywords:
Alkaline protease,
Optimization,
Bacillus sp.,
Solid state fermentation

ABSTRACT

Optimization of the fermentation medium for maximum alkaline protease production was carried out. Fifteen positive isolates were examined for their extent of alkaline protease production. The most potent producer was identified as *Bacillus* sp. The solid substrate screening showed that the combination of wheat straw and bean husk was the best one. The initial screening by using Plackett–Burman's design demonstrated that among the tested factors, casein, ammonium sulphate and pepton as the nitrogen sources and glucose, lactose and sucrose as carbon sources, glucose and casein significantly ($P < 0.05$) enhanced the protease production in combinatory solid state fermentation (SSF). Further optimization of protease production by *Bacillus* sp. strain k7 on different factors such as incubation time (84 h), inoculums size (64%), initial moisture content (97%), buffer volume (4.9%) in SSF by applying RSM was achieved.

1. Introduction

Alkaline proteases are one of the most widely studied groups of enzymes because of their use in food, pharmaceutical, leather and detergent industries (Singh et al., 2004). Alkaline proteases are produced by a wide range of microorganisms including bacteria, mould, yeast and also mammalian tissues. Currently, a large proportion of commercially available alkaline proteases such as other bio-industrial products are derived from many fungal and bacterial strains (Anvari and Khayati, 2009a). Bacteria of the genus *Bacillus* are active producers of extracellular alkaline proteases (Singh et al., 1999). They accounted for 40% of the total worldwide enzymes sales and 89% of the total protease sale and this trend are expected to increase in near future (Ellaiah et al., 2002). Proteases have been produced in submerged (SmF) and solid-state fermentations (SSF) (Sandhya et al., 2005).

Each organism or strain has its own special conditions for maximum enzyme production. Therefore, optimization of medium composition has to be carried out to maintain a balance between the various medium components, thus minimizing the amount of unutilized

[*]Corresponding author: Dr. Anvar
E-mail address: anvari@iaurasht.ac.ir

components at the end of fermentation (Manivannan and Kathiresan, 2007). The conventional method of optimization involves varying one parameter at a time and keeping the others constant. This often does not bring about the effect of interaction of various parameters as compared to factorial design (Anvari and Khayati, 2009b).

The Plackett–Burman factorial designs allow the screening of main factors from a large number of process variables, and these designs are thus quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes. Response surface methodology (RSM) is a useful model for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. In addition, RSM is an efficient strategic experimental tool by which the optimal conditions of a multivariable system may be determined (Khayati and Kiyani, 2012). During the recent years, efforts have been directed to explore the means to reduce the enzyme production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural byproducts as substrate for protease production (Mukherjee et al., 2008).

Iran is one of the largest wheat producers in the world. Also Guilan and Mazandaran provinces are the most important proven in bean production in the country. There is 15 million tons wheat production annually with 20% waste and 5 million tons bean with 25% waste (Khayati and Kiyani, 2012). These are the locally available, inexpensive agro-wastes and kitchen wastes byproducts as optimal fermentation materials for alkaline protease production under SSF.

The present investigation aimed to study on bacterial alkaline protease production through submerged and solid-state fermentation and a comparative evaluation on the enzyme productivity by fermentation systems. Therefore, another objective of this study was statistical optimization of process parameters for enhancing the protease (fibrinolytic enzyme) yield by selected bacterium in submerged fermentation using Plackett–Burman's design followed by RSM.

2. Materials and Methods

2.1. Organisms

Alkalotrophic bacterial strains were isolated, from saline-alkali soils of the saline-alkali soils of the Guilan Iran. All the strains were screened for their alkaline protease production on milk agar medium containing (g/l): skim milk powder 28, yeast extract 2.5, agar 15,dextrose 1, pH 8.0 (adjusted after autoclaving). Fifteen isolates found positive for alkaline protease were tested again for their enzyme production.

