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Application of Rotatable Central Composite Design for Optimization of Extracellular Alkaline Protease Production from newly isolated *Bacillus pseudofirmus* MSB4

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Abstract

Introduction: Microbial extracellular alkaline proteases are one of the most important industrial enzymes with a wide range of applications in various industries. In the present study, the production of an alkaline protease by *Bacillus pseudofirmus* MSB4 was evaluated using response surface methodology (RSM).

Materials and methods: Four significant independent variables were selected, based on the results of one-factor-at-a-time (OFAT) approach (xylose concentration, beef extract concentration, pH and temperature), for rotatable central composite design (RCCD) to analyze the response pattern and to establish a model. The design consisted of total 30 runs, at five levels for each factor, with four replications of the center points. In addition, the presence of extracellular alkaline protease genes was evaluated by using PCR.

Results: Maximum protease production (185.397 U/ml), 2.2 times higher than that of OFAT method and 3.7 times higher than the unoptimized conditions, was obtained by using 3% w/v beef extract, at pH 9 and 37ºC according to the results of RCCD. Furthermore, the genes *sub I, II* and *apr* with the expected size (319, 486 and 194 bp, respectively) corresponding to the extracellular alkaline serine protease and metalloprotease were detected in MSB4 by using PCR.

Discussion and conclusion: The experimental data well fitted the model (Adj R^2: 0.9982) and the established quadratic model has a great ability to predict responses for new observations (Pred R^2: 0.9967). The results showed that the OFAT and RSM strategies were a useful screening and optimization method for enhancing protease production of MSB4, respectively.

Key words: *Bacillus pseudofirmus* MSB4, Alkaline protease, One-factor-at-a-time strategy, Response surface methodology, Rotatable central composite design

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Introduction

Microbial alkaline proteases (EC.3.4.21-24, 99) are one of the most commercially important industrial enzymes because of their stability and activity under harsh conditions such as high temperatures, high pH and in the presence of surfactants or oxidizing agents (1). Currently, the major proportion of commercially available alkaline proteases such as Subtilisin obtained from *Bacillus* strains due to their activity and stability at high pH (9-11) and temperature (50-60°C). In addition, the bacilli are generally regarded as safe strains (2). Because of their broad substrate specificity, proteases especially alkaline proteases have a wide range of applications. Leather processing, detergent formulations, baking, brewery, meat tenderization, peptide synthesis, cheese manufacture, soy sauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, recovery of silver from waste photographic film, as well as analytical tools in basic research are some of their applications (3-5).

The production of microbial extracellular proteases is influenced by the incubation time, pH, temperature, carbon and nitrogen sources. Even small improvements in biotechnological enzyme production processes have been significant for commercial success (6). A number of optimization methods could be used for this purpose. Statistical approaches offer ideal ways for optimization studies of enzyme production. Time consuming, requirement of more experimental data sets and missing the interactions among parameters are some of the obstacles in the conventional optimization procedures like 'one-factor at a time' (OFAT) (7). On the other hand, statistical methods have some advantages basically due to utilization of fundamental principles of statistics, randomization, replication and duplication (8). Response surface methodology (RSM) is one of the useful methods in the optimization procedures, mainly developed based on the central composite design (CCD). RSM is a collection of mathematical and statistical techniques that are applicable for modeling and analysis where the objective is to optimize a response and the response is influenced by several variables (9).

The present study aimed to find the optimum medium component and physicochemical parameters which influence alkaline protease production by *Bacillus pseudofirmus* MSB4. For this purpose, a two-step optimization strategy was established: (1) screening of the most significant medium components and physicochemical parameters affecting the production of the alkaline protease using the conventional approach, *i.e.,* OFAT strategy and (2) optimization of significant factors using RSM. This two-step methodology was also selected by previous researchers (10 & 11). In addition, we have evaluated extracellular alkaline serine protease and metalloprotease genes in MSB4 using PCR.

Materials and methods

Microorganism and protease production: The microorganism used in the present study was isolated from a sausage factory wastewater (Isfahan, Iran), screened using a skim milk agar plate (pH 10) and later in alkaline skim milk broth medium (pH 10). The strain MSB4 was identified by employing morphological and biochemical characteristics and confirmed through molecular characterization. Briefly, the genomic DNA was extracted and nearly

full length 16S rRNA sequence (1432 bp) was amplified by using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (12). 0.5µl each primer, 0.5µl dNTP (10mM), 2.5 µl PCR buffer, 1µl $MgCl_2$ (50mM), 3µl template DNA, 0.2 µl Taq DNA polymerase (Cinnagen, Iran) and 17 µl dH2O in a final volume of 25 µl were used. PCR was performed under the following conditions; 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 54°C for 45s and 72°C for 1.5 min, with a final 5 min extension at 72°C. The PCR product was sequenced in both directions using an automated sequencer by Bioneer Company (Korea). The 16S rRNA gene sequence of the strain has been submitted to the GenBank database (accession number KT006758). The phylogenetic analysis was done by using the software package MEGA, version 6 (Tamura, Stecher, Peterson, Filipski, and Kumar 2013) after obtaining multiple alignments of data by Muscle program (Fig 1).

The inoculum was prepared by adding a loop full of pure culture into 5 ml of sterile pre-culture medium *viz*., Horikoshi agar I (HK) medium containing (g/l): 10 g glucose, 5 g peptone, 5 g yeast extract, 1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 5 g Na_2CO_3 (pH: 10) and incubated at 30 °C overnight. The production medium, *i.e.,* 1٪ w/v skim milk broth medium (50-ml in a 250-ml Erlenmeyer flask) was inoculated with 1% of inoculum (OD 600 nm: 0.1) and incubated at 30 °C under shaking conditions (180 rpm). After incubation designated for each experiment, the culture broth was centrifuged at 10,000 rpm for 10 min at 4 °C and enzyme activity was determined in the cell-free supernatant.

