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Research Article

Statistical design for the optimization of alkaline protease production by local isolate of Bacillus amyloliquefaciens NRC69

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Abstract

The production of inexpensive proteolytic enzymes not only solves environmental problems, but also promotes the economic value and application of these enzymes in different industrial processes. In this study optimization of alkaline protease production using *Bacillus amyloliquefaciens* by submerged fermentation was investigated. The effect of some physiological parameters such as (fermentation medium, pH, fermentation time, inoculums size as well as carbon and nitrogen sources) were tested using both one variable optimization and multivariable optimization processes. Response Surface Methodology (RSM) was employed to optimize these physiochemical parameters for protease production. Seven parameters were selected for the (Plackett Burman) factorial design involving temperature, pH, incubation time, glucose, peptone, $MgSO_4.7H_2O$ and K_2HPO_4. The results showed that pH, temperature as well as $MgSO_4.7H_2O$ had impact on the production of alkaline protease by the selected strain. Accordingly, the design (RSM) was employed to study the level of significance to the 3 significant variables on the enzyme production as well as their interactive action. On using the above design the results reflected the interactive effect of the most influential parameters resulted in 1.63 fold increase on the enzyme productivity.

Key words: production ; alkaline protease; statistical design; Bacillus

Introduction

Proteases are the groups of enzymes which catalyze the hydrolysis of protein through peptide bond cleavage [1]. They have large variety of applications and numerous functions specially in detergent, food, pharmaceutical and leather industries. It was produced by different ways plant, animal and microorganisms. Among the previousy mentioned different proteases sources, alkaline proteases produced by microorganisms are of main interest from a biotechnological perspective [2].Bacterial proteases are the most significant compared with animal and fungal proteases and it has wide range of industrial applications.

One of the main applications of the alkaline proteases is in the laundry detergents, where they help in removing protein-based stains from clothing during washing. The target on using protease as detergent additives, it should be stable and active in the presence of other detergent ingredients, such as surfactants, builders, bleaching agents, fillers, fabric softeners and various other formulations [3,4].

The main objective of a statistically designed optimization study were to confirm effects and interactions of different variables, estimate quadratic effects, and determine optimal settings of the significant factors. The present study focused on statistical optimization of some fermentation parameters to find out the critical contribution of each factor. Both Placket Burman model (PBM) and Response Surface Methodology (RSM) were used in sequence to optimize the process for production of alkaline proteases. PBM determines the linear correlation factors, while RSM determines the interactions of various factors and their influence on the production.

Material and Methods

Microorganism

The bacterial strain used in the current work was local isolate of *Bacillus amyloliquefaciens NRC69.* It was provided by Chemistry of Natural and Microbial Products Department Culture collection, National Research Centre (NRC) , Dokki, Cairo, Egypt.

Maintenance and inoculum preparation

The experimental microorganism *B. amyloliquefaciens* was maintained by sub-culturing on nutrient agar slants at 37°C. The inoculum was prepared by adding a loop full of pure culture into 50 ml of sterile nutrient broth in 250 ml conical flasks and then incubated using a rotary shaker (160 rpm) at 28 °C for 24 h.

Production process

Fifty ml of the production medium containing (g/L) glucose, 5.0; peptone, 7.5; $MgSO_4.7H_2O$, 5.0; KH_2PO4, 5.0; and $FeSO4.7H_2O$, 0.1, pH 8.0 was inoculated by using 10 % of the previously prepared inoculum. The fermentation process was incubated using a rotary shaker (160 rpm) at 28°C for 3 days. The whole fermentation broth was centrifuged at 10,000 rpm at 4°C, and the clear supernatant was recovered and used as crude enzyme.

Alkaline protease assay

Protease activity of the culture supernatant was determined according to the method of [5] by using casein as substrate. A mixture of 500 µl of 1% (w/v) of casein in 50 mM phosphate buffer, pH 8 and 200 µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. Then, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. The reaction mixture was centrifuged to separate the un-reacted casein at 10,000 rpm for 5 minutes at $4C^o$. The supernatant mixed with 2.5 ml of 0.4M Na_2CO_3. 1 ml of 3-fold diluted Follin Ciocalteus phenol reagent was adding. The resulting solution was incubated at room temperature in the dark for 30 minutes and absorbance of the blue color developed 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 per minute under the standard conditions of supernatant solution.

Fermentation parameters affecting alkaline protease production (one variable process)

The effect of some fermentation parameters on protease activity were examined e.g different pH value (8-11) of the fermentation medium, fermentation period (12, 24, 48, 72, and 96h),the incubation temperature (25, 30, 35, 40, and 45^oC). Also the effect of different monosaccharide (glucose, fructose, mannose, and galactose), as well as different organic and in organic nitrogen sources (peptone, casein, urea, beef extract, yeast extract, ammonium sulphate, and potassium nitrate) were also tested.