2.2. Inoculum preparation

Inoculum was prepared by transferring 5ml suspension prepared from a 24 h old slant culture, into 250 ml Erlenmeyer flask containing 45 ml of sterile inoculum medium comprising glucose (2.0 g/L), casein (0.5 g/L), peptone (0.5 g/L), yeast extract (0.5 g/L) and salt solution (50 ml) (salt solution containing KH2PO4 5 g/L, MgSO4.7H2O g/L and FeSO4 .7 H2O, 0.1 g/L). The flask was kept on a rotary shaker at 220 rpm at 37°C.

2.3. Solid-state fermentation and optimization of process condition

Wheat Straw and bean husk were obtained from Ardebil and Guilan provinces respectively. The substrates were ground into coarse powder with a blender. 5 g of coarse substrate was taken in a 500 ml Erlenmeyer flask and a predetermined quantity of 50mM magnassium phosphate buffer, pH 9.4 was added, mixed thoroughly and autoclaved at 121°C, 15 psi pressure for 15 min.

In the next step, a preliminary screening study was conducted to explore the best co-source of carbon (glucose, lactose and sucrose) and nitrogen (casein, ammonium sulphate, yeast extract and pepton) those have stimulatory impact on protease production by Plackett–Burman's factorial design. Based on Laboratory design, each factor was examined at two levels: -1 for low level and +1 for high level, and a

center point was run to evaluate the linear and curvature effects of the variables (Table 1).

Plackett–Burman's experimental design is based on the first order polynomial model (Khayati and Kiyani, 2012):

$$Y = \beta_{\circ} + \sum_{i=1}^{k} \beta_i x_i \quad (1)$$

where Y is the predicted response, β0 and βi are constant coefficients, and xi is the coded independent variables or factors. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response.

2.4. Statistical optimization of protease production using response surface methodology

RSM was used to determine the optimum condition for protease production. The effect of four independent variables including incubation time (X1), initial moisture content (X2), inoculum size (X3), and buffer volume (X4) on the response variable (%Y, the enzyme activity) was evaluated using central composite design (CCD) (Table 2). The five coded levels of each variable were incorporated into the design and were analyzed in 31 experimental trails (Table 3). The central point of the design was repeated seven times to calculate the reproducibility of the method (Montgomery, 2001). For each experimental trail of the independent variables in the experimental design, the dependent parameter (the protease activity) was determined. The effect of these independent variables X1, X2, X3 and X4 on the response Y was investigated using the second-order polynomial regression equation. This equation, derived using RSM for the evaluation of the response variable which is as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x^2 + \sum_{i \neq j}^{k} \beta_{ij} x_i x_j \quad (2)$$

where β0 is defined as the constant, βi the linear coefficient, βii the quadratic coefficient and βij the interaction coefficient. xi and xj are the independent variables while k equals to the number of the tested factors (k =4). The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were analyzed statistically by computing the P-value at a probability of 0.05. The regression coefficients were then used to make statistical calculations to generate response surface and contour maps from the regression models. The analysis of data and the optimizing process were generated using Minitab statistical software version 15.

2.5. Enzyme extraction

The enzyme from the fermented bacterial Broth was extracted twice with 20 ml carbonate/ bicarbonate buffer (0.01 M; pH:10) and filtered. The pooled filtrate of two extractions was centrifuged (10.000×g for 15 min; 4°C) and used as the source of enzyme for assays (Uyar and Baysal, 2004).

2.6. Protease assay

Protease activity was determined by modified method using casein as substrate. Fifty microliters of crude protein was added to 450 µl of substrate solution (1%, v/v, casein with 50mM Tris–HCl buffer; pH 8.0) and incubated at 30°C for 30 min independently with respective to controls. The reaction was stopped by adding 750µl of 5% TCA mixture (5% TCA, 9% Na–acetate, 9% acetic acid) followed by 30 min at room temperature followed by centrifugation at 10,000 rpm for 15 min. The absorbance of supernatant was measured at 280 nm. One unit of enzyme activity was defined as the amount of enzyme which releases 1µmol of tyrosine per minute under the assay conditions. The amount of tyrosine was determined from the tyrosine standard curve (Potumarthi et al., 2007).