Detection of bacterial alkaline metalloprotease and serine protease genes: In order to detect extracellular alkaline metalloprotease and serine protease genes, apr and sub primer sets were used, respectively (Table 1). PCR amplification was employed by apr and sub I and II primer sets with some modifications. The amplification reaction contained l0 µl distilled water, l0 µl PCR Master Mix (2X) (Jena bioscience, Germany), 5 µl DNA, 0.5 µl forward primer (100 pmol) and 0.5 µl reverse primer (100 pmol). The following conditions were used in the amplification of alkaline protease genes: 95°C for 5 min, followed by 30 cycles of 94°C for 30s, 53°C for 30s and 72°C for 20s, with a final 10 min extension at 72°C. The PCR products were then checked on 1% agarose gel with ethidium bromide staining.

Table 1- Oligonucleotides used as primers for detection of genes for alkaline metallopeptidases (*apr*) and serine peptidases (*sub*) (13).

Oligonucleotide	Composition (5′ to 3′)	*Tm (°C)*	Length of amplicon (bp)
apr- forward	TAYGGBTTCAAYTCCAAYAC	52–60	194
apr-reverse	VGCGATSGAMACRTTRCC	52–60	
sub I-forward	ATGSAYRTTRYYAAYATGAG	50–56	319
sub I- reverse	GWGWHGCCATNGAYGTWC	52–58	
sub II-forward	GNACHCAYGTDGCHGGHAC	58–62	486
sub II- reverse	GWGWHGCCATNGAYGTWC	52–58	

Enzyme activity assay: Enzyme activity was measured by the method previously described with some modifications (14). 0.5 ml of enzyme solution was incubated with 2.5 ml of 0.65% w/v casein solution in 20 mM carbonate-bicarbonate buffer (pH 10) at 37°C for 10 min. Then, the undigested protein was precipitated by adding 2.5 ml of 10% w/v trichloroacetic acid (TCA). The terminated reaction mixture was incubated at 37°C for 30 min. The precipitates were removed by centrifugation at 10000 rpm for 10 min at 4°C and discarded. Then, 2.5 ml of 0.5 M sodium carbonate and 1 ml of 10% v/v Folin-Ciocalteau reagent were added to 1 ml of the supernatant to yield a blue color. The colored mixture was incubated at 37°C for 20 min before absorbance was measured at 660 nm. The blank was prepared in the same manner except the buffer was used instead of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme liberating 1µg of tyrosine/min/ml under the assay conditions. The values represented are the mean of at least three independent experiments. The difference between values did not exceed 5%.

Growth kinetics and enzyme production: The kinetics of growth and enzyme production was followed at different time intervals (6, 8, 12, 18, 24, 36, 48, 60, 72 and 96 h) to find the optimum time for the maximum protease production. At each time interval, culture samples were withdrawn aseptically and cell density along with enzyme activity was monitored as described before.

Effect of pH and temperature on enzyme production: pH and temperature are important physical parameters which significantly influence enzyme production yield. Therefore, these parameters are normally intended to keep constant at their optimal values throughout the fermentation process (2). In order to investigate the influence of pH on enzyme production, MSB4 was grown in SMB medium at varying pH (7-12). After 48 h of incubation at 30°C under shaking conditions (180 rpm) enzyme activity was quantified. Also, the effect of temperature on protease production was investigated by incubating culture flasks at various temperatures ranging from 25° to 55°C in a rotary incubator shaker (180 rpm).

Effect of carbon and nitrogen sources on enzyme production: Different carbon sources (glucose, soluble starch, sucrose, mannitol, lactose and xylose) were employed in an initial fermentation medium to find the suitable carbon source for alkaline protease production by MSB4. All these carbon sources were studied at 1% w/v initial concentration. To investigate the effect of various nitrogen sources on protease production, two categories, *viz.* organic nitrogen sources (yeast extract, beef extract, peptone and urea) and inorganic nitrogen sources ($NaNO_3$ and NH_4Cl) were employed. The initial fermentation medium was supplemented with different nitrogen sources, each at a concentration of 1% w/v. Enzyme activity was monitored after 48h of incubation at 37°C (180 rpm).

Optimization of enzyme production by RSM: The significant independent variables based on OFAT results were used to determine the optimum levels of selected parameters by using RSM. According to the primary results of OFAT experiments, the most significant and effective variables were found to be pH, temperature, carbon source (xylose concentration) and nitrogen source (beef extract concentration). The

response surface approach involving a rotatable central composite design (RCCD) was used to optimize MSB4 protease production. Each factor varied over 5 levels (-2, -1, 0, +1, +2), that is, plus and minus alpha (axial point), plus and minus one (factorial point) and zero (center point). Coded and actual values of the four variables are presented in Table 2. A set of 60 experiments was carried out in Erlenmeyer flasks containing skim milk broth medium that had been inoculated with *B. pseudofirmus* MSB4. For all the experiments, samples were harvested from the flasks aseptically after 48h of incubation and protease activity was measured under the assay conditions.

The mathematical relationship of the independent variables and the response (MSB4 alkaline protease production) can be calculated with a quadratic (second degree) polynomial equation:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{44}X_4^2+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+b_{23}X_2X_3+b_{24}X_2X_4+b_{34}X_3X_4$$

where Y is predicted value, b_0 is constant, X_1 is temperature (°C), X_2 is carbon source (xylose concentration), X_3 is pH and X_4 is nitrogen source (beef extract concentration), b_1, b_2, b_3 and b_4 are linear coefficients, b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are cross product coefficients and b_{11}, b_{22}, b_{33} and b_{44} are quadratic coefficients.

