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Statistical, Optimization, And Thermodynamic Studies On The Production of Alkaline Protease Using New Local Isolate Of *Bacillus Sp*

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Abstract

Investigation of alkaline protease production under different fermentation conditions using one factor at a time involving temperature, pH, incubation time as well as different carbon and organic nitrogen sources. The results showed that the maximum activity (470U/ml) was obtained at peptone 7.5 (g/L), glucose 5 (g/L), pH 10, 24h incubation time and 35ºC. Further more, Two sequential statistical designs were used to optimize these physiochemical parameters (pH, temperature, incubation time, glucose, peptone, and $MgSO_4.7H_2O$, K_2HPO_4) for enzyme production. Firstly, the Plackett Burman factorial design was applied followed by Box-Behnken experimental design. The results showed that the best protease production (580 U/ml) was obtained at peptone 10 (g/L), glucose 12.5 (g/L), pH 11, 24h incubation time and 35ºC. The level of significance was studied using the three significant variables on the enzyme production (pH, glucose and peptone conc.) and the results indicated the integration of these parameters on protease production. The thermodynamic parameters of crude enzyme were also investigated and they proved the superior thermal stability of the *B. amyloliquefaciens* protease

Keywords: Production, Alkaline protease, *Bacillus*, Statistical design, Thermodynamics

1. Introduction

The production of alkaline proteases enzymes not only solves certain environmental problems, but also enhanced the economic value of some industrial processes. Proteases include groups of enzymes which catalyze peptide bond cleavage and the hydrolysis of protein [1]. They have many applications and functions especially in pharmaceutical drugs, food, leather, and detergent industries. Proteases can be produced by animals, plants, and microorganisms. Among these different sources of proteases, microbial proteases have a main interest in biotechnological applications especially bacterial proteases [2].

Most of the alkaline proteases applied for the industrial purposes face some limitations due to low activity and stability towards surfactants and oxidants as well as temperature changes. *Bacillus* strains have the ability to secrete industrially significant proteases which are stable and compatible with various detergent components. Both Placket Burman model (PBM) and Box-Behnken experimental design were used. PBM determines the linear correlation factors, while Box Behken Design determines the interactions of various factors and their influence on the production [3, 4].

The objectives of this study were to investigate a statistically designed optimization production process, testing the interactions of different variables, detect the quadratic effects, and determine optimal settings of the significant factors applying Placket Burman and Box-Behnken designs.

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Material and Methods

Microorganism

The microorganism used in this study was a local isolate of *Bacillus amyloliquefaciens* NRC-IB-1. The strain was provided by Chemistry of Natural and Microbial Products Department Culture collection, National Research Centre (NRC), Dokki, Cairo, Egypt.

Maintenance and inoculum preparation

The used bacterial isolate was grown and maintained by sub-culturing on nutrient agar slants at 37°C. To prepare the inoculum a loop full of bacterial culture was transferred to sterile nutrient broth (50 ml) then incubated at 35°C, 160 rpm for 24 h. Bacterial strain were routinely grown on nutrient broth medium at 35°C for 24 h and preserved at -80°C in 50%(v/v) glycerol and cultured every 3 months for maintenance.

Protease production process

The previously prepared inoculum (10%) was inoculated into Fifty ml of the production medium containing (g/L) glucose 5.0; peptone 7.5; $MgSO_4.7H_2O$ 5.0; K_2HPO4 5.0; and $FeSO4.7H_2O$ 0.1, at pH 8.0. Then the cultures were incubated in shaking incubator adjusted at 160 rpmfor 72 hat 35°C. To obtain the crude enzyme; the fermentation broth was centrifuged at 10,000 rpm for 15 min at 4°C after that the supernatant was used as a crude enzyme solution.

Alkaline protease assay

Protease activity was determined in the culture filtrate using casein as a substrate, by the method of Tsuchida *et al*. [5]. 0.2 ml of both enzyme solution and substrate solution (1% w/v casein in 50 mM Sodium phosphate buffer pH 8) were mixed and incubated in a water bath at 40°C for 20 minutes.

The reaction stopped by 1ml of 10% (w/v) trichloroacetic acid then incubated at room temperature for 15 minutes. The insoluble proteins were precipitated by centrifugation and the acid soluble supernatant was neutralized by adding 2.5 ml of the alkaline $Na_2\ CO_3$ solution (0.4 M). Finally, 1 ml of threefold diluted Folin Ciocalteau reagent was added then the mixture was incubated at room temperature for 30 minutes. The absorbance was measured at 660 nm. against a reagent blank using a tyrosine standard [6]. One unit of protease is defined as the amount of enzyme that releases 1 µg of tyrosine/ ml per min under the standard conditions of supernatant solution.