3. Results and Discussion

3.1. Screening of significant variables using Plackett–Burman design

Classical approach of medium optimization is a time-consuming and labor-intensive process. The Plackett–Burman experimental design proved to be a valuable tool for the rapid evaluation of the effects of the various medium components (Mukherjee et al., 2011). A total of seven variables were analyzed with regard to their effects on protease production using a

Plackett–Burman design (Table 1). The design matrix selected for the screening of significant variables for protease production and the corresponding responses are shown in Table 4. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's t-test for ANOVA (Table 5). Factors evidencing p-values of less than 0.05 were considered to have significant effects on the response. Casein, with a probability value of 0.010, was determined to be the most significant factor, followed by glucose (0.027). The lower probability values indicate the more significant factors on the production of alkaline protease. Both of the two significant screened variables, glucose and casein, exerted a positive effect. All other insignificant variables were neglected.

3.2. Optimization of variables using response surface methodology

3.2.1. Effects of *independent variables on responses*

Response surface were used to illustrate the effect of inoculums size, buffer volume, incubation time and initial moisture content on the protease production. The response surfaces and contour plots of enzyme production conditions are shown in Figures 1–3.

The effect of initial moisture and incubation time on the enzyme production is shown in Figure 1. The enzyme production was increased with the incubation time increasing to a certain value (approximately 70 h) and thereafter was constant. The incubation time is governed by characteristics of the culture and is based on growth rate and enzyme production. Many workers have reported a rapid decline in the enzyme yield after the optimum incubation time due to depletion of nutrients available to microorganisms or denaturation and/or decomposition of protease as a result of interactions with other compounds in the fermented medium (Uyar and Baysal, 2004). A prolonged incubation time beyond this period did not increase the enzyme yield.

The effect of inoculum size and incubation time on the enzyme production is shown in Fig. 2. A similar effect was observed to that of inoculum size (Figs. 2-3). With the increasing of inoculums size the enzyme production also was increased and then gradually was decreased. The higher enzyme production (4847U/g) was obtained at 64.4 (v/w) inoculums level for wheat straw and bean husk. However Sen (1995) reported a 10% inoculum level for the production of alkaline protease by *Bacillus licheniformis* S40. With the increase in inoculum level, the production of enzyme declined due to exhaustion of nutrients in the fermentation mash. In addition, the free excess liquid present in an unabsorbed form will give rise to an additional diffusion barrier together with that imposed by the solid nature of the substrate and lead to a decrease in enzyme production and growth (Nutan et al., 2002).

There was a direct relation between incubation time and enzyme production. Most production resulted in 84 h after incubation (Figs. 1-2).

A major crucial factor in the SSF system that influences the microbial growth as well as product yield is the initial moisture content of the substrate (Ramachandran et al., 2004). Since growth of microbes and product formation takes place at or near the surface of moist solid substrate (Pandey et al., 2007), thus, for achieving maximum yield of the desirable product, it is the most crucial step to optimize the moisture content that controls the wa of the fermenting substrate (Prakasham et al., 2006).

The effect of inoculum size and initial moisture on the enzyme production is shown in Fig. 3. With an increase in the initial moisture content of the substrate from 25% to 100% alkaline protease production was concomitantly enhanced, and further increase in the moisture content of substrate resulted in a steady decline in protease yield.

The effects of production conditions on the enzyme production by the regression coefficients of fitted second-order polynomial are presented in Table 6. It was evident that all the interaction terms and the linear terms except for buffer volume were significant (P-value < 0.05). The results indicated that the incubation time was the major contributing factor to protease production. Within the experimental range, however, buffer volume had no

significant effects (P-value > 0.05) on the protease production (Table 6).

3.3. Fitting the model and Response surface analysis

RSM was used to optimize protease production conditions and the experimental results were presented in Table 3. The enzyme production was analyzed to get a regression model. The mathematical model representing that as a function of the independent variables within the region under investigation was expressed by the following equation:

$$Y = -15384.4 + 272.8X1 + 33.1X2 + 136.8X3 - 1.5X11 - 0.1X22 - 1.4X33 - 51.9X44 + 1.5X24 \quad (4)$$

where Y is the enzyme production, whereas X1, X2, X3 and X4 are the independent variables for inoculums size, buffer volume, incubation time and initial moisture content, respectively.