Table 2- Experimental range and levels of the independent variables in terms of actual and coded factors

Actual factors levels at coded level						
Factor	Name	-2	-1	0	1	2
X_1	Temperature (°C)	23	30	37	44	51
X_2	Xylose (% w/v)	0	1.5	3	4.5	6
X_3	pH	5	7	9	11	13
X_4	Beef extract (% w/v)	0	1.5	3	4.5	6

Statistical analysis: Design Expert 7.0 (Stat-Ease, Minneapolis, USA) was used for designing the experiments, data analysis, regression coefficients and response surface diagrams. Data were analyzed using Analysis of Variance (ANOVA) with 95% confidence interval. The quality of fit of the second-order polynomial model equation was expressed with the determination coefficient (R^2) and adjusted R^2 (Adj R^2). The fitted polynomial equation was then expressed in terms of three-dimensional (3D) surface plots in order to illustrate the relationship between the responses and the experimental levels of each variable utilized in this study. All experiments were carried out in triplicate and average values were reported

Results

Detection of bacterial extracellular alkaline metalloprotease and serine protease genes: Two extracellular proteolytic enzyme genes, alkaline metalloprotease (apr) and subtilisin-like serine protease (sub) were analyzed in this study. As shown in Fig. 2, the fragments of the expected size were amplified (319 and 486 bp) for subtilisin-like serine protease and (194 bp) alkaline metalloprotease genes.

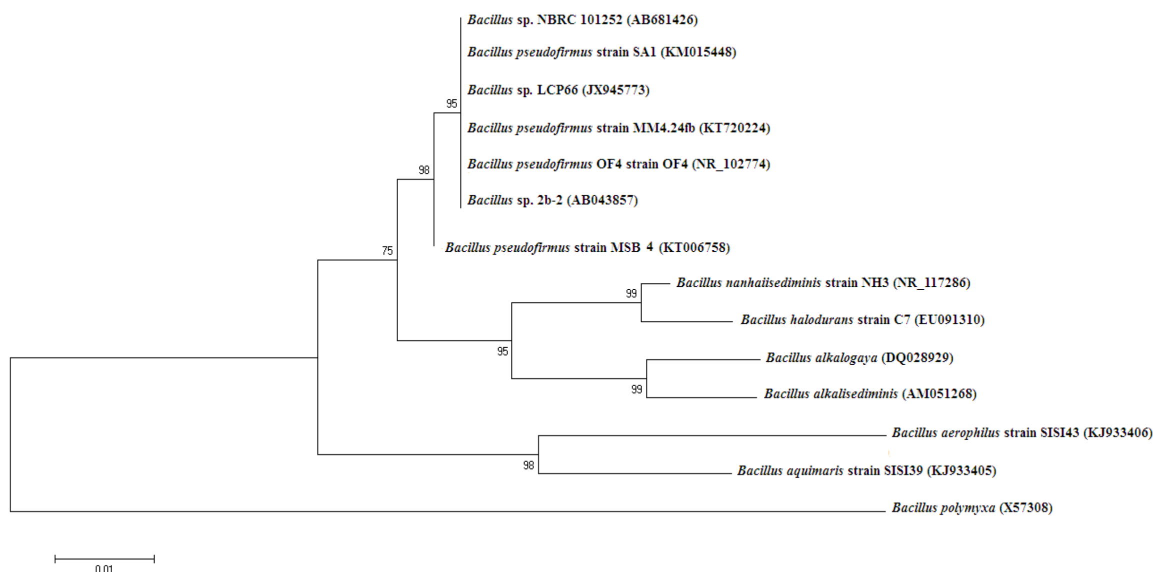

Fig. 1- Phylogenetic tree showing the position of *Bacillus pseudofirmus* MSB4, based on the 16S rRNA sequence comparison, obtained by the maximum likelihood method. The accession numbers of the reference strains are included in brackets. Bootstrap values are indicated on the branches.

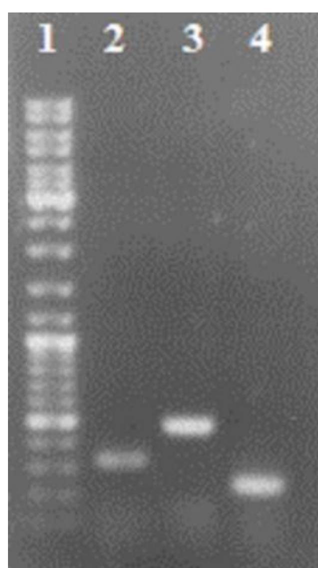

Fig. 2- Gel electrophoresis of PCR products with the primers specific for the genes for bacterial serine peptidases (*sub I,II*) and alkaline metalloproteases (*apr*). 100 bp DNA ladder (lanes 1), amplification product with primers *sub I* (lane 2) and primers *sub II* (lane 3), amplification product with primers *apr* (lane 4).

Effect of different incubation times on enzyme production: The yield of protease production by *B. pseudofirmus* MSB4 was significantly related to the time of incubation (*p* value= 0.00). Fig. 3 shows that protease production was weak after only 8h of incubation (10.51 U/ml) and increased gradually by the time until it reached its maximum value (49.99 U/ml) after 48 h of incubation. At this incubation time, the strain was in the stationary phase, according to the result of growth kinetics study, which is compatible with the result of the previous study (15). In many bacilli, protease production is during the early stationary phase of growth (16).

Effect of pH and temperature on enzyme production: The strain was able to produce protease at pH range 7-12. As illustrated in Fig. 4, protease production increased gradually by increasing the initial pH to the optimum value of 10, at which the highest protease activity (49.32 U/ml) was achieved. Strain MSB4 showed the maximum enzyme production at 37ºC (51.15 U/ml) (Fig. 4). The effects of temperature and pH on protease production by MSB4 in the present study were statistically significant (P value=0.00).