Statistical analysis

The Plackett-Burman experimental design is a factorial design recommended when more than five factors are under investigation [7]. This design is practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or near optimal responses. In this study, the design was used to reflect the importance of some medium com-

ponents and fermentation conditions on alkaline protease production. Seven independent variables were screened, organized according to the Plackett Burman 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:

$$E_{xi} = (M_{i+} - M_{i-}) / N$$

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

In the second phase of medium formulation for optimum protease activity, the Box-Behnken experimental design was applied [8]. In this model, the most significant independent variables, glucose conc. (X_1), peptone (X_2) and pH (X_3), all were treated as independent variables were 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:

$$Y=b_o+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2$$

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). If 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.

Results and Discussion

The constituent of the fermentation medium clearly affected the protease production. The results showed that the maximum enzyme production (339 U/ml) was obtained by using fermentation medium containing (g/l) glucose 5, peptone 7.5, K_2HPO_4 5, $MgSO_4.7H_2O$ 5, $FeSO_4.7H_2O$ 0.1 at pH 10.

The data in Table (1) revealed that the maximum protease production (421U/ml) was obtained at pH 10. These result indicated the tendency of the obtained enzyme to be more active in alkaline medium. These results suggested that there is a stimulation of enzyme production at the alkaline pH which could be an indicative of the alkalophylic nature of the used strain [9,10].

On the other hand, the period of the fermentation process affected the activity of the enzyme. The results presented in Fig.(1) showed that the maximum enzyme activity (421) U/ml) at 72 hr. Meanwhile, at the longer fermentation periods (96h) a reduced activity was obtained (247.7 U/ml).

Table 1: Effect of different initial pH value on the production of alkaline protease by B. amyloliquefaciens

Initial pH	Final pH	Dry weight (g/ml)	Enzyme activity (U/ml)
8	9.71	0.0072	339
9	9.19	0.0099	374
10	9.71	0.0099	421
11	10.30	0.0081	334

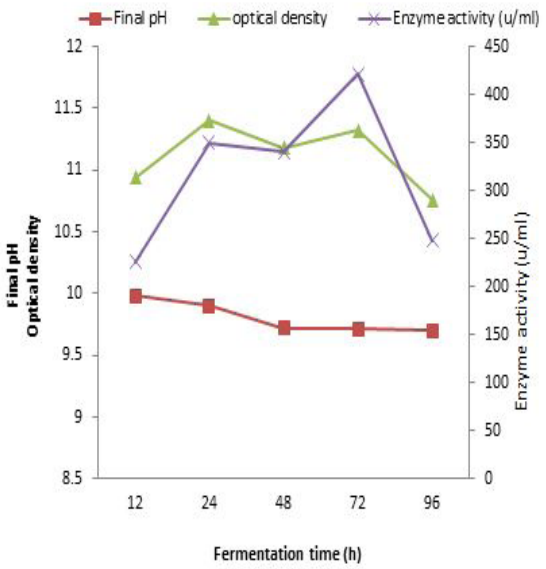


Figure 1: Effect of different fermentation time on the production of alkaline protease

However, the results in Fig.(2) indicated that increasing the temperature led to an enhancement of enzyme activity (458U/ml at 35^{0}C), Meanwhile, any increase in temperature more than 35^{o}C was accompanied by reduction in enzyme activity (62.8U/ml) at 45^{o}C. The fermentation temperature influenced the extracellular enzyme production, possibly by changing the physical properties of the cell membrane [11]. The finding of optimum protease production at 35 ^{o}C was stated also by Singh *et al*., (2001).