In general, exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits a good fit, which makes checking of the model adequacy essential (Liyana-Pathirana and Shahidi, 2005). The P-value of the model was less than 0.001 (Table 7). This value confirmed that the model fitness was acceptable. Coefficient of determination (R2) is defined to be the ratio of the explained variation to the total variation and is a measurement of the degree of fitness (Nath and Chattopadhyay, 2007). The closer of R2 value to unity, the better the empirical models fits the actual data (Khayati and Kiyani, 2012). On the other hand, the lesser R2 value the less relevance the dependent variables in the model have in explaining the behavior of variations (Khayati and Kiyani, 2012). By analysis of variance, the R2 value of this model was determined to be 0.903. Therefore, the developed model could adequately represent the real relationship among the chosen parameters.

The enzyme production predicted values were calculated using the regression model and compared with the experimental values (Fig. 4). The figure showed that there was good agreement between model (Eq.4) and experimental data.

Conclusion

The statistical approach with rapid identification of important factors and development of a polynomial model showed significant results for optimizing protease production by isolated *Bacillus* sp. from wheat straw and bean husk. These data showed that protease activity influenced significantly by the glucose as carbon source and casein as nitrogen source in the medium. Maximum protease activity from the experimental was determined to be 4847 (U/g substrate) under the optimal conditions. SSF system showed its superiority for enzyme production and also revealed the possibile of effective utilization of wheat bran (other agro-industrial residues) matter of value in Biotechnology. Such processes would not only of help in reducing the cost of production, but also Solid waste management in order to give effective.

Table 1. Experimental variables at different levels used for the production of alkaline protease by *Bacillus* sp. RKY3 using Plackett–Burman design

Variables uncode	Units Lower	Symbol Higher	Experimental values	
glucose	(%w/w)	X_1	0.5	1.0
lactose	(%w/w)	X_2	0.5	1.0
sucrose	(%w/w)	X_3	0.5	1.0
caseine	(%w/w)	X_4	0.1	0.5
ammunium sulfate	(%w/w)	X_5	0.1	0.5
yeast extract	(%w/w)	X_6	0.1	0.5
pepton	(%w/w)	X_7	0.1	0.5

Table 2. Independent variables and their coded and actual levels

Independent variables (factors)	Symbol	Actual values of coded levels				
		-α	-1	0	+1	+α *
incubation time (h)	x1	36	48	60	72	84
initial moisture content (%)	x2	25	100	175	250	325
inoculum size (%)	x3	20	40	60	80	100
buffer volume (%)	x4	2.5	5	7.5	10	12.5

* α = 2.0 (star point for orthogonal CCD for the case of 4 independent variables)

Table 3. Central composite experimental design and responses

Run	Independent variables				response (Y %)
	x_1	x_2	x_3	x_4	
1	-1	-1	-1	-1	1525
2	1	-1	-1	-1	4025
3	-1	1	-1	-1	1850
4	1	1	-1	-1	1550
5	-1	-1	1	-1	2200
6	1	-1	1	-1	3825
7	-1	1	1	-1	650
8	1	1	1	-1	1950
9	-1	-1	-1	1	2325
10	1	-1	-1	1	2850
11	-1	1	-1	1	1800
12	1	1	-1	1	1950
13	-1	-1	1	1	1150
14	1	-1	1	1	2400
15	-1	1	1	1	1525
16	1	1	1	1	2375
17	-2	0	0	0	3850
18	2	0	0	0	3550
19	0	-2	0	0	3975
20	0	2	0	0	4075
21	0	0	-2	0	1700
22	0	0	2	0	4700
23	0	0	0	-2	2300
24	0	0	0	2	1400
25	0	0	0	0	1775
26	0	0	0	0	1850
27	0	0	0	0	2850
28	0	0	0	0	2625
29	0	0	0	0	3275
30	0	0	0	0	4375

