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Statistical Optimization of Conditions for Protease Production from *Bacillus* sp. and Its Scale-up in a Bioreactor

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Abstract

A statistical approach, response surface methodology (RSM), was used to study the production of extracellular protease from *Bacillus* sp., which has properties of immense industrial importance. The most influential parameters for protease production obtained through the method of testing the parameters one at a time were starch, soybean meal, $CaCl_2$, agitation rate, and inoculum density. This method resulted in the production of 2543 U/mL of protease in 48 h from *Bacillus* sp. Based on these results, face-centered central composite design falling under RSM was employed to further enhance protease activity. The interactive effect of the most influential parameters resulted in a 1.50-fold increase in protease production, yielding 3746 U/mL in 48 h. Analysis of variance showed the adequacy of the model and verification experiments confirmed its validity. On subsequent scale-up in a 30-L bioreactor using conditions optimized through RSM, 3978 U/mL of protease was produced in 18 h. This clearly indicated that the model remained valid even on a large scale. RSM is a quick process for optimization of a large number of variables and provides profound insight into the interactive effect of various parameters involved in protease production.

Index Entries: *Bacillus* sp.; fermentation; protease; response surface methodology; face-centered central composite design.

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Introduction

Proteases are a complex group of enzymes collectively known as peptidyl peptide hydrolase. They account for about 60% of the worldwide sale of enzymes, and this percentage is expected to increase further *(1–3)*. Proteases have had a long history of use in the detergent, food, leather, dairy, and textile industries. Recently, these enzymes have attracted attention and are being employed in new sectors such as in the enzymatic conversion of protein wastes and in the development of therapeutic agents *(4–7)*. Protease production is an inherent property of all microorganisms; however, only those microbes that produce a substantial amount of this extracellular enzyme are of industrial importance. A major portion of the commercially available alkaline protease is derived from *Bacillus* sp. *(8–12).*

Because of the importance of proteases, an extensive screening program was carried out, which resulted in the isolation of a *Bacillus* sp. (isolate no. SBP-66) as a potential protease producer. Quantitative estimation showed that this isolate initially produced 242 U/mL of protease in modified minimal medium supplemented with 1% casein. Characterization of this protease showed that the enzyme has a wide range of both pH (6.0–12.0) and temperature tolerance (4–100°C), with an optima at 9.0 and 60°C, respectively. The protease has a residual activity of 33% at pH 12.0 even after 12 h of incubation, and 15% residual activity at 100°C even after 30 min of incubation. It is stable for 6 mo at room temperature. Owing to these properties, this enzyme has shown its importance in the formulation of detergents and degumming of raw silk fiber (unpublished data).

Based on the aforementioned properties and applications, it was deemed worthwhile to develop an economically viable fermentation process for the maximum production of this enzyme. It was established that extracellular production of protease in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources *(13–16)*, and physical factors such as pH, temperature, agitation rate, inoculum density, and incubation period *(16–19)*. Therefore, optimization of these parameters is paramount in obtaining maximum production of this enzyme.

Response surface methodology (RSM), a statistical approach, is an efficient tool for optimizing different physicochemical parameters. It has an advantage over the traditional approach of dealing with one variable at a time because the latter is a time-consuming process and does not account for the combined interactions among various physicochemical parameters *(20–25).* Statistical optimization is carried out to determine the influence of variables on enzyme units and to optimize them for achieving maximum yield under the best possible economic conditions.

In the present study, we employed face-centered central composite design (FCCCD) falling under RSM to optimize conditions for obtaining maximum protease production. Subsequently, we conducted the production of this enzyme in a 30-L bioreactor using the optimized conditions obtained through RSM.

Materials and Methods

Chemicals

Casein for protease assay was purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade and procured from the local market.

Organism and Growth Conditions

Bacillus sp. (SBP-66), a potent protease producer, was isolated from the soil at 37°C by enrichment and selective screening on a skimmed milk agar plate. The isolate is a Gram-positive bacillus. It was cultivated at 37 ± 1°C in a bacteriologic incubator for 24 h and subsequently maintained at 4°C in a B.O.D. incubator (Yorko, Deluxe-10) by routine transfers on nutrient agar slants at pH 7.0.