Protease production optimization using one factor at a time

Optimization process was carried out through testing the effect of some physiological and biochemical parameters involving initial pH value (8, 9, 10 and 11), incubation time (12, 24, 48, 72 and 96 h), temperature (25, 30, 35, 40 and 45^{o}C). Selection of the optimum fermentation parameters were carried out, in addition, the effect of different monosaccharide (fructose, glucose, galactose and mannose) as carbon source, as well as different organic nitrogen sources (casein, peptone, urea, yeast extract and beef extract,) were also studied.

Multi factorial Experimental design and optimization

Both plakett Berman model (PBM) and response surface methodology (RSM) were used to optimize the production process of alkaline protease. PBM determine the linear correlation factors while, RSM interaction of various factors and their influence on the production process. It was preferred when more than five factors are under investigation [7]. This design is practical, when a large number of factors were tested and the investigator is unsure which settings are likely to produce optimal or near optimal responses. In our study, that design was applied to reflect the importance of fermentation conditions and some medium components on alkaline

protease production. Seven independent variables were screened, organized according to the PlackettBurman design matrix described in the results section. For each variable, a high level (+) and low level (-) was tested. All trials were performed in duplicates and the averages of products percentage were treated as the responses. The main effect of each variable was determined by the following equation:

$$E_{xi} = (M_{i+} - M_{i-}) / N \quad \text{equation (1)}$$

where $E_{xi}$is the variable main effect, M_{i+} and M_{i-} are protease activity in trials, the independent variable (x_i) was present in high and in low levels, respectively, and N is the number of trials divided by 2.

Box-Behnken Design

Box-Behnken design was applied to study the most significant independent variables [8]. Glucose conc. (X_1), peptone (X_2) and pH (X_3),all were treated as independent variables included and each factor was examined at three different levels, low (-), high (+) and central or basal (0). These factors included thirteen combinations and their observations (shown in the results section) were fitted to the following second order polynomial mode:

$$Y=b_o+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2 \quad \text{eq.(2)n}$$

where Y is the dependent variable (protease activity); X_1, X_2 and X_3 are the independent variables; b_o is the regression coefficient b_1, b_2 and b_3 are linear coefficients; b_{12}, b_{13} and b_{23} are second-order interaction coefficients; and b_{11}, b_{22} and b_{33} are quadratic coefficients. The values of the coefficients were calculated and the optimum concentrations were predicted using JMP 8 software. The quality of the fit of the polynomial model equation was expressed by R_2 (regression coefficient). The proposed model is adequate, as revealed by the diagnostic tests provided by an analysis of variance (ANOVA). The 3D graphs were generated to understand the effect of the selected variables individually and in combination to determine their optimum level and to study the response surface and locate the optimum operational conditions for maximal production of protease. The F-test was calculated to determine factors having a significant effect.

Temperature profile and thermodynamic studies

The protease assay was performed as described earlier; however, the assay temperature was altered from 30 to 65°C. The highest activity attained was set as the 100% activity and all the remaining activities were displayed as percentages from this activity. Moreover, the activities attained at the temperatures 30-50°C were expressed as (r) (mol L^{-1} sec^{-1}). Ln(r) was then plotted against 1/temperature (in Kelvin scale) in order to construct the Arrhenius plot. The slope of this plot was equivalent to $-E_a/R$ where E_a was the activation energy of the enzyme and R was the universal gas constant 8.314 JK^{-1} mol^{-1}.

As regards to the enzyme's thermal inactivation and its thermodynamic parameters, the experiments were performed as previously shown [9]. The enzyme was incubated at various temperatures (53 to 69°C) for up to 1h. At specific time intervals, the activity was assayed as described earlier and this activity was expressed as a percent from the enzyme's activity at 0 min residence at the respective temperature (residual activity percent). Log residual activity percent was then plotted against time. The slope of the resulting straight line was utilized to calculate the first order thermal denaturation rate constant (k_d) at each temperature. The K_d values were then utilized to calculate the half lives ($t_{1/2}$) and decimal reduction times (D-values) at each temperature as follows:

$t_{1/2}=\ln 2/k_d$ Equation 3

D-value$=\ln 10/k_d$ Equation 4

The activation energy for protease denaturation (E_d) was also estimated. The E_d was estimated from the

slope of the plot of the lnk_d versus 1/temperature (in Kelvin scale) where this slope amounted to $-E_d/R$. The change in enthalpy (ΔH), Gibb's free energy (ΔG) and entropy (ΔS) for protease thermal denaturation were estimated as follows:

$\Delta H=E_d - RT$ Equation 5

$\Delta G= -RT \ln (k_d*h/k_B*T)$ Equation 6

$\Delta S= (\Delta H - \Delta G)/T$ Equation 7

T was the temperature in K, R was the universal gas constant ($8.314 Jmol^{-1}K^{-1}$), h was the Planck constant ($11.04 \times 10^{-36} Jmin$), and k_B was the Boltzman constant ($1.38x10\text{-}23JK^{-1}$).