Table 4. Twelve-trial Plackett–Burman design matrix for seven variables with actual values along with the observed and predicted protease activity

Run order	Experimental values							Protease activity (U/g)	
	x_1	x_2	x_3	x_4	x_5	x_6	x_7	Observed	Predicted
1	+1	-1	+1	-1	-1	-1	+1	324	192.83
2	+1	+1	-1	+1	-1	-1	-1	2025	1718.17
3	-1	+1	+1	-1	+1	-1	-1	225	231.83
4	+1	-1	+1	+1	-1	+1	-1	1284	1559.83
5	+1	+1	-1	+1	+1	-1	+1	550	856.83
6	+1	+1	+1	-1	+1	+1	-1	275	268.17
7	-1	+1	+1	+1	-1	+1	+1	775	668.17
8	-1	-1	+1	+1	+1	-1	+1	700	662.17
9	-1	-1	-1	+1	+1	+1	-1	1724	1592.83
10	+1	-1	-1	-1	+1	+1	+1	400	262.17
11	-1	+1	-1	-1	-1	+1	+1	125	231.83
12	-1	-1	-1	-1	-1	-1	-1	925	1087.17

Table 5. Estimated effect, regression coefficient and corresponding *t* and *P* values for protease activity in eight variable Plackett–Burman design experiment

Variables	Effect	Coefficient	Standard error	*t*-value	*P*-value
glucose	64.0	32.0	87.61	0.37	0.027
lactose	-230.3	-115.2	87.61	-1.31	0.259
sucrose	-361.0	-180.5	87.61	-2.06	0.108
caseine	797.3	398.7	87.61	4.55	0.010
ammunium sulfate	-264.0	-132.0	87.61	-1.51	0.206
yeast extract	-27.7	-13.8	87.61	-0.16	0.882
pepton	-597.3	-298.7	87.61	-3.41	0.733

Table 6. Significance of regression coefficients of the fitted second-order polynomial model for response (Y)

Term	Regression Coef.	SE Coef.	*P*- value
β_0	-15384.4	4393.18	0.004
Linear			
β_1	272.8	90.83	0.009
β_2	33.1	12.02	0.016
β_3	136.8	46.92	0.011
β_4	20.7	2.02	0.107
Quadratic			
β_{11}	-1.5	0.64	0.039
β_{22}	-0.1	0.02	<0.001
β_{33}	-1.4	0.23	<0.001
β_{44}	-51.9	14.67	0.003
Interaction			
β_{12}	-0.3	0.13	0.062
β_{13}	0.6	0.50	0.282
β_{14}	-4.9	4.00	0.241
β_{23}	0.0	0.08	0.798
β_{24}	1.5	0.64	0.034
β_{34}	-1.4	2.40	0.559

Table 7. Analysis of variance (ANOVA) of the regression parameters for the response surface model

Source	D. F.	Sum of Squares	Mean Square	*F*-value	*P*-value
Model	14	29842135	2131581	9.25	0.001
Residual	14	3227615	230544		
Lack of fit	10	2466990	246699	1.30	0.432
Pure error	4	760625	190158		
Total	29	33377917			

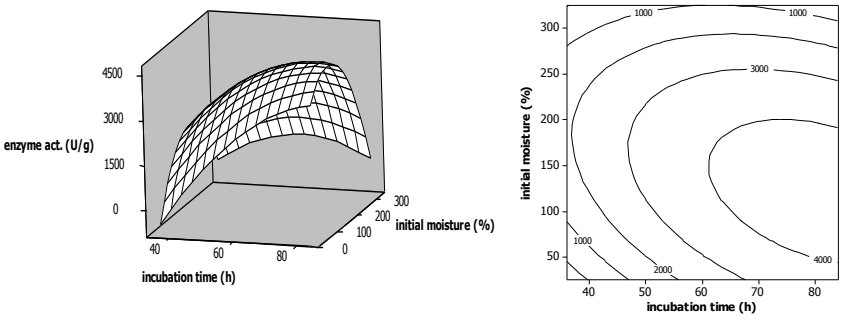

Fig 1. Response surface plot (upper) and its contour plot (lower) for the effects of incubation time (h) and initial moisture (%) on protease activity (*Y*)