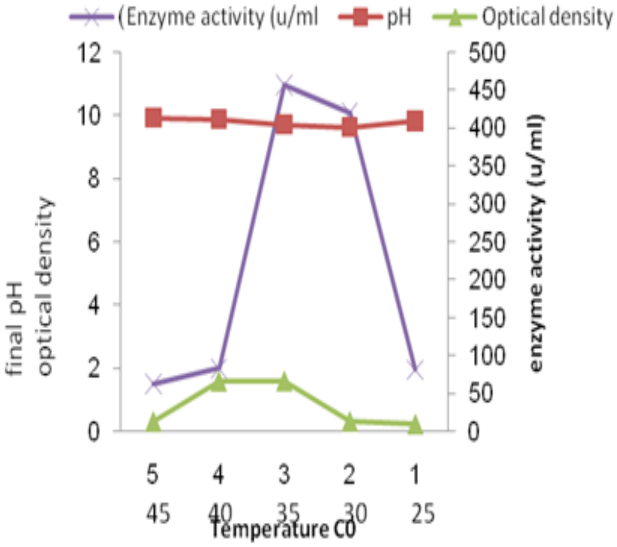


Fig.(2) Effect of different temperature on the production of alkaline protease

Figure 2: Effect of different temperature on the production of alkaline protease

The production of alkaline protease is highly depen-

Table 2: 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

dent on both carbon and nitrogen sources. Using of some monosaccharide could influence the production of enzyme [13], the addition of different glucose concentrations were investigated , the data in Table (2) revealed that (5g/l) glucose produced increase in protease activity to (465U/ml), the enhancement effect of glucose may be attributed to its simplicity to be used during cell metabolism [14]. The alkaline protease production was improved using some organic nitrogen sources more than the inorganic sources [15].The results presented in Table (2) also showed that (7.5g/l) peptone was the best nitrogen source on the production process where, it gave (470U/ml) compared to the other nitrogen sources used the enhancement effect of peptone on protease production was stated by Singh et al., (2001) .However, the agitation rate also affected the protease activity, the results in fig.(3) showed that the best enzyme activity (550 U/ml) was obtained at agitation rate 160 rpm.

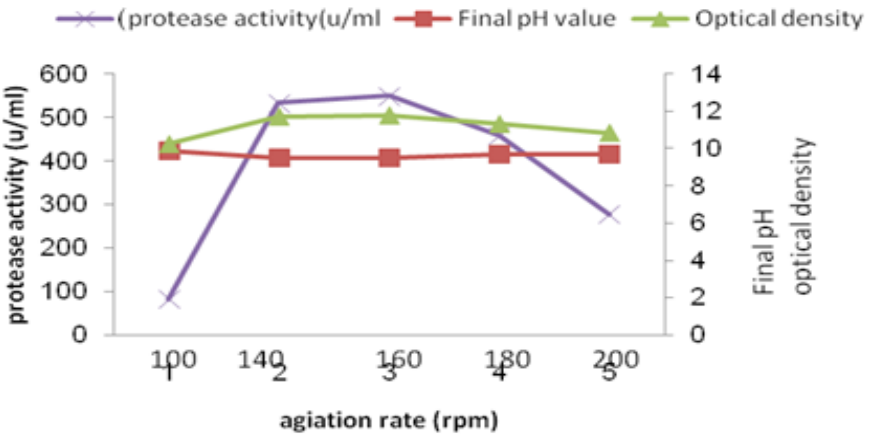


Fig.(3) Effect of different agitation rate on the production of alkaline protease

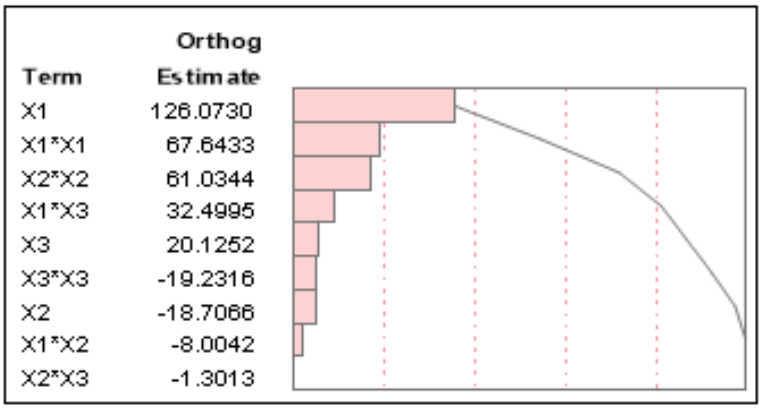
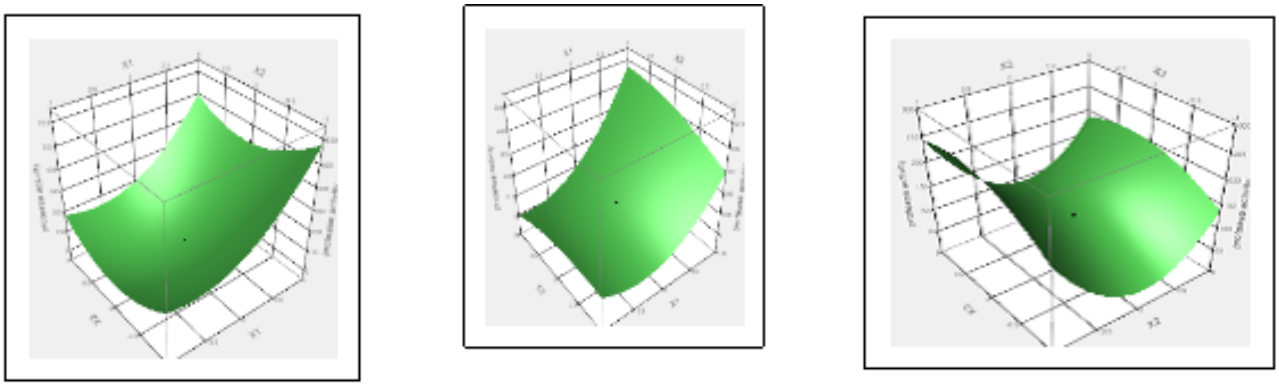


Figure 3(a): Pareto Plot of Transformed Estimates protease



a x1 x2 **b x1x3** **c x2x3**

Figure 3(b): Response of protease activity against the selected variables.