Results and Discussion

The results indicated that protease activity affected by different parameters. The incubation time was found to affect the enzyme activity Figure (1). The maximum enzyme activity (387U/ml) and specific activity (1548U/mg) reached at 24h. Whereas, after 96h of incubation; the activity and specific activity reduced to 142.5U/ml and 1158.5U/mg respectively while, Shumi *et al.* [10, 11] who observed that the maximum production of protease from *Bacillus* sp. was detected at 48 to 72 h of incubation.

Furthermore, Figure (2) and Table (1) showed that the maximum activity (470U/ml)was obtained at high alkaline pH reached 10 , glucose (5.0 g/l) as a carbon sources, and peptone (7.5 g/l) as a nitrogen sources indicating that the enzyme was more active in alkaline conditions. It was observed that the enzyme production was enhanced in alkaline conditions which could be an indicative of the alkalophylic nature of the microorganism as previously mentioned by [12].While, Boughachiche *et al.* [13] found that the optimum pH for protease production by *Streptomyces* sp.was at 8.

The production of alkaline protease is highly dependent on the main components of the medium such as carbon and nitrogen sources (Table1). It was found that some monosaccharides affects on the production of protease enzyme [14] in the present investigation, glucose as sole carbon source increased protease activity to 465 U/ml. This may be due to that glucose is a simple monosaccharide which can be used by the organism during cell metabolism [15]. Glucose was found to be better than other saccharides; and the greater concentration of glucose was> 2% (w/v) showed an inhibitory effect on the protease production as well as cell growth [16]. On the other hand, some studies have shown a positive effect of fructose on acid protease production by *Aspirgllus* sp. [17].

Moreover, Table (1) showed that the most suitable nitrogen source was peptone which gave enzymatic activity reached 470 U/ml compared to other nitrogen sources used. The elevation of protease production was stated by Singal *et al*. [18]. On the other hand, the fermentation medium supplemented with yeast extract resulted in the highest protease activity followed by soybean meals [19, 20].

The results of temperature effects revealed that the optimum temperature was at 35^{o}C giving enzyme activity reached 458U/ml and specific activity 1387.8U/mg and beyond that degree at 45^{o}Cthere was an inhibition effect on the enzyme activity and specific activity reached 62.8U/ml and 628U/mg respectively Figure (3). It indicated that high temperature can affect the enzyme activity due to the effect on cell membrane properties [21]. Both the rate of chemical reaction and the rate of enzyme deactivation were enhanced with increasing temperature till certain degree at moderate temperature; deactivation rate is negligible and can be ignored. In that context, Rajput *et al*. [22] stated that most of proteases produced by mesophillic

organisms were thermostable within the range of 25-50ºC.

Statistical analysis

Plackett-Burman Factorial Design.

Plackett-Burman design was applied to screen different parameters with neglect of their interaction effects. Seven factors namely pH (X_1), temperature (X_2), incubation time (X_3), glucose (X_4), peptone (X_5), $MgSO_4.7H_2O$ (X_6) and K_2HPO_4 (X_7)were selected to test their effect on alkaline protease production (Table 2). Three different levels (-, 0, +) of each parameter were studied. Furthermore, Table 3 showed a set of 9 trials were carried out to detect the enzyme production under different combinations. The main effect of each variable was calculated (Table 4). Glucose, peptone, pH and temperature have positive effect on the production process, the three variable Glucose, peptone and pH were statistically optimized using Box Behnken design while the other variables showed negative effects

Box-Behnken design

In Box-Behnken design, three main effective factors called glucose (X_1), peptone (X_2) concentrations and pH (X_3) (Table 5) and their interactions were studied (Table 6). Each parameter was studied at three different levels (- , 0, +). The maximum activity (580 U/ml) was shown in trial number 2 with F- and *p*- values< 0.01 were used to determine the significance of model. Low values of *p* indicate high significance of the corresponding coefficient while large t and F values indicate the significance of corresponding coefficients [17]**.**

Table 8 reveals the ANOVA analysis for our model. The variability of the observed response can be affected by the applied parameters and their interactions and this relationship can be measured by the correlation coefficient (R^2) as mentioned by Tapasi and Uma Ghosh [23]. R^2 of the model is 0.70% and the predicted R^2 is in acceptable agreement with the adjusted R^2 as shown by the actual predicted plot.

The linear model represents $Y_{activity}$ (U/ml) as a function of (X_1) , (X_2) and (X_3). The production of protease enzyme $Y_{activity}$ (U/ml) was predicted by the following model equation:

Protease conc. = -493.34291 + 65.27675 * Glucose conc. -17.59412 * Peptone conc.+30.08013 * pH

A verification experiment was performed in triplicates, the results showed an actual maximum protease production (1.68fold) increase when compared to that produced by basal condition.