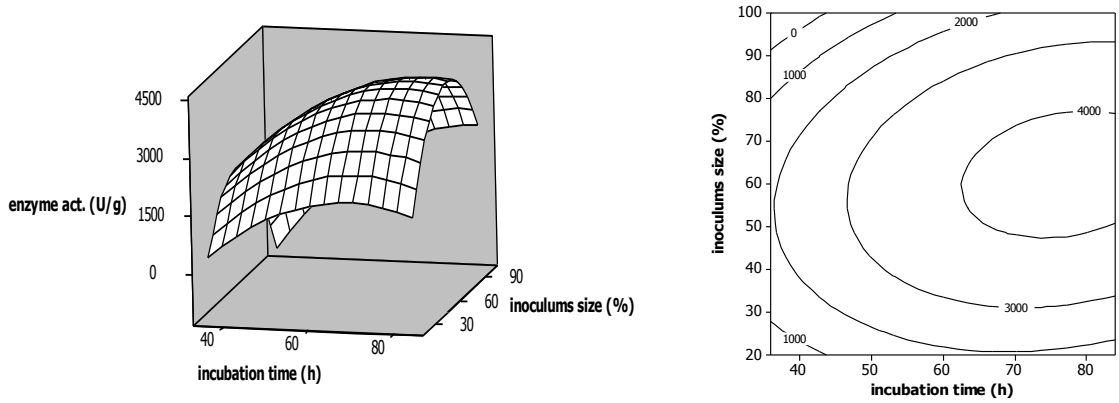

Fig 2. Response surface plot (upper) and its contour plot (lower) for the effects of incubation time (h) and inoculums size (%) on protease activity (*Y*)

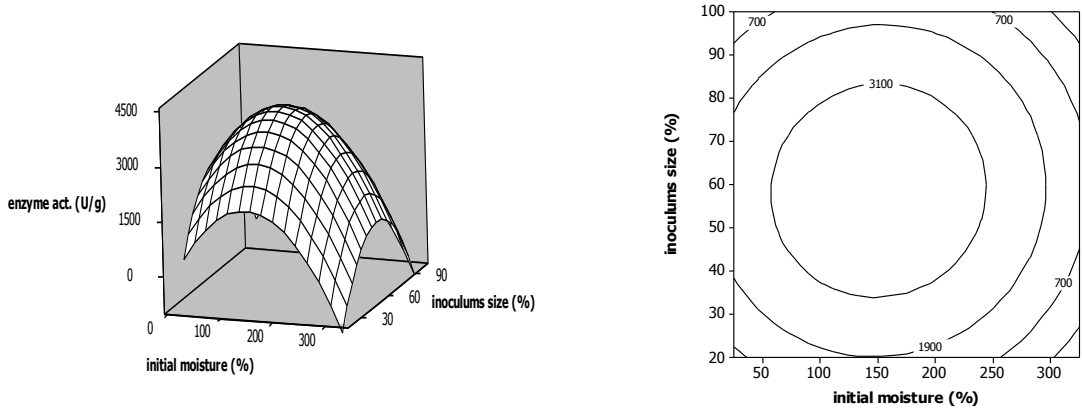

Fig 3. Response surface plot (upper) and its contour plot (lower) for the effects of initial moisture (%) and inoculums size (%) on protease activity (*Y*)