Production of Enzyme and Harvesting of Culture

The modified basal medium (pH 7.0) used for protease production contained glucose (variable), casein (variable), 1.0 g/L of KH_2PO_4, 3.0 g/L of K_2HPO_4, 2.0 g/L of Na_2SO_4, and 0.10 g/L of $MgSO_4 \cdot 7H_2O$. The production medium (50 mL) was inoculum with 2.0% inoculum with an OD_{660} of 0.65. The culture was incubated at 37 ± 1°C at 200 rpm in an incubator shaker (NBS Shaker, G-25R; Edison, NJ). After the desired incubation period, the culture was centrifuged at 8000*g* for 20 min at 4°C (Sorvall, RC-5C plus; DuPont, Ratingen, Germany) to obtain protease-rich broth. The cell pellet was dis-carded and the supernatant preserved at 4°C for enzyme analysis.

Protease Assay

Protease activity was measured as described by Meyers and Ahearn *(26)* with some modifications. A total of 0.5 mL of glycine NaOH buffer (pH 10.0, 0.2 *M*) was added to 0.5 mL of appropriately diluted enzyme and was then incubated with 1 mL of 1% casein solution (prepared in glycine NaOH buffer, pH 10.0) for 15 min at 60°C. The reaction was stopped by the addition of 4 mL of 5% (v/v) trichloroacetic acid. The contents were centrifuged after 1 h at 3000*g* for 10 min, and the filtrate was used to measure protease activity on the basis of color development. For color development, 5 mL of 0.4 *M* sodium carbonate solution was added to 1 mL of the filtrate and kept for 10 min. To this, 1:1 diluted Folin's Ciocalteau Phenol reagent was added and kept in the dark for 30 min, and the optical density was recorded at 660 nm. One unit of protease was equivalent to the amount of enzyme required to release 1 mg/(mL·min) of tyrosine under standard assay conditions.

RSM: FCCCD as Experimental Design

The statistical approach RSM was employed to study the interaction of the most influential parameters (i.e., starch, soybean meal, $CaCl_2$,

Table 1
Experimental Range of Levels of Five Independent Variables Used in RSM in Terms of Actual and Coded Factors

	Range of level					
Variable	Actual	Coded	Actual	Coded	Actual	Coded
Starch (g/L)	0.25	–1	0.5	0	0.75	+1
Soybean meal (g/L)	15	–1	20	0	25	+1
CaCl2 (m*M*)	3	–1	4	0	5	+1
Agitation rate (rpm)	150	–1	200	0	250	+1
Inoculum density (%)	1	–1	2	0	3	+1

agitation rate, and inoculum density) obtained using the method of testing the parameters one at a time. FCCCD was performed because it incorporates replication of the medial point as (000). Table 1 provides the levels of the five variables—starch as carbon source (*A*), soybean meal as nitrogen source (*B*), $CaCl_2$ as metal ion (*C*), agitation rate (*D*), and inoculum density (*E*)— selected for this study.

The statistical software package Design-Expert® 6.0 (Stat-Ease, Minneapolis, MN) was used to analyze the experimental design. A set of 32 experiments was generated. Table 2 presents the design matrix with five variables set at three levels (–1, 0, +1). The ranges of these variables were decided according to the ranges determined by the one-at-a-time method. Other variables were set at their optimum level. Experiments were performed in triplicate, and the average of the three readings was incorporated into the Design-Expert software to avoid any practical error.

Statistical Analysis and Modeling

The data on protease production were subjected to analysis of variance (ANOVA). The mathematical relationship of the independent variable and the response (protease production) was calculated by the second-order polynomial equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2 + \beta_{12} AB + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD$$

in which Y is the predicted response; β_0 is the intercept; β_1, β_2, β_3, β_4, and β_5 are linear coefficients; β_{22}, β_{33}, β_{44}, and β_{55} are squared coefficients; and β_{12}, β_{14}, β_{23}, and β_{24} areinteraction coefficients. To validate the model, six sets of experiments were randomly generated by the software within the design space.

Protease Production in a Bioreactor

The final scale-up and verification of the statistical model for protease production was carried out in a 30-L bioreactor (Scigenics, Chennai, India).