Temperature profile and thermodynamic studies

The results in (figure 4) revealed that the upmost activity of the *B. amyloliquefaciens* protease was expressed at 45-50°C. This optimum was higher than the 40°C temperature optimum reported for the crude *Aspergillusamari* protease [24, 25]. Based on the disclosed *B. amyloliquefaciens* protease optimum temperature, the temperature 50°C was the upmost temperature utilized to construct the Arrhenius plot (figure 5). From the slope of the Arrhenius plot, the activation energy (E_a) of the *B. amyloliquefaciens* protease was calculated as 24.85kJmol^{-1}. This E_a was lower than that reported for the crude *Aspergillusamari* protease (34.2 kJmol^{-1}), the *B. Subtilis* metallo protease (37.57 kJ mol^{-1}), and the *A. amaries* protease (72.6 kJmol^{-1}) [26, 27]. The low E_a value of the *B. amyloliquefaciens* protease implied that it required low energy in order to construct the activated complex for casein hydrolysis. Thus it could be seen that it possesses a high hydrolytic ability.

The heat inactivation of *B. amyloliquefaciens* protease was studied within a 53-69°C temperature range. The plot of log (%residual activity) against time yielded straight lines which implied the first order kinetics for the *B. amyloliquefaciens* protease figure (6). From the slope of these plots, the first

order thermal deactivation rate constants (k_d) were estimated at each temperature (Table 8). The k_d values were then employed to calculate the half-life ($t_{1/2}$) and the D-values of the *B. amyloliquefaciens* protease at each temperature (Table 8). The $t_{1/2}$ and the D-values are the durations that the enzyme resides at a particular temperature till its activity declines to 50% and 10% of its initial activity, respectively. The $t_{1/2}$ of protease decreased progressively from 70.95 min to 50.84 min with escalating temperature from 53 to 69°C. In a similar manner, the D-value declined from 235.71 min to 168.88 min with escalating temperature from 53 to 69°C. Nevertheless, these results were superior to those attained by the crude *A. Amari* protease whose $t_{1/2}$ and D values dropped from 39.8 to 31.8 min and from 132.3 to 105.7 min, respectively, upon escalating the temperature from 60 to 70°C [25]. The $t_{1/2}$ attained by the *B. Subtilis* metallo protease was also lower than those reported here where 40.76 and 26.63 min were the $t_{1/2}$ at 50 and 60°C, respectively [26]. The higher $t_{1/2}$ and D-values attained by the *B. amyloliquefaciens* protease reflected its higher thermostability.

The slope of the plot of lnkdvs 1/T (Figure 6) allowed us to calculate the activation energy of denaturation (E_d) of the *B. amyloliquefaciens* protease, which amounted to 18.11kJmol^{-1}. This E_d was lower than that estimated for the *B. licheniformis* alkaline protease (32.8-48.5kJmol^{-1}) [25]. The E_d was then utilized to calculate the enthalpy (ΔH) which is the parameter that indicates that entire amount of energy necessary to denature the molecules of the enzyme. Table 8 revealed that the values of ΔH were decreased from 15.40 to 15.26 kJ mol^{-1} upon escalating the temperature from 53 to 69°C. This indicated that lower amounts of energy were necessary to denature the molecular structure of the enzyme at escalated temperatures [27]. The ΔH values reported here for the *B. amyloliquefaciens* protease were higher than those reported for the *Geotrichumcandidum* protease which attained a ΔH of 5.04 kJ mol^{-1} at 65°C [28]. The entropy (ΔS) was also estimated for the *B. amyloliquefaciens* protease, and it exhibited negative values at all the tested temperatures (Table 8). Similarly, the crude *A. Amari* protease displayed negative ΔS values at all the tested temperatures. They debated that this implied even a somewhat less disordered transition state as compared to the native enzyme form. This reflected the thermo stability of the inspected protease [25].

Gibbs free energy (ΔG) was another investigated parameter as it involved contribution of both the enthalpic and entropic processes. ΔG increased gradually from 103.77 to 108.05 kJmol^{-1} upon escalating the temperature from 53 to 69°C. Similarly, the ΔG of the crude *A. amari*protease increased from 92.6 to 96.6 kJmol^{-1} upon escalating the temperature from 50 to 80°C [28]. The higher ΔG values attained by the *B. amyloliquefaciens* protease implied that it was more resistant to denaturation and more thermostable [25].

Table (1): Effect of different carbon and nitrogen sources on the production of alkaline protease

Carbon source	Final pH	Enzyme activity (U/ml)	Nitrogen source	Final pH	Enzyme activity (U/ml)
Glucose	9.50	465	Peptone	9.50	470
Fructose	9.70	367	Casein	9.56	432
Mannose	9.80	117	Urea	9.60	371
Galactose	9.99	0.6	Beef extract	9.60	422
Lactose	9.90	356	Yeast extract	9.90	30

Table (2): Actual values of the process variables

Variable	Symbol	-	0	+
pH	X1	9	10	11
Temp	X2	30	35	40
Time	X3	12	24	36
Glucose	X4	2.5	5	7.5
Peptone	X5	5	7.5	10
MgSO4	X6	3	5	7
K2HPO4	X7	3	5	7

Table (3): Plackett-Burman experimental design for evaluation of factors

Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Protease Activity (U/ml)
1	-	-	-	+	+	+	-	359
2	+	-	-	-	-	+	-	2.4
3	-	+	-	-	-	-	+	1.29
4	+	+	-	+	+	-	-	544
5	-	-	+	+	-	-	+	415
6	+	-	+	-	+	-	+	3.9
7	-	+	+	-	-	+	-	3.4
8	+	+	+	+	+	+	+	493
9	0	0	0	0	0	0	0	396.1

Table (4): Main effect of variables

Variables	Main effect
pH	44.4
Temp	44.1
Time	-10.2
Glucose	236.3
Peptone	133.5
$MgSO_4$	-13.29
K_2HPO_4	-10.8

Table (5): Actual values of the process variables

Variable	**Symbol**	**-**	**0**	**+**
Glucose	X_1	7.5	10	12.5
Peptone	X_2	10	12	14
pH	X_3	10	11	12

Table (6): Box-Behnken factorial design for three independent

Trial	Glucose X_1	Peptone X_2	pH X_3	Protease Activity (U/ml)
1	-	-	0	135.24
2	+	-	0	580
3	-	+	0	149.2
4	+	+	0	500.9
5	-	0	-	150
6	+	0	-	263.6
7	-	0	+	167.9
8	+	0	+	563.39
9	0	-	-	315.17
10	0	+	-	213.6
11	0	-	+	283.27
12	0	+	+	168.46
13	0	0	0	138.48

Table (7): ANOVA for Response Surface Linear Model of Box-Behnken design

Source	Sum of Squares	df	Mean Square	F Value	P-value Prob>F
Model	2.523E+005	6	42043.85	3.47	0.0779
A-Glucose conc.	*2.131E+005*	*1*	*2.131E+005*	*17.57*	*0.0057*
B- Peptone conc.	*9905.70*	*1*	*9905.70*	*0.82*	*0.4009*
C. pH	*7238.51*	*1*	*7238.51*	*0.60*	*0.4691*
AB	*2165.27*	*1*	*2165.27*	*0.18*	*0.6873*
AC	*19857.04*	*1*	*19857.04*	*1.64*	*0.2479*
BC	*43.88*	*1*	*43.88*	*3.619E-003*	*0.9540*
Residual	*72746.94*	*6*	*12124.49*		
Cor Total	*3.250E+005*	*12*			

The Model F-value of 3.47 implies the model is non significant. There is only a 0.88% chance that a "Model F-Value" this large could occur due to noise.

Std. Dev.	102.64		R-Squared	0.7083
Mean279.18		Adj R-Squared	0.6110	
C.V. %	36.76		Adeq Precision	6.969
PRESS	1.888E+005			

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The current ratio of 6.969 indicates an adequate signal. The model can be used to navigate the design space.

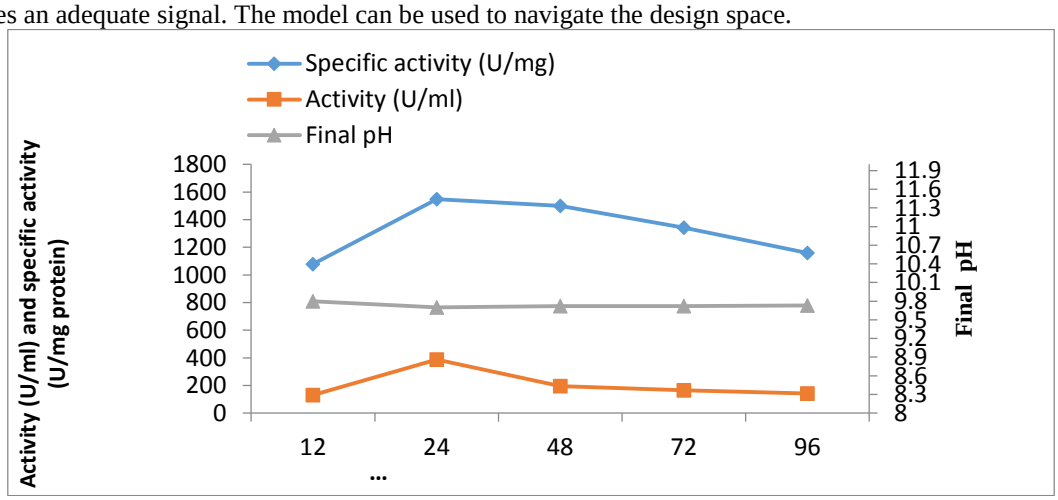

Fig .1 Effect of different incubation time on the production of alkaline protease by *B. amyloliquefacien*

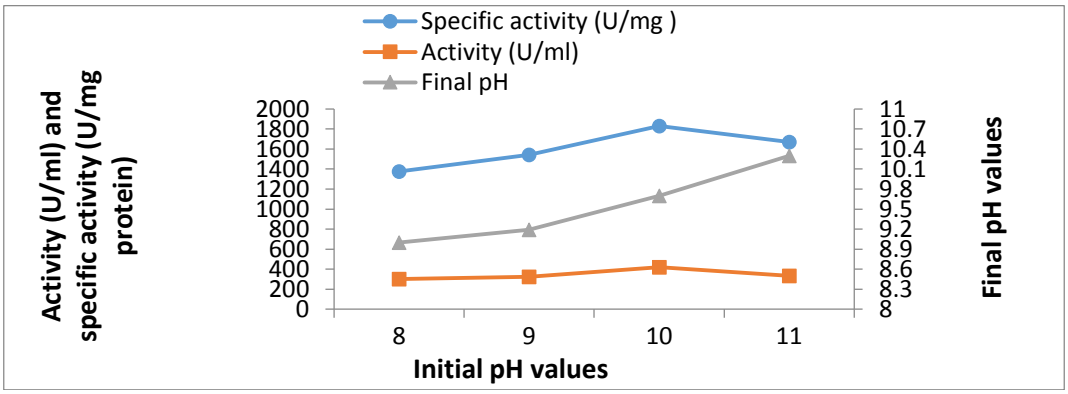

Fig .2 Effect of different initial pH value on the production of alkaline protease *by B. amyloliquefaciens*

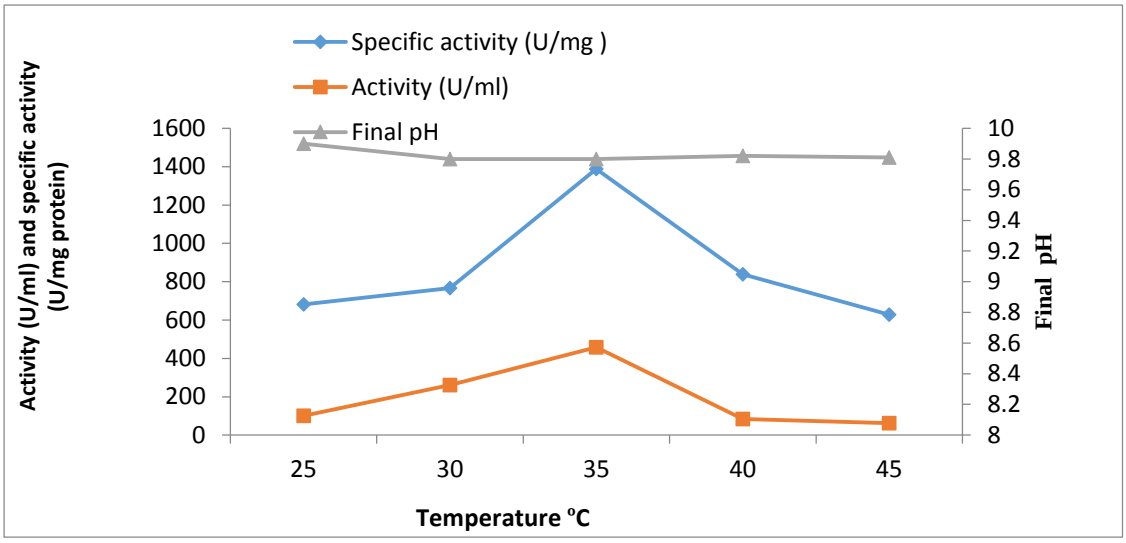

Fig .3 Effect of different temperature on the production of alkaline protease by *B. amyloliquefaciens*

Table (8): The thermostability parameters of the *B. amyloliquefaciens* protease.

Temperature (°C)	K_d (min^{-1})	$t_{1/2}$ (min)	D-value (min)	ΔH ($kJmol^{-1}$)	ΔG ($kJmol^{-1}$)	ΔS ($Jmol^{-1}K^{-1}$)
53	0.00977	70.95	235.71	15.40	103.77	-270.97
57	0.01087	63.77	211.85	15.36	104.79	-270.86
61	0.01091	63.54	211.06	15.33	106.08	-271.59
65	0.01220	56.83	188.78	15.30	107.07	-271.40
69	0.01363	50.84	168.88	15.26	108.05	-271.20

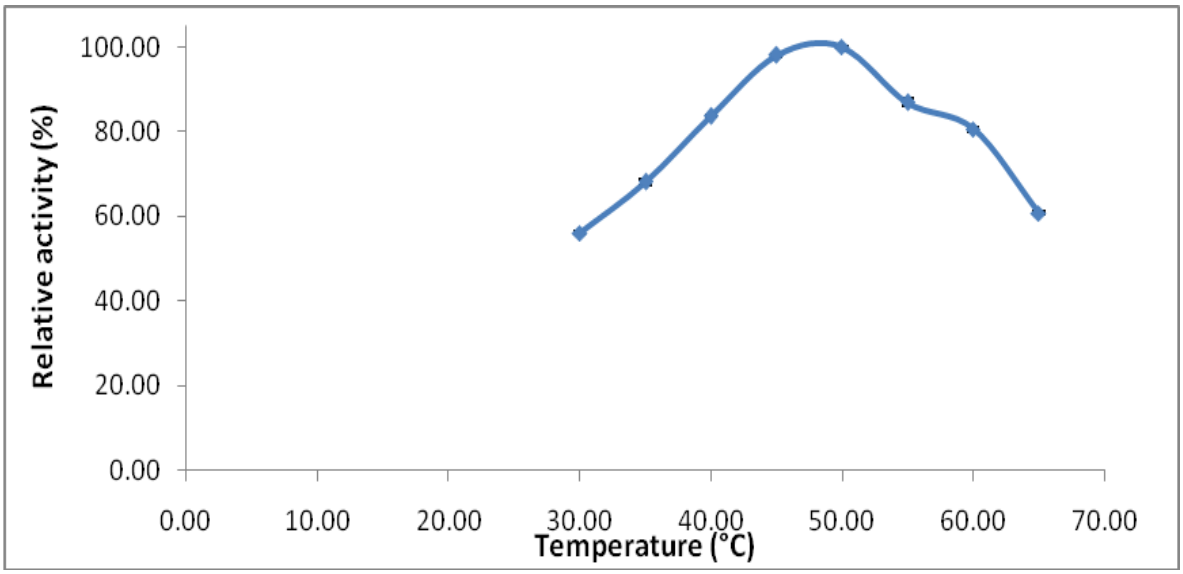

Fig. (4): Effect of temperature on activity of *B. amyloliquefaciens* protease

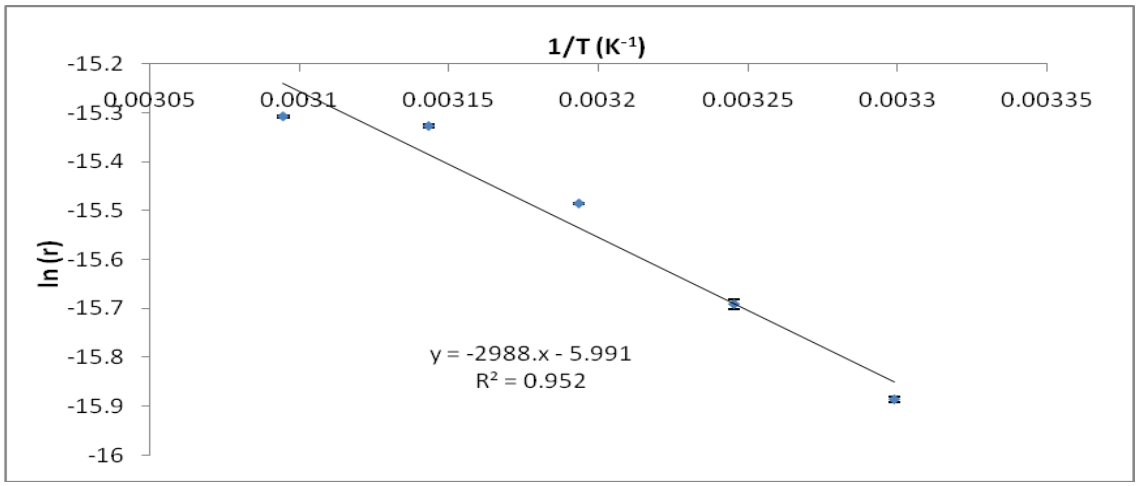

Fig. (5): Arrhenius plot to estimate the Ea

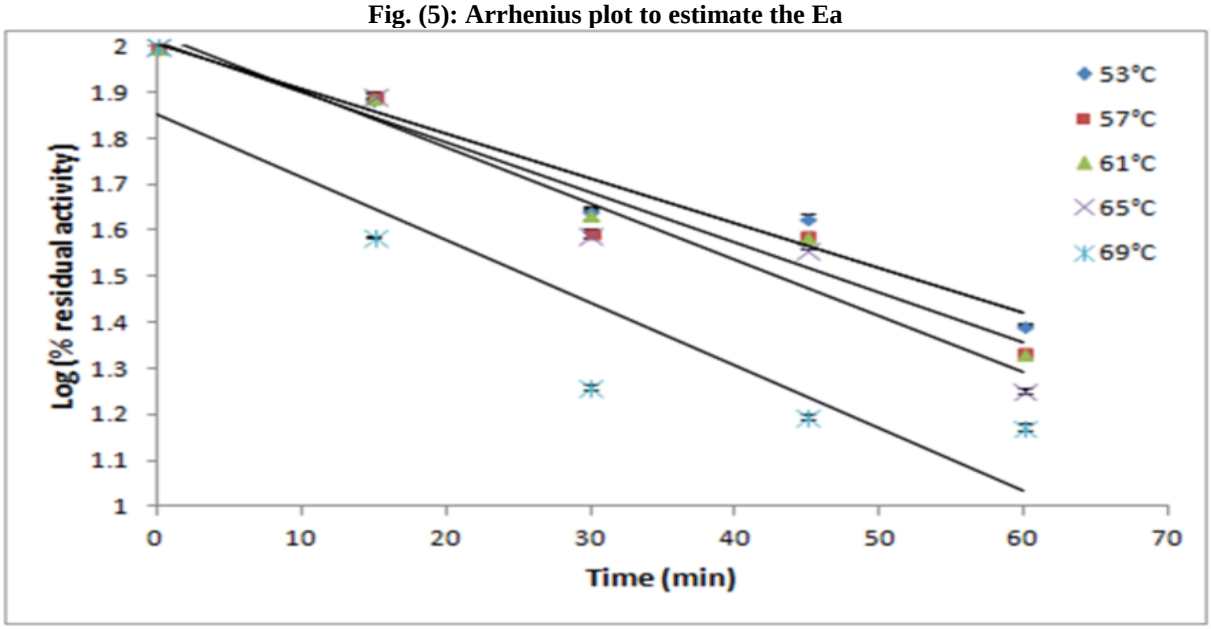

Fig. (6): Log residual activity percent plotted against time

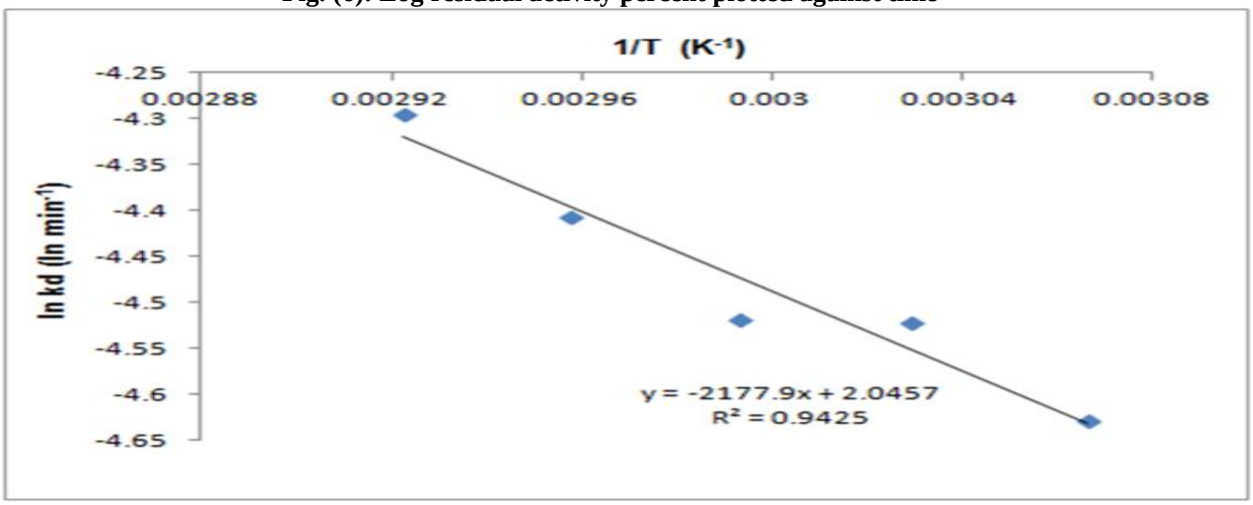

Fig. (7): plot of lnkdvs 1/T

Conclusion

The present study gave an insight for the enhancement of protease activity under different physiological and biochemical paramet. The results showed the obvious enhancement effects of the pH , temprature, as well as and carbon and nitrogen source on the activity of the enzyme. Statistical model designed curtained the significance of glucose on the enzyme activity which indicated the significance of glucose on the enzyme activity. The thermodynamic parameters of crude enzyme proved the superior thermalstability of the *B. Amyloliquefaciens* protease and the low Ea could be seen when it possesses a high hydrolytic ability of the *B. Amyloliquefaciens* protease. The higher ΔG implied that it was more resistant to denaturation and it was thermostable. From the previous results it could be concluded that *B. Amyloliquefaciens* protease could be used in different industrial aspects.

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