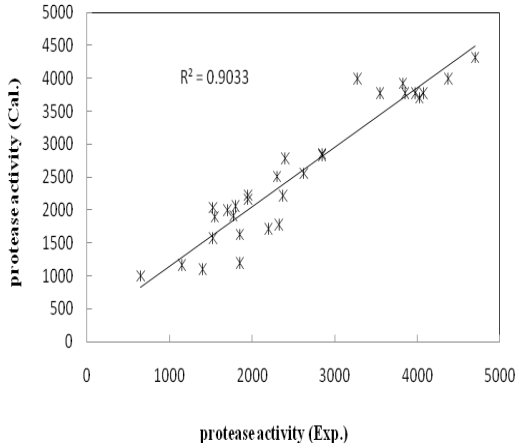

Fig 4. The relationship between the calculated protease activity and experimental data

Refereces

Anvari, M., and Khayati, G., 2009a. In situ recovery of 2,3-butanediol from fermentation by liquid–liquid extraction, J. Ind. Microbiol. Biotechnol. 36, 313–317.

Anvari, M., and Khayati, G., 2009b. Optimization of 2,3-Butanediol Production by Klebsiella pneumoniae PTCC 1290 Using Taguchi Methodology, Asian J. Chem. 21(3), 2131-2140.

Ellaiah, P., Srinivasulu, B., and Adinarayana, K., 2002. A review on microbial alkaline proteases, J. Sci. Ind. Res. 61, 690-704.

Khayati, G., and Kiyani, F., 2012. A statistical approach for optimization of lipase production by using rice straw: analysis of different inducers and nitrogen sources effect, Minerva Biotec. 24,83-89.

Liyana-Pathirana, C., and Shahidi F., 2005. Optimization of extraction of phenolic compounds from wheat using response surface methodology, Food Chem. 93, 47−56.

Manivannan, S., and Kathiresan, K., 2007. Alkaline protease production by Penicillum fellutanum isolated from mangrove sediment, Inter. J. Biological Chem. 1(2), 98-103.

Montgomery, D.C., 2001. Design and analysis of experiments (5th ed.), New York: John Wiley and Sons.

Mukherjee, A.K., Adhikari, H., and Rai, S.K., 2008. Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using Imperata cylindrical grass and potato peel as low-cost medium: Characterization and application of enzyme in detergent formulation, Biochem. Eng. J. 39, 353–361.

Mukherjee, A.K., and Rai, S.K., 2011. A statistical approach for the enhanced production of alkaline protease showing fibrinolytic activity from a newly isolated Gram-negative Bacillus sp. strain AS-S20-I, New Biotechnol. 28(2), 182-189.

Nath, A., and Chattopadhyay, P.K., 2007. Optimization of oven toasting for improving crispness and other quality attributes of ready to eat potato–soy snack using response surface methodology. J. Food Eng. 80, 1282−1292.

Nutan. D., Ulka, S.P., Kulbhushan, B.B., Jayant, M.K., and Digamber, V.G., 2002. Production of acidic lipase by Aspergillus niger in solid state fermentation. Process Biochem. 38, 715-721.

Pandey, A., Soccol, C.R., and Mitchell, D., 2000. New developments in solid-state fermentation I. Bioprocesses and applications. Process Biochem. 35, 1153–1169.

Potumarthi, R., Subhakar, C., and Jetty, A., 2007. Alkaline protease production by submerged fermentation in stirred tank reactor using Bacillus licheniformis NCIM-2042: Effect of aeration and agitation regimes. Biochem. Eng. J. 34, 185–192.

Prakasham, R.S., Rao, C.S., 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.

Ramachandran, S., Patel, A.K., Nampoothiri, K.M., Francis, F., Szakacs, G., and Pandey, A., 2004. Coconut oil cake—a potential ray material for the production of α- amylase. Bioresour. Technol. 93, 169–174.

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

Sen, S., Alkaline protease of a moderate thermophile *Bacillus licheniformis* S40. Ph.D. Thesis, University of Delhi, 1995.

Singh, J., Vohra, R.M., and Sahoo, D.K., 1999. Alkaline protease from a new obligate alkalophilic isolate of Bacillus sphaericus, Biotechnol. Lett. 21, 921-924.

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.

Uyar, F., and Baysal, Z., 2004. Production and optimization of process parameters for alkaline protease production by a newly isolated Bacillus sp. under solid state fermentation, Process Biochem. 39, 1893–1898.