

Optimization of Alkaline Protease Production by *Bacillus* sp. Using Taguchi Methodology

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Received September 15, 2003; Revised July 20, 2004;

Accepted August 13, 2004

Abstract

Optimization of alkaline protease production parameters by *Bacillus* sp. was investigated using Taguchi methodology. The pH of the medium was observed to be the most significant factor among all selected optimization parameters at an individual level. The combinatorial influence of least significant factors, inoculum level and salt solution concentration (at the individual level), resulted in an interacting severity index of 76%, suggesting their interactive role in the regulation of protease production in this microbial species. Protease production could be improved more than 100% with Taguchi's optimized conditions of the medium composition by this microorganism.

Index Entries: Alkaline protease; *Bacillus* sp.; production; optimization; Taguchi methodology.

Introduction

Alkaline proteases are gaining considerable industrial importance because of their stabilized catalytic activity at elevated pH conditions. They are classified as hydrolases (EC 3.4.21.14), which are primarily used as detergent additives. They are also widely used on a commercial level for multiple applications in several industrial sectors such as leather processing, silver recovery, food processing, pharmaceuticals, feeds, chemicals, and water processing (1). In addition, proteases are known to contribute to the development of enzyme-added digestion-derived high-value products.

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The present commercial turnover is increasing logarithmically, with more than \$700 million in business at present.

Although several microbial species are known to produce the enzyme, protease (2–8), only those microbial organisms have importance in the industrial sector, which produces substantial quantities of enzyme (9). Alkaline protease-producing organisms are widely distributed in nature and can be found in almost every environment (1). The physicochemical and kinetic properties of enzymes vary with the microbial source. A source-dependent variation in physical/biochemical/molecular properties, such as optimum pH, temperature, metal ion requirement, substrate specificity, inhibition pattern, and molecular weight, of protease enzymes produced by different microbial species has been observed (10–17). This created considerable interest in the exploitation of proteases, which have the capability to catalyze reactions in a nonconventional environment (1). In fact, major industrial companies and the scientific world are continuously involved in efforts to isolate and identify the microbial biosystems that produce enzymes that have potential in industrial as well as commercial application directly and indirectly (1).

Enzyme production, in general, is highly regulated by the microbial cell metabolism (18), which, in turn, is regulated by media composition, such as carbon and nitrogen sources (10,19), metal ion, and their concentrations (8,20), and type of carbon/nitrogen source and their ratio. In addition, the production properties of enzymes depend on physiologic factors such as pH (21), incubation temperature (22), aeration, and agitation (3,23) of the fermentation process. Therefore, it is essential that any isolated organism be provided with optimal growth conditions to obtain maximum enzyme productivity (1). The culture conditions that promote optimum enzyme production differ significantly with the biochemical nature of the microbial strain (1,3,8,10,19,20,23). Hence, optimization of the components of the fermentation medium and physiologic growth conditions is essential to optimizing product production pattern.

Conventional optimization of product production, by altering each factor one at a time, is a time- and labor-consuming process and does not provide the combinatorial effect of studied factors effectively. To deal with such a situation, statistical approaches have been developed using a combination of mathematical and analytical tools (24). In this respect, recently developed orthogonal array (OA) optimization methodology provides offline quality control of system, parameter, and tolerance designs that help in the identification of key factors and their levels for best performance (25). In addition, the tolerance design provides fine-tuning of the factors for optimum productivity. Moreover, the variant design enables one to focus on the main factors to enhance efficacy and reproducibility. Several microbial product and process parameters have been optimized with significant success using this Taguchi methodology (26–30). In the present study, our goal was to optimize alkaline protease production using an isolated *Bacillus*

Table 1
Factors and Their Levels Assigned to Different Columns

Serial no.	Factor	Level 1 (–)	Level 2 (+)
1	pH	8.0	12.0
2	Glucose (w/v [%])	0.50	1.00
3	Peptone (w/v [%])	0.50	1.50
4	Inoculum level (v/v [%])	3.00	8.00
5	Salt solution (v/v [%])	3.00	8.00

strain. We found significant improvement in enzyme production under optimized conditions.

Materials and Methods

Organism and Growth Conditions

An isolated bacterial strain that has potential to produce protease at alkaline pH growth conditions was used. The microbial strain was preliminarily characterized as *Bacillus* strain. The organism was grown in medium consisting of 0.75% glucose, 1.0% peptone, and 0.5% salt solution; salt solution was prepared by dissolving 0.5 g of MgSO_4 , 0.5 g of KH_2PO_4 , and 0.01 g of $\text{Fe}(\text{SO}_4)_2$ in 100 mL of distilled water) at pH 10.0. The sterile medium was inoculated with 5% inoculum aseptically and grown by incubating at 200 rpm at $32 \pm 2^\circ\text{C}$. The organism was maintained on agar-based growth medium slants incubated at $32 \pm 2^\circ\text{C}$, stored at 4°C until further use, and subcultured at regular intervals.