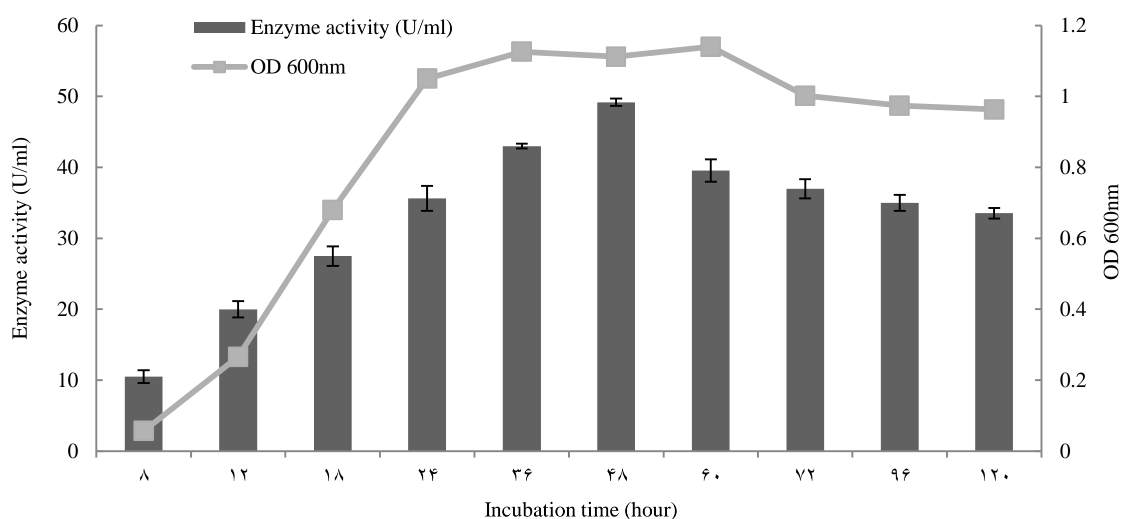

Fig. 3- Kinetics of growth and protease production of *Bacillus pseudofirmus* MSB4 in the basal medium. Results represent the means of three experiments, and *bars* indicate ± standard deviation.

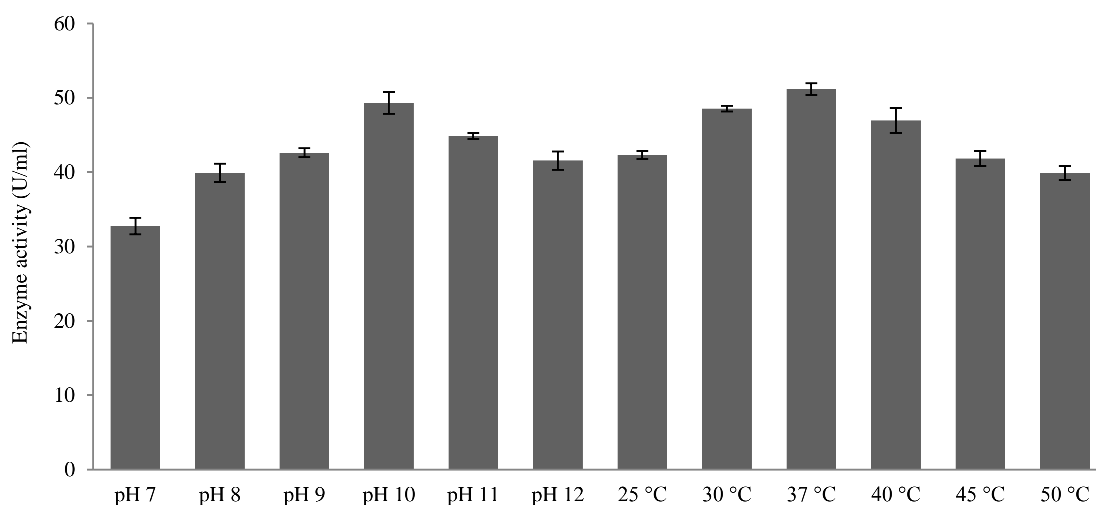

Fig. 4- Effect of pH and temperature on protease production of *Bacillus pseudofirmus* MSB4 in the basal medium. Values are mean±S.D. of triplicate determinations after 48 h of incubation.

Effect of carbon and nitrogen sources on enzyme production: Production of proteases strongly depends on the availability of both carbon and nitrogen sources in the fermentation medium and an excess or their deficiency may cause suppression or reduction of protease synthesis by bacteria (17). Among different carbon sources tested in this study, xylose was found to be the best for protease production (69.24 U/ml) as shown in Fig. 5. In fact, the addition of 1% w/v xylose enhanced enzyme production by 37.83 %. However, there are contradictory reports about catabolite repression of enzyme production by glucose, this effect was not observed in this study (18-20).

In the present study, the effect of organic and inorganic nitrogen sources on protease production was examined and the results compared with those of the initial production medium which had skim milk as

the only carbon and nitrogen source (Fig. 5). Beef extract proved to be the best nitrogen source for protease production by MSB4 (83.24 U/ml) which increased enzyme production by 65.69%. The enzyme production was reduced in the presence of ammonium chloride, sodium nitrate and urea. These results were in agreement with the previous studies which reported that complex nitrogen sources are better for protease production and enzyme synthesis was repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion (15, 21 & 22). However, the influences of both carbon and nitrogen sources were statistically significant (*p*-value= 0.00) in this study, nitrogen source had more positive effect on enzyme production.

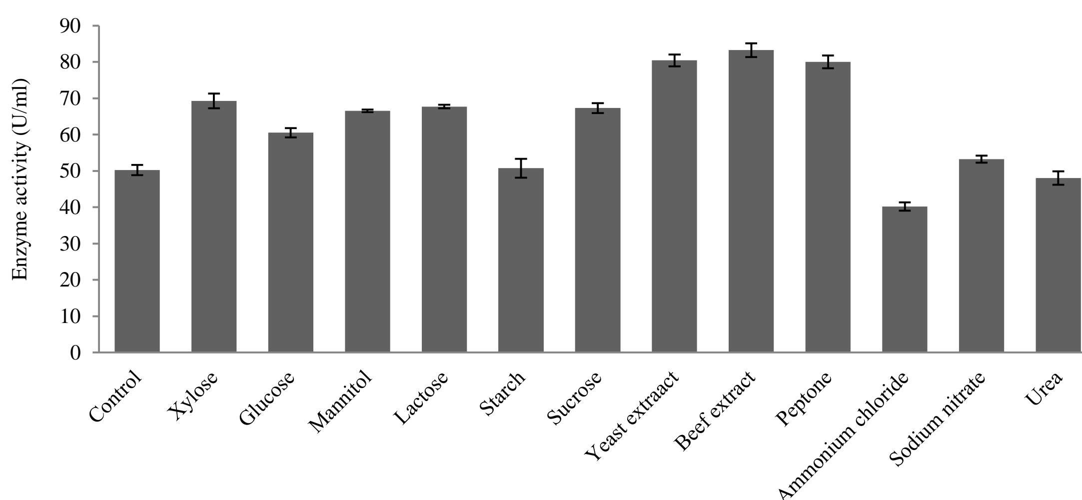

Fig. 5- Effect of different carbon and nitrogen source on protease production of *Bacillus pseudofirmus* MSB4 in the basal medium at a concentration of 1٪ w/v. Values are mean±S.D. of triplicate determinations after 48 h of incubation at 37°C.

Optimization of enzyme production by RSM: In order to search for the optimum formulation of the medium and culture conditions, a CCD with four factors, including 30 experiments in duplicates was employed to fit a second order polynomial model. The significant independent variables (xylose, beef extract, pH and temperature) were selected based on the preliminary results. The design matrix, experimental and predicted responses are shown in Table 3. The results were analyzed by analysis of variance (ANOVA). Values of "Prob > *F*" less than 0.05 indicate model terms are significant. ANOVA analysis indicated that the linear effects of all tested variables were statistically significant. The linear effects of xylose and beef extract (*P value* < 0.0001) were determined to be more significant than the effects of other variables. These results indicate that the concentration of these variables bears a direct relationship to protease production. In addition, the interactions between pH and xylose concentration (0.0479), xylose and beef extract concentration (<0.0001), and temperature and beef extract concentration (0.0109) were statistically significant, as shown by the interaction terms *P* value.

ANOVA was repeated after removing non-significant model terms (‘‘prob>F’’higher than 0.05).

Using the experimental data, the polynomial model for enzyme production yield (Y) was regressed only by considering the significant terms. The regression coefficients were calculated and the data were fitted to a second-order polynomial equation. The response, enzyme production, was expressed in terms of the following regression equation:

Enzyme activity (U/ml)= $172.34 + (0.36*X_1) - (3.71*X_2) + (0.41*X_3) - (1.65*X_4) - (0.56*X_1X_4) + (0.41*X_2X_3) - (3.37*X_2X_4) - (13.24*X_1^2) + (1.60*X_2^2) - (6.28 *X_3^2) - (12.21*X_4^2)$

Table 3- Rotatable CCD matrix for four variables with actual and predicted protease activity

Run No.	X_1 Temperature (°C)	X_2 Xylose (% w/v)	X_3 pH	X_4 Beef extract (% w/v)	Protease yield (U/ml)	
					Observed	Predicted
1	30.00	1.50	7.00	1.50	143.99	143.64
2	44.00	1.50	7.00	1.50	145.27	144.48
3	30.00	4.50	7.00	1.50	141.62	141.44
4	44.00	4.50	7.00	1.50	144.02	143.69
5	30.00	1.50	11.00	1.50	144.05	143.61
6	44.00	1.50	11.00	1.50	145	145.02
7	30.00	4.50	11.00	1.50	143.36	143.05
8	44.00	4.50	11.00	1.50	145.67	145.88
9	30.00	1.50	7.00	4.50	149.02	148.46
10	44.00	1.50	7.00	4.50	146.98	147.08
11	30.00	4.50	7.00	4.50	133.01	132.78
12	44.00	4.50	7.00	4.50	132.71	132.81
13	30.00	1.50	11.00	4.50	147.78	147.90
14	44.00	1.50	11.00	4.50	147.25	147.09
15	30.00	4.50	11.00	4.50	133.42	133.87
16	44.00	4.50	11.00	4.50	134.33	134.47
17	23.00	3.00	9.00	3.00	118.17	118.64
18	51.00	3.00	9.00	3.00	120	120.08
19	37.00	0.00	9.00	3.00	185.39	186.14
20	37.00	6.00	9.00	3.00	171.52	171.32
21	37.00	3.00	5.00	3.00	145.57	146.41
22	37.00	3.00	13.00	3.00	148.33	148.04
23	37.00	3.00	9.00	0.00	125.99	126.79
24	37.00	3.00	9.00	6.00	120.45	120.20
25	37.00	3.00	9.00	3.00	171.69	172.34
26	37.00	3.00	9.00	3.00	173.36	172.34
27	37.00	3.00	9.00	3.00	173.21	172.34
28	37.00	3.00	9.00	3.00	173.03	172.34
29	37.00	3.00	9.00	3.00	171.53	172.34
30	37.00	3.00	9.00	3.00	171.22	172.34

in which X_1 is temperature (°C), X_2 is xylose concentration, X_3 is pH and X_4 is beef extract concentration.

The statistical significance was evaluated by performing F-test and ANOVA analysis as shown in Table 4. The Model *F*-value of 1145.87 with *P*-value of < 0.0001 implies the model is significant. There is only a 0.01% chance that a "Model *F*-Value" this large could occur due to noise. The "Lack of Fit *F*-value" of 0.46 implies the Lack of Fit is not significant relative to the pure error and the obtained experimental data well fitted with the model (*p* value= 0.8642). There is a 86.42% chance that a "Lack of Fit F-value" this large could occur due to noise.

Table 4- Regression analysis for enzyme production from *Bacillus pseudofirmus MSB4* for quadratic response surface model fitting (ANOVA)

Source	Sum of Squares	Degrees of freedom	Mean square	*F*-value	*p*-Value prob>*F*	Coefficient estimate
Model[a]	9415.56	14	672.54	1145.87	< 0.0001	
Intercept						+172.34
pH (X_1)	3.11	1	3.11	5.30	0.0361	+0.36
Peptone (X_2)	329.60	1	329.60	561.56	< 0.0001	-3.71
Glucose (X_3)	3.97	1	3.97	6.76	0.0201	+0.41
Temperature (X_4) (X_4) Temperature	65.21	1	65.21	111.10	< 0.0001	-1.65
X_1X_2	2.00	1	2.00	3.41	0.0846	+0.35
X_1X_3	0.33	1	0.33	0.56	0.4645	+0.14
X_1X_4	4.95	1	4.95	8.43	0.0109	-0.56
X_2X_3	2.72	1	2.72	4.64	0.0479	+0.41
X_2X_4	181.71	1	181.71	309.60	< 0.0001	-3.37
X_3X_4	0.28	1	0.28	0.48	0.4996	-0.13
X_1^2	4811.49	1	4811.49	8197.77	< 0.0001	-13.24
X_2^2	70.03	1	70.03	119.32	< 0.0001	+1.60
X_3^2	1081.16	1	1081.16	1842.08	< 0.0001	-6.28
X_4^2	4089.72	1	4089.72	6968.02	< 0.0001	-12.21
Residual	8.80	15	0.59			
Lack of Fit	4.20	10	0.42	0.46	0.8642	
Pure error	4.61	5	0.92			
Cor total	9424.36	29				

Std. Dev. (0.77); Mean (148.23); C.V.% (0.52); PRESS (30.81); R-Squared (0.9991); Adj R-Squared (0.9982); Pred R-Squared (0.9967); Adeq Precision (124.606)

*significant

The fit of the model was also checked by the co-efficient of determination (R^2) which was 0.9991 revealing a good correlation between the experimental and predicted values. This means that variability above 99.91% in the response could be explained by the established model. The R^2 value is always between 0 and 1 and when it is closer to 1 the model is strong (11 & 23). The predicted determination coefficient (Pred R^2 = 0.9967) and adjusted determination coefficient (Adj R^2 = 0.9982) were also satisfactory to confirm the significance of the model. Unlike R^2, the adjusted R^2 increases only if the new term improves the model more than that would be expected by chance. The adjusted R^2 can be negative and will always be less than or equal to R^2. In the present study, adjusted R^2 was slightly less than R^2, which confirms the excellent correlation between independent variables and the fitted model. Predicted R^2 determines how well the model predicts responses for new observations. Therefore, larger values of predicted R^2 indicate models of greater

predictive ability. Predicted R^2 can prevent over-fitting the model and can be more useful than adjusted R^2 for comparing models because it is calculated using observations not included in model estimation. A very low value of coefficient of variation (C.V., 0.52 %) indicates better precision and reliability of the experiments executed. The adequate precision value measures signal to noise ratio. The higher ratio indicates an adequate signal and also proves that model can be used to navigate the design space. Its desired value is 4 or more. The adequate precision value of 124.606 indicates an adequate signal. So, this model can be used to navigate the design space.

3D response surface plots and two dimensional contour plots were generated by plotting the response, *i.e.,* alkaline protease production on the *Z* -axis against any two independent variables on the X- and Y-axes, while keeping other variables constant at their optimal levels. 3D response surface graphs plotting aimed to show a better description of interactions between variables (Fig 6). Fig. 7 shows the normal probability plot. Some scatter along the line indicated that the residuals follow a normal distribution. Therefore, the model satisfies the assumptions of the ANOVA that reflected the accuracy and applicability of RSM to optimize the process for enzyme production (U/ml). Perturbation plot shown in Fig. 8 represents a comparison of the effect of all the factors at the midpoint (coded 0) in the design space. A steep slope or curvature with pH, temperature, carbon and nitrogen sources shows that the response is sensitive to these factors. Finally, the model was validated for all four variables used in the present study by performing a set of 6 experiments. The results are summarized in Table 5. The experimentally determined values of enzyme production in these experiments were in close agreement with the statistically predicted values within 5% of predicted error, confirming the model's authenticity.

Overall, the RSM results revealed that pH, temperature and concentration of carbon and nitrogen sources had a significant effect on enzyme production, which is in agreement with the results of OFAT strategy. According to the CCD results, the highest protease production was achieved at 37ºC, pH 9, 0% w/v xylose, 3% w/v beef extract (185.39 U/ml), which was 2.2 times higher than that of the OFAT method.

Because each organism and even each strain have its own nutritional requirement for maximum enzyme production, the optimization of culture conditions of each strain is unique. There is scarce information about the statistical optimization of protease production by using other strains of *Bacillus pseudofirmus*. Abdel-Fattah *et al.,* studied the effect of pH, incubation time and peptone on the production of an alkaline protease by *Bacillus pseudofirmus* Mn6 using RSM (24). Their results showed that pH 9.5, 2% peptone and incubation time of 60h increased the enzyme production about 6.4 times with respect to the basal medium. Also, Sen *et al*., optimized pH of the medium, temperature and rpm of the shaking incubator to increase the production of *Bacillus pseudofirmus* SVB1 alkaline protease (15). Their obtained results were pH 9.2, 27.3 °C and 195 rpm which improved the enzyme production by only 36.23%. The optimum pH obtained in the study of Abdel-Fattah *et al.,* and Sen *et al*., was in agreement with

our results. Interestingly, MSB4 produced its optimum enzyme amount in a shorter period of time than Mn6. The improvement of enzyme production by using statistical optimization in the present study was more than Sen *et al*., and lower than Abdel-Fattah *et al*.,. The reasons that may be explained this observation, are the differences between the strains and the initial fermentation medium.

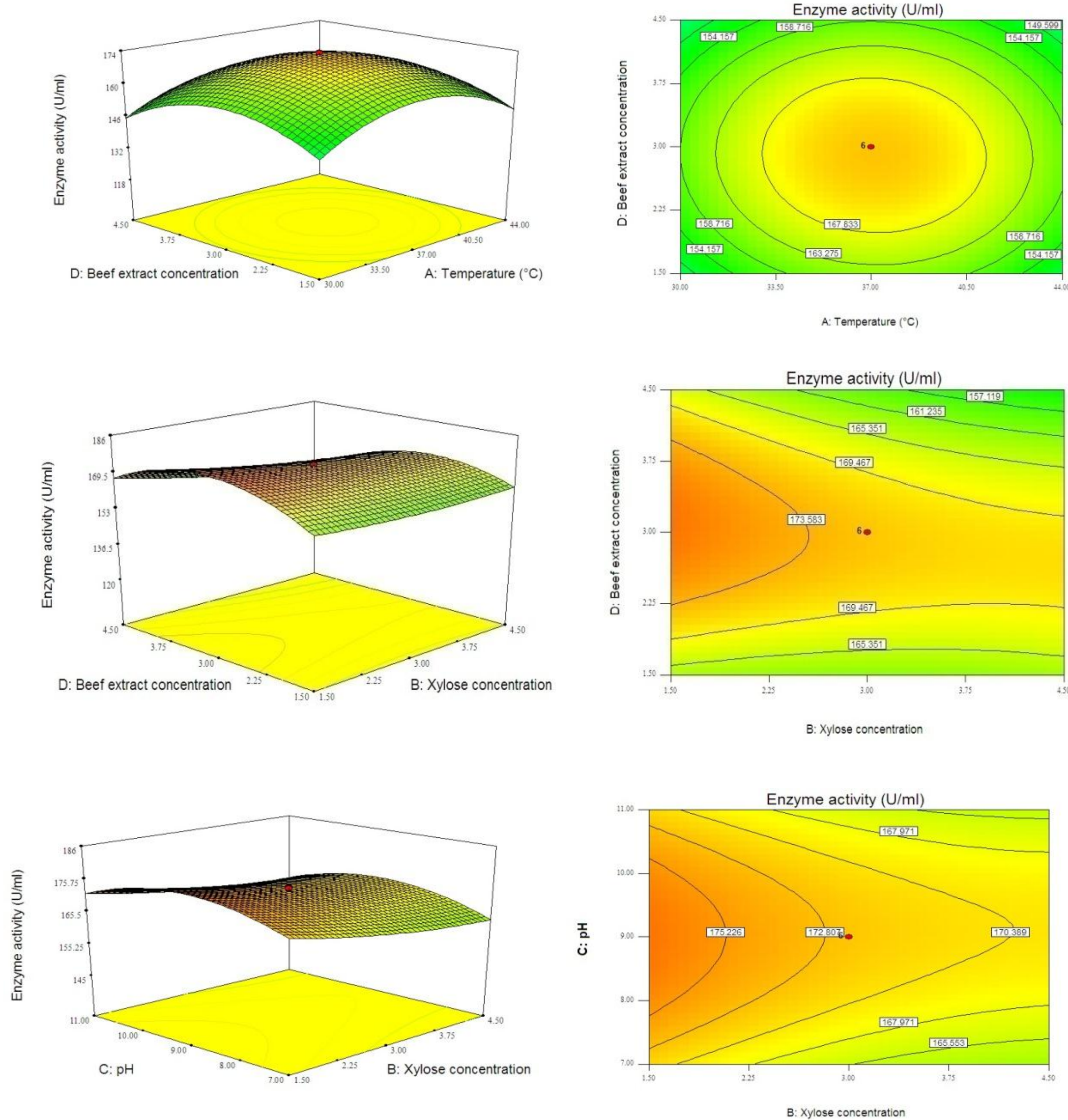

Fig. 6- Contour plots and 3D response surface curves of enzyme activity (U/ml) by *Bacillus pseudofirmus* MSB4 as a function of temperature (A), xylose concentration (B), pH (C), and beef extract concentration (D) in design space.

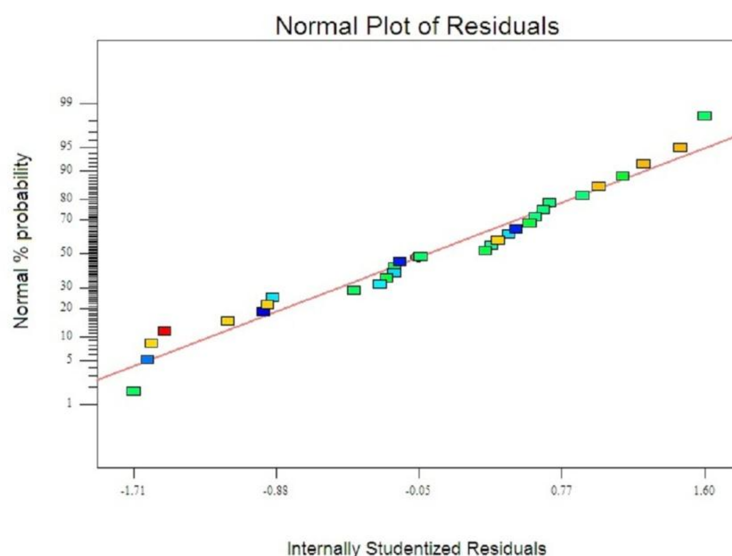

Fig. 7- Normal plot of residual for protease activity (U/ml) by *Bacillus pseudofirmus* MSB4

Table 5- Validation of quadratic model within the design space

Number	Temperature	Xylose	pH	Beef extract	Actual	Predicted	Predicted
1	37	1.5	8.97	3.11	174.37	177.703	1.88
2	37.03	1.5	9.03	3.04	172.49	177.677	2.92
3	37.2	4.43	9.13	2.7	166.95	170.783	2.24
4	37.3	1.5	9.36	3.16	173.54	177.457	2.21
5	36.5	1.5	8.39	3.24	174.33	176.972	1.49
6	37.06	1.5	8.98	3.12	172.12	177.702	3.14

[a] Predicted error (%) = (actual value - predicted value) × 100/predicted value

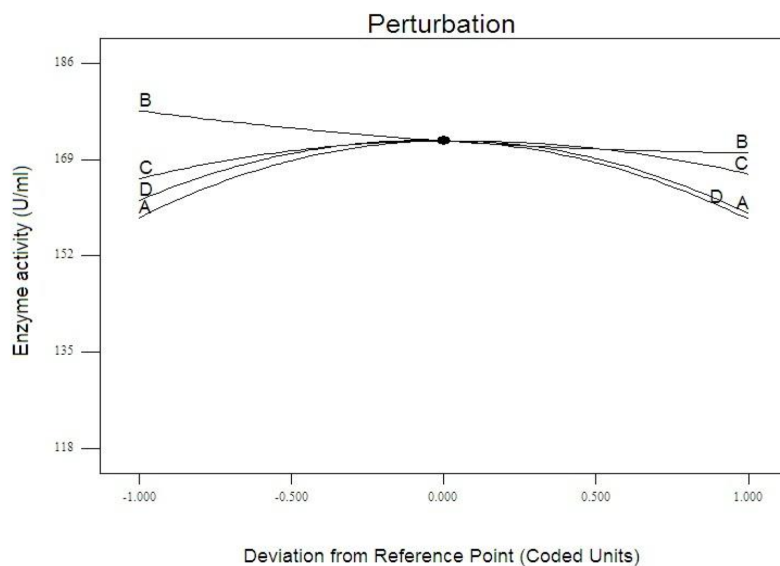

Fig. 8- Perturbation plot of enzyme activity by *Bacillus pseudofirmus* MSB4 as a function of temperature (A), xylose concentration (B), pH (C), and beef extract concentration (D).

Discussion and conclusion

A significant improvement (two-fold) in the production of alkaline protease by MSB4 was accomplished using CCD with respect to OFAT strategy. According to the agreements between OFAT and RSM results, regarding the significant effects of the variables tested in the present study, the OFAT strategy was a useful screening method for the selection of effective variables. In addition, RSM results showed that the enzyme production was enhanced using different values of variables with respect to OFAT results. However, maximum protease activity (185.397 U/ml) was low when compared with the protease activity of many *Bacillus* sp. used industrially; it should be taken into account that industrial strains activities were obtained from purified enzymes and after strain improvement steps. PCR analysis revealed that the strain MSB4 contained both extracellular serine and metalloprotease genes.

Acknowledgment

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کاربرد طرح مرکب مرکزی چرخش پذیر به منظور بهینه سازی تولید آنزیم آلکالین پروتئاز خارج سلولی توسط سویه جداسازی شده جدید *باسیلوس پسودوفیرموس* MSB4

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چکیده

مقدمه: پروتئازهای قلیایی خارج سلولی با منشا میکروبی، یکی از مهم‌ترین آنزیم‌های صنعتی بوده که کاربردهای فراوان در صنایع مختلف دارند. در این مطالعه، تولید پروتئاز قلیایی باکتری *باسیلوس پسودوفیرموس* MSB4 با استفاده از روش‌شناسی سطح پاسخ افزایش یافت.

مواد و روش‌ها: چهار متغیر مستقل موثر، بر اساس نتایج به دست آمده از روش تک عاملی (غلظت زایلوز، غلظت عصاره گوشت، pH و دمای انکوباسیون)، به منظور تجزیه و تحلیل الگوی پاسخ و ساخت مدل با استفاده از روش طرح مرکب مرکزی چرخش پذیر انتخاب شدند. طراحی آزمایش، شامل ۳۰ آزمایش دارای ۴ تکرار در نقطه مرکزی می شد و هر متغیر در ۵ سطح مختلف بررسی شد. به علاوه حضور ژن‌های پروتئازهای قلیایی خارج سلولی در سویه MSB4 با استفاده از PCR ارزیابی شد.

نتایج: حداکثر مقدار تولید آنزیم (۱۸۵/۳۹۷ U/ml) که حدود ۲/۲ برابر بیشتر از روش تک عاملی و ۳/۷ برابر بیشتر از شرایط غیر بهینه است، بر طبق نتایج طرح مرکب مرکزی چرخش پذیر با استفاده از ۳ درصد عصاره گوشت، اسیدیته ۹ و دمای ۳۷ درجه سانتی‌گراد به دست آمد. همچنین ژن های sub I, II و apr با سایز مورد انتظار (به ترتیب با ۳۱۹، ۴۸۶ و ۱۹۴ زوج باز) مسؤول متالوپروتئاز و سرین پروتئاز قلیایی خارج سلولی در سویه MSB4 با استفاده از PCR تشخیص داده شدند.

بحث و نتیجه‌گیری: نتایج آزمایشگاهی به خوبی با مدل به دست آمده سازگاری دارند (Adj R^2: ۰/۹۹۸۲) و این مدل توانایی زیادی برای پیش بینی متغیر پاسخ برای مشاهدات جدید خواهد داشت (Pred R^2: ۰/۹۹۶۷). نتایج این مطالعه نشان داد که روش تک عاملی و سطح پاسخ به ترتیب روش مفیدی برای غربال‌گری و بهینه‌سازی تولید پروتئاز در سویه MSB4 بودند

واژه‌های کلیدی: *باسیلوس پسودوفیرموس* MSB4، پروتئاز قلیایی، روش تک عاملی، روش‌شناسی سطح پاسخ، طرح مرکب مرکزی چرخش پذیر

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