Table 2
Design Matrix of FCCCD for Protease Production

Run no.	Glucose	Soybean meal	$CaCl_2$ (m*M*)	Agitation rate (rpm)	Inoculum density (%)	Mean observed (U/mL)	Mean predicted (U/mL)
1	–1	–1	–1	+1	–1	3008	3011
2	0	0	0	0	0	2546	2550
3	+1	–1	+1	+1	–1	2131	2143
4	0	0	0	0	0	2550	2548
5	0	0	0	0	0	2543	2553
6	–1	–1	–1	+1	0	3747	3742
7	0	0	–1	0	0	2601	2608
8	+1	–1	–1	+1	+1	2772	2779
9	0	0	+1	0	0	2389	2385
10	–1	–1	–1	–1	+1	2246	2254
11	+1	–1	–1	–1	–1	2842	2837
12	0	0	0	+1	0	2731	2726
13	0	0	0	–1	0	2423	2419
14	–1	–1	+1	+1	+1	2775	2781
15	–1	0	0	0	0	2619	2625
16	+1	+1	+1	+1	+1	3088	3093
17	0	0	0	0	0	2549	2557
18	–1	–1	+1	–1	–1	2627	2631
19	+1	+1	–1	+1	–1	3206	3200
20	0	0	0	0	+1	2444	2451
21	–1	+1	+1	+1	–1	2950	2942
22	0	–1	0	0	0	3031	3027
23	0	+1	0	0	0	2488	2493
24	–1	+1	+1	–1	+1	3022	3017
25	+1	0	0	0	0	2399	2396
26	+1	+1	+1	–1	–1	2635	2641
27	–1	+1	–1	–1	–1	2746	2740
28	+1	–1	+1	–1	+1	2325	2329
29	0	0	0	0	0	2545	2548
30	0	0	0	0	0	2549	2553
31	0	0	0	0	–1	2123	2119
32	+1	+1	–1	–1	+1	3054	3049

The optimized medium was sterilized *in situ* at 121°C for 20 min and inoculated with an appropriate amount of the seed inoculum (OD_{660nm}· 0.600). Fermen-tation was carried out at 37 ± 1°C for 24 h with the pH controlled at 7.0. The impeller speed was initially adjusted to 350 rpm, and compressed sterile air was sparged into the medium at a constant rate of 5 vvm. The dissolved oxygen (DO) was not allowed to fall below a fixed set point of 25% by cascading. Samples were withdrawn at 2-h intervals and analyzed for protease production.

Results and Discussion

The most influential parameters optimized by the one-at-a-time method were starch (0.5 g/L), soybean meal (20 g/L), $CaCl_2$ (4 m*M*),

agitation rate (200 rpm), and inoculum density (2%), resulting in the production of 2543 U/mL of protease in 48 h.

A statistical design approach using RSM was employed to study the interactive effects of different nutritional and physical factors on protease production. Table 2 summarizes the response (i.e., protease production) of the experiment (FCCCD) for each individual run along with the predicted responses. By employing RSM, a maximum protease production of 3746 U/mL was achieved in 48 h of incubation at a starch concentration of 0.25 g/L, a soybean concentration of 15 g/L, a $CaCl_2$ concentration of 3 m*M*, an agitation rate of 250 rpm, and an inoculum density of 2.0%.

The results obtained after FCCCD were then analyzed by standard ANOVA, which gave the following regression equation (in terms of coded factors):

$$Y = +2551.62 - 81.69*A + 513.35*B - 90.35*C + 173.35*D + 23.69*E + 4.54*B^2 - 9.09*C^2 + 3.54*D^2 + 4.79*E^2 + 6.84*A*B - 3.41*A*D + 7.84*B*C - 23.97*B*D$$

in which Y is the protease produced as a function of starch (A), soybean meal (B), $CaCl_2$ (C), agitation rate (D), and inoculum density (E).

The regression equation obtained from the ANOVA indicated that the coefficient of determination (R^2) was 0.9851 (Table 3); a value >0.75 indicates aptness of the model *(27)*. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approx 98% of the variability in the dependent variable (response) could be explained by this model. For a good statistical model, the R^2value should be close to 1 *(25,28)*. The PROB-value was equal to 0.0001 and the model was significant. Nine model terms (A, B, C, D, B^2, C^2, AB, BC, BD) were most significant with the PROB-F value <0.0001. The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.9746. The model F-value was 218.80 and the lack of fit F-value was 3.24. High F-value and nonsignificant lack of fit indicated that the model was a good fit (Table 3). The predicted sum of square (PRESS), a measure of how a particular model fits each point in the design, was 0.9654. The adequate precision value, which measures the signal-to-noise ratio, was 58.731, indicating an adequate signal. A ratio >4 is desirable *(27)*. Thus, this model could be used to navigate the design space.

Three-dimensional (3D) response surface curves were plotted to determine the optimum concentration of each factor for maximum protease production. Figure 1 shows the relative effects of three different sets of factors—starch and soybean meal (Fig. 1A), soybean meal and $CaCl_2$ (Fig. 1B), and soybean meal and agitation rate (Fig. 1C)—when all the other factors are kept at their optimum levels. The results clearly showed a fairly strong degree of curvature of 3D surface, from where the optimum was determined.

Six validation experiments (Table 4) clearly showed that the experimentally determined production value of 3703 U/mL was in close agreement

Table 3
ANOVA for Response Surface Quadratic Model

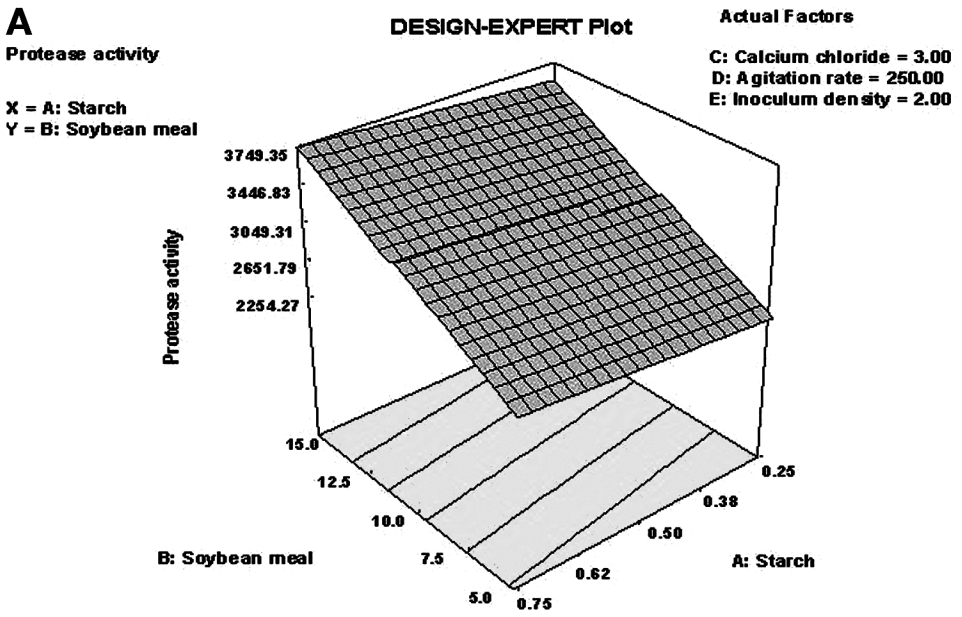
Model term	Value
R^2	0.9851
Adj R^2	0.9746
Pred R^2	0.9654
Adeq precision	58.731
Model *F*-value	218.80
Lack of fit *F*-value	3.24

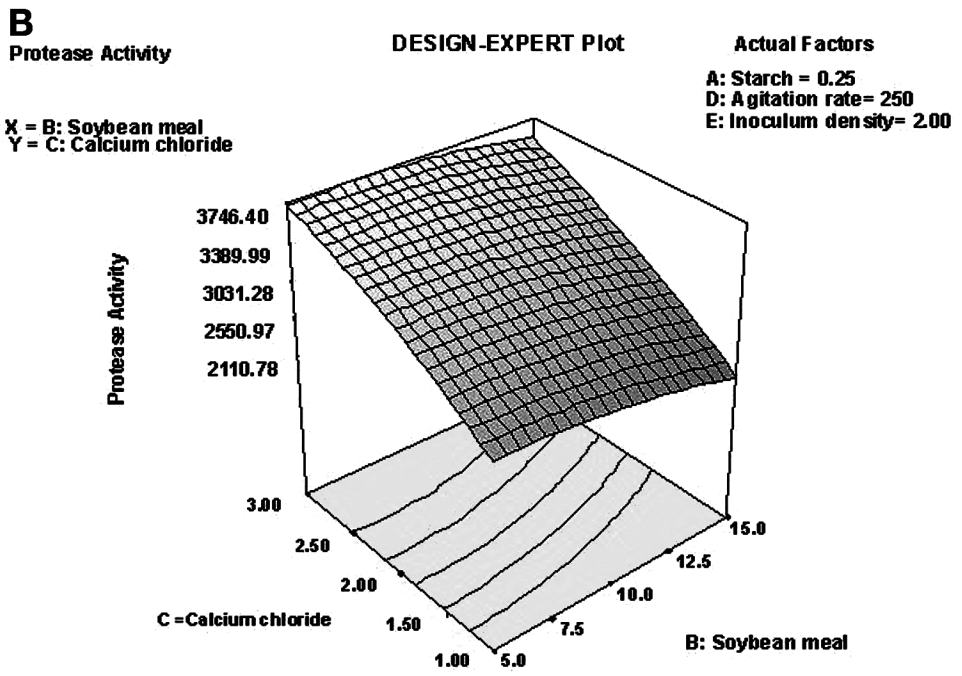
with the statistically predicted one, 3710 U/mL. The results verify that the previous model with soybean meal (15.0 g/L), $CaCl_2$ (3 m*M*), and agitation rate (250 rpm) had the best combination of factors for obtaining maximum protease production, thereby confirming the model's accuracy.

Verification of Model in a 30-L Bioreactor

The conditions obtained as optimum through RSM were finally examined and verified in a 30-L bioreactor with a working volume of 22.5 L. Protease production started early, within 4 h (lag phase of growth); however, there was no significant increase in the production up to 8 h (exponential phase of growth). At this time, as the organism was in the exponential phase, a significant fall (below 25%) in DO was observed. To provide the DO, the agitation reached up to 700 rpm through cascading. The exponential phase was followed by the stationary phase, during which maximum protease activity was observed, resulting in the production of 3978 U/mL of protease in 18 h (Fig. 2). Thereafter, a decline in protease production was observed in the bioreactor. Moon and Parulekar *(29),* Chu et al. *(30)*, and Gupta et al. *(31)* also reported a similar cessation in protease production once a maximum amount of the enzyme was produced during the run. There are several theories, such as autoproteolysis *(32)* and protease degradation by some proteolytic activity on the cell surface *(30),* that could explain this cessation of protease production; however, the exact mechanism is yet to be clearly known.

During fermentation, aeration rate also influences the mixing of medium components and thus affects the nutrient availability to microorganisms *(29,33).* In the present model, the high agitation rate (>200 rpm) favored maximum production, and a decrease in the agitation rate drastically lowered the total yield of protease. Protease production at high agitation rate up to 360–600 rpm has been reported by Moon and Parulekar *(29)* while working with *Bacillus firmus* and by Jang et al. *(32)* while working with *Bacillus* sp. B21-2. A similar result was found in the present investigation: when the agitation rate increased from 350 to 700 rpm, the organism grew at a fast rate, achieving maximum protease (3978 U/mL) in a minimal time of 18 h in the bioreactor.


A
Protease activity
X = A: Starch
Y = B: Soybean meal
DESIGN-EXPERT Plot
Actual Factors
C: Calcium chloride = 3.00
D: Agitation rate = 250.00
E: Inoculum density = 2.00
Protease activity
3749.35
3446.83
3049.31
2651.79
2254.27
B: Soybean meal
15.0
12.5
10.0
7.5
5.0
A: Starch
0.25
0.38
0.50
0.62
0.75


B
Protease Activity
X = B: Soybean meal
Y = C: Calcium chloride
DESIGN-EXPERT Plot
Actual Factors
A: Starch = 0.25
D: Agitation rate= 250
E: Inoculum density= 2.00
Protease Activity
3746.40
3389.99
3031.28
2550.97
2110.78
C =Calcium chloride
3.00
2.50
2.00
1.50
1.00
B: Soybean meal
15.0
12.5
10.0
7.5
5.0

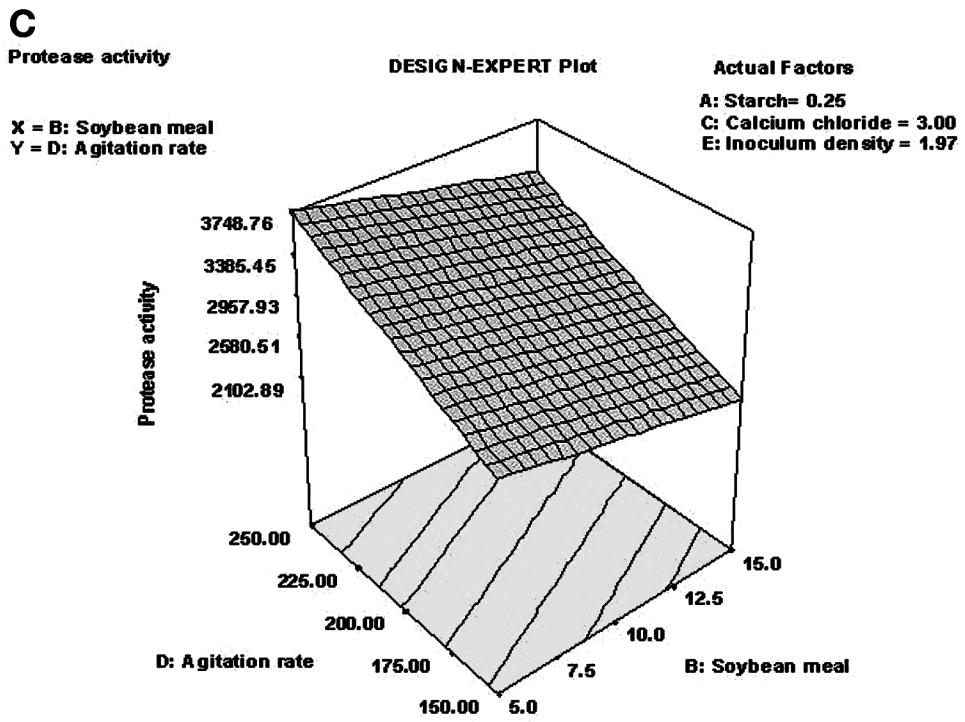


Fig. 1. Response surface curves of protease production from *Bacillus* sp. showing **(A)** interaction between starch and soybean meal and **(B)** interaction between soybean meal and $CaCl_2$; and **(C)** interaction between soybean meal and agitation rate.

Table 4
Validation of FCCCD Using Different Levels of Soybean Meal, $CaCl_2$, and Agitation Rate

Run no.	Soybean meal (g/L)	$CaCl_2$ (m*M*)	Agitation rate (rpm)	Protease activity (U/mL)	
				Predicted	Observed
1	1.25	3.25	250	3327	3321
2	0.85	3.00	175	2580	2577
3	1.50	2.75	275	3559	3562
4	1.75	2.50	250	3486	3482
5	1.60	3.00	240	3710	3703
6	1.50	3.50	225	3621	3618

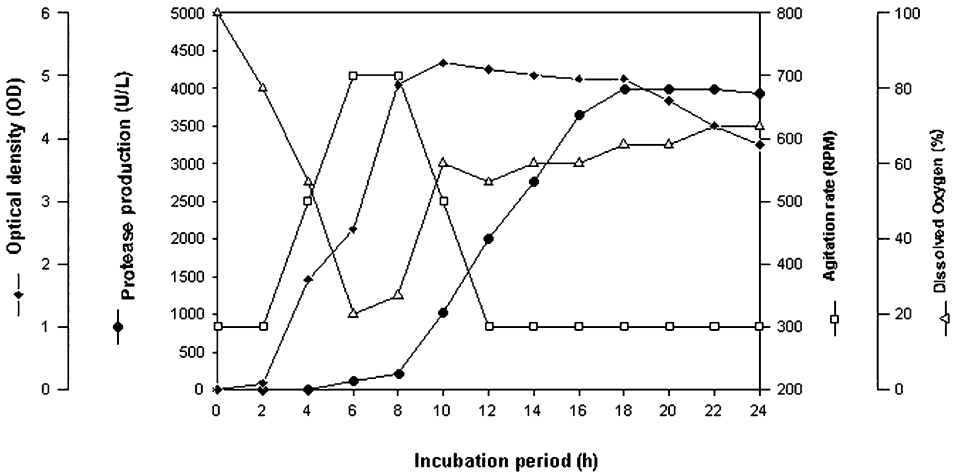


Fig. 2. Fermentation profile of protease production by *Bacillus* sp. in a 30-L bioreactor.

Conclusion

It is evident that by employing RSM *Bacillus* sp. (SBP-66) could produce 3978 U/mL of protease in a 30-L bioreactor and 3746 U/mL in shake flasks in 18 and 48 h, respectively. This is an approx 1.5-fold increase in the yield of protease compared to the one-at-a-time method, for which 2543 U/mL of the enzyme was obtained in 48 h. By employing this statistical approach, there is not only an increase in the protease yield but also a reduction of 5 g/L in the concentration of soybean meal from 20 to 15 g/L, and a slight reduction in the concentration of starch from 0.5 to 0.25 g/L and $CaCl_2$ from 4 to 3 m*M*. The statistical approach has an advantage over the classic approach because it demonstrates the effect of interaction(s) of various parameters. With the increase in the yield and simultaneous reduction in the cost as well as time, protease production using RSM is economically attractive. It will certainly enable researchers to develop a procedure that may be useful in many industries seeking potential hydrolases.

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