Analytical Procedure

Alkaline protease activity was measured in cell-free broth (enzyme source) using a modified casein hydrolysis method. A total of 2 mL of reaction mixture contained 1.0 mL of 1% (w/v) casein dissolved in glycine-NaOH buffer (0.02 M, pH 11.0) and 0.9 mL of 70°C preincubated glycine-NaOH buffer (0.02 M, pH 11.0). The enzyme reaction was initiated by adding 0.1 mL of enzyme source and incubated at 70°C for 30 min. The reaction was then terminated by adding 2 mL of 10% trichloroacetic acid. The precipitated unhydrolyzed casein was removed by filtration using Whatman filter paper no. 1 and absorbance of the filtrate was measured at 280 nm. The protease activity was measured in terms of tyrosine released using a tyrosine standard curve. One unit of the alkaline protease activity was defined as the amount of enzyme required to liberate $1\text{ }\mu\text{g}$ of tyrosine/mL under experimental conditions.

Design of Experiments

An L16 OA was used consisting of 16 different experimental trials. The total degrees of freedom available in an OA was equal to the number

Table 2
OA (L16) Experimental Design for Alkaline Protease Production by Isolated *Bacillus* sp.

Experiment no.	Selected optimization factors and levels ^a					Experimental protease production (U/mL)
	pH	Glucose (w/v [%])	Peptone (w/v [%])	Inoculum level (v/v [%])	Salt solution (v/v [%])	
1	-	-	-	-	+	6.67
2	-	-	-	+	-	5.53
3	-	-	+	-	-	19.9
4	-	-	+	+	+	1.66
5	-	+	-	-	-	10.02
6	-	+	-	+	+	9.03
7	-	+	+	-	+	18.99
8	-	+	+	+	-	25.38
9	+	-	-	-	-	27.98
10	+	-	-	+	+	32.98
11	+	-	+	-	+	46.53
12	+	-	+	+	-	68.01
13	+	+	-	-	+	81.99
14	+	+	-	+	-	45.44
15	+	+	+	-	-	67.44
16	+	+	+	+	+	89.23

^aA minus sign indicates the level 1 selected parameter concentrations, and a plus sign indicates the level 2 selected parameter concentrations in Table 1.

Table 3
Main Effects of Selected Factors on Alkaline Protease Production

Serial no.	Factor	Level 1	Level 2	L2–L1
1	pH	11.772	57.424	45.651
2	Glucose (w/v [%])	25.757	43.440	17.682
3	Peptone (w/v [%])	39.357	26.840	–9.517
4	Inoculum level (v/v [%])	27.430	41.767	14.337
5	Salt solution (v/v [%])	37.807	31.389	–6.419

of trials minus one. Each column consisted of a number of conditions depending on the levels assigned to each factor. All five selected factors and their assigned levels and the experimental design along with protease production data are represented in Tables 1 and 2, respectively.

Results and Discussion

To achieve the maximum alkaline protease productivity by the *Bacillus* strain, the following parameters were selected for optimization: pH of the medium, glucose (w/v [%]), peptone (w/v [%]), salt solution (v/v [%]) concentration and inoculum level (v/v [%]). Each factor was assigned two levels: minimum and maximum values. Table 3 indicates the average effects of the studied factors and their interactions at the assigned levels on alkaline protease production under experimental conditions. The relative differential influence of each factor on enzyme protease production at level 2 and level 1 against average value is shown in column 5 of Table 3. The data denote that the selected parameters showed mixed influence on the production of protease by this organism. The pH of the medium was found to have the strongest impact on the protease production pattern, whereas peptone, a medium component, had the least influence among the selected optimization factors. The order of impact of selected optimization parameters was observed to be pH < glucose < inoculum level < salt solution < peptone concentration at the assigned levels. This suggests that hydrogen ion concentration, carbon source, and initial inoculum level play a vital role in alkaline protease production in this microbial species compared to that of nitrogen source and salt solution concentration, indicating the importance of growth pH in metabolic reactions leading to alkaline protease production in this bacterial strain.

Further analysis of these results with respect to fine-tuning a broader range of selected parameters revealed that the optimization parameters along with their assigned levels had a mixed impact on protease production. The assigned levels of inoculum level, glucose concentration, and pH values revealed a positive effect whereas the other two parameters showed a negative influence on protease production when factor-assigned values increased from level 1 to level 2 (Fig. 1), and among all selected optimization parameters, pH showed the maximum influence on protease produc-

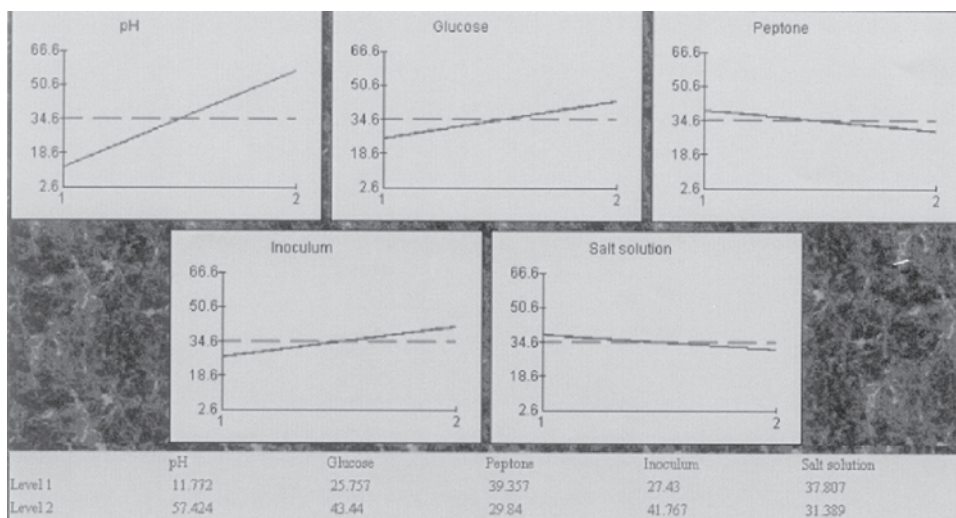


Fig. 1. Multiple graphs of main effects on alkaline protease production by *Bacillus* sp.

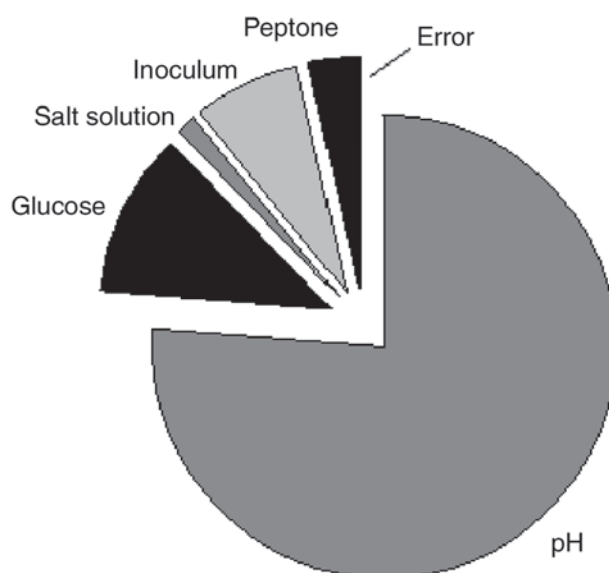


Fig. 2. Significant factors and interaction influences on alkaline protease production by *Bacillus* sp.

tion. The relative influence of selected factors and their interactions in terms of significant factor and interaction influence are depicted in Fig. 2. Here, too, the results reveal that the least significant factor was salt solution concentration and the most significant factor was pH.

Table 4
Estimated Interaction of Severity Index for Selected Parameters^a

Serial no.	Interacting factor pairs	Columns	SI %	Col	Opt
1	Inoculum × salt solution	4 × 5	76.10	1	[2,1]
2	Glucose × peptone	2 × 3	72.08	1	[2,1]
3	pH × peptone	1 × 3	27.91	2	[2,1]
4	pH × salt solution	1 × 5	23.89	4	[2,1]
5	Peptone × inoculum	3 × 4	22.79	7	[1,2]
6	Glucose × salt solution	2 × 5	22.56	7	[2,1]
7	pH × glucose	1 × 2	15.02	3	[2,2]
8	pH × inoculum	1 × 4	10.69	5	[2,2]
9	Peptone × salt solution	3 × 5	04.37	6	[1,1]
10	Glucose × inoculum	2 × 4	02.17	6	[2,2]

^aColumns = the column locations to which the interacting factors are assigned; SI = interaction severity index (100% for 90 ° angle between the lines, 0% for parallel lines); Col = shows the column that should be reserved if this interaction effect were to be studied (only 2-L factors); Opt = the factor levels desirable for the optimum condition (based strictly on the first two levels). If an interaction is included in the study and found significant (in ANOVA), the indicated levels must replace the factor levels identified for the optimum condition without consideration of any interaction effects.

Analytical data on different factors and their interactions indicate that the least influential factors had a major role in the production of alkaline protease compared with their contribution at individual levels. It can be clearly seen from Table 4 that the combination of inoculum level and salt solution resulted in the maximum severity index (76.1%), whereas the pH (the strongest factor at the individual level) interaction with the least influential factor at the individual level, peptone, showed a severity index of nearly 75% less than the inoculum level vs salt solution interaction on protease production. Maximum productivity interaction was seen for four factors (i.e., inoculum level vs salt solution and carbon source [glucose] vs peptone) of five selected factors, as indicated by severity index values (Table 4). These data suggest that although the pH had the highest influence at the individual level, it did not show much of an effect at the interaction level. In addition, except inoculum vs salt solution and glucose vs peptone, all other interaction pairs showed a severity index between 28 and 2%, thus indicating that the influence of each factor on alkaline protease production was dependent on the level and condition of the other factors in the protease optimization process.

Analysis of variance of the data (ANOVA) was performed to determine the percentage contribution of each factor (Table 5). The results suggest that the pH of the medium during fermentation was the most significant factor in alkaline protease production by the studied microbial strain. The percent contribution of this factor to protease production was observed to be 76%, whereas all other investigated factors contributed only 24%. Among all studied factors, glucose was the next most significant con-

Table 5
Results of ANOVA

Serial no	Factor	df (f)	Sum of squares (S)	Variance (V)	F ratio (F)	Pure sum (S')	Percent (P[%])
1	pH	1	8336.602	8336.602	833,660,211.559	8336.602	76.226
2	Glucose (w/v [%])	1	1250.684	1250.684	125,068,476.014	1250.684	11.435
3	Peptone (w/v [%])	1	362.332	362.332	36,233,251.102	362.332	3.313
4	Inoculum (v/v [%])	1	822.256	822.256	82,225,694.662	822.256	7.518
5	Salt solution (v/v [%])	1	164.738	164.738	16,473,854.395	164.738	1.506

Table 6
ANOVA Table After Pooling

Serial no	Factor	df (f)	Sum of squares (S)	Variance (V)	F ratio (F)	Pure sum (S')	Percent (P[%])
1	pH	1	8336.602	8336.602	189.802	8292.679	75.824
2	Glucose (w/v [%])	1	1250.684	1250.684	28.474	1206.672	11.034
3	Peptone (w/v [%])	(1)	(362.332)		Pooled (CL = 99.96%)		
4	Inoculum (v/v [%])	1	822.256	822.256	18.72	778.334	7.116
5	Salt solution (v/v [%])	(1)	(164.738)		Pooled (CL = 99.99%)		

CL, Confidence level.

Table 7
Optimum Conditions and Performance

Serial no.	Factor	Level description	Level	Contribution
1	pH	12	2	22.826
2	Glucose (w/v [%])	1.0	2	8.841
3	Peptone (w/v [%])	0.5	1	4.758
4	Inoculum level (v/v [%])	8.0	2	7.168
5	Salt solution (v/v [%])	3.0	1	3.208

tributor to alkaline protease production. These results are in accordance with earlier observations in which the pH of the medium was found to be one of the important factors in optimal production of enzyme, especially with an alkalophilic microorganism in which pH is known to strongly influence many enzymatic processes and the transport of various compounds across the cell membrane (1,3,30–35).

To reduce the error term, the ANOVA data for protease production were fine-tuned by pooling the data until the degrees of freedom became close to half the total degrees of freedom; the data are depicted in Table 6. The least significant factors among all selected factors, peptone and salt solution, were pooled for this purpose. It was found that the significance of pH, glucose, and inoculum level were changed to 76, 11, and 7%, respectively, after pooling.

Based on the analysis of the data using Taguchi's optimization procedure, the optimum conditions and their performance were estimated and are presented in Table 7. The total contribution of all selected parameters was observed to be 46.801, and the current grand average performance of the experimental factors was 34.598. The data suggest that among all investigated factors, increasing the pH of the medium toward alkalinity was found to be more significant for alkaline protease production by this isolated *Bacillus* strain followed by glucose concentration. The studied factors of peptone level and salt solution (%) should be used at a lower level, 0.5 and 3%, respectively, for optimum production.

Figure 3 presents the influence of the optimized conditions and their performance in alkaline protease production over current experimental conditions. With the optimized conditions, the expected protease production could be increased more than 100% over the studied conditions, thus indicating that the regulation of hydrogen ion concentration during fermentation drastically improves alkaline protease production with optimized conditions. This finding coincides with previously observed values of alkaline protease production by *Bacillus* sp. (3).

Conclusion

A combination of parameters and their level influenced the production of alkaline protease by isolated *Bacillus* sp. Optimized conditions