Table 3: Actual values of the process variables

Variable	Symbol	-	0	+
pH	X_1	9	10	11
Temp	X_2	30	35	40
Time	X_3	12	24	36
Glucose	X_4	2.5	5	7.5
Peptone	X_5	5	7.5	10
$MgSO_4$	X_6	3	5	7
K_2HPO_4	X_7	3	5	7

Statistical Optimization of alkaline protease production

Plackett-Burman Factorial Design.

Screening of different parameters was done by Plackett-Burman design with respect to their main effects and not to their interaction effects. For the alkaline protease production, seven factors were selected, namely, pH (X1), temperature (X2), incubation time (X3), glucose(X4), peptone(X5), MgSO4(X6) and K2HPO4 (X7). Each parameter was studied at three different levels (- , 0 , +,) table (3). On the other hand, Table (4) represents a set of 9 trials carried out to determine alkaline protease production under different combinations.

Box-Behnken design

The effect of the three main effective factors namely glucose (X1), peptone (X2) concentrations and pH (X3) and their interactions were studied. Each parameter was studied at three different levels (- , 0 , +, table 3).

Box- Behnken designed results were shown in Table (4), maximum protease activity (544U/ ml)was shown in trial number 4 with the F-value (Fischer' s statistical analysis) and p- value (>0.01) were used for determining the significance of model. Low values of p indicate high sig

Table 4: 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)
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 5: Main effect of variables

Variables	Main effect
pH	44.4
Temp	44.1
Time	-10.2
Glucose	236.3
Peptone	133.5
$MgSO_4$	-197.2
K_2HPO_4	-10.8

nificance of the corresponding coefficient while large t and F values indicate the significance of corresponding coefficients [16]. Subjecting our model to ANOVA analysis Table 4 . The correlation coefficient (R2) measures how much the variability of the observed response can be explained by the experimental parameters and their interactions [17]. R2 of the model is 0.84 %. The predicted R2 is in acceptable agreement with the adjusted R2 as shown by the actual predicted plot. The F-value of 2.94 is significant for the model and similarly the model P- value of the effect of each parameter on protease activity and interaction between the three variables were illustrated in Table (7).

Table 6: 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 7: Box-Behnken factorial design for three independent

Trial	**Glucose X_1**	**Peptone X_2**	**pH X_3**	**Protease Activity (U/ml)**
1	-	-	0	102.6
2	+	-	0	650
3	-	+	0	113.2
4	+	+	0	598.6
5	-	0	-	113.8
6	+	0	-	162.06
7	-	0	+	127.4
8	+	0	+	427.4
9	0	-	-	239.1
10	0	+	-	162.08

Table 8: Sorted Parameter Estimates

Term	Estimate	t Ratio	t Ratio	Prob>|t|
X1	172.6325	3.99		0.0104*
X1*X1	141.3675	2.22		0.0769
X2*X2	119.6725	1.88		0.1187
X1*X3	62.935	1.03		0.3503
X3	27.5575	0.64		0.5517
X3*X3	-38.7625	-0.61		0.5689
X2	-25.615	-0.59		0.5792
X1*X2	-15.5	-0.25		0.8099
X2*X3	-2.52	-0.04		0.9687

11	0	-	+	214.9	
12	0	+	+	217.8	
13	0	0	0	105.06	

3D aided in the visual determination of the maximum levels of each of the three parameters when they interact. It is well known that the 3D responses have either elliptical or circular representation which is considered either Significance or negligible. Table (8) showed that, as the level of these parameters changes, their interactive effect on the response also varies. Interaction between glucose (X1) and peptone (X2) was examined while maintaining the pH (X3) was constant (Fig.3 a). In Fig. (3b), protease activity was tested revealing the effect of the interaction between the glucose (X1) and peptone (X2) . While Fig.(3) c , showed the effect of interaction between glucose (X1) & pH (X3) on protease activity .

The quadratic model represents Y activity(U/ml) as a function of (X1) , (X2) and (X3). The production of protease enzyme Y activity(U/ml)was predicted by the following model equation :

Y = 105.06+172.63X1-25.61X2+ 27.555X3-15.5X1X2-62.93X1X3-2.52X2X3+141.36X12+119.67X22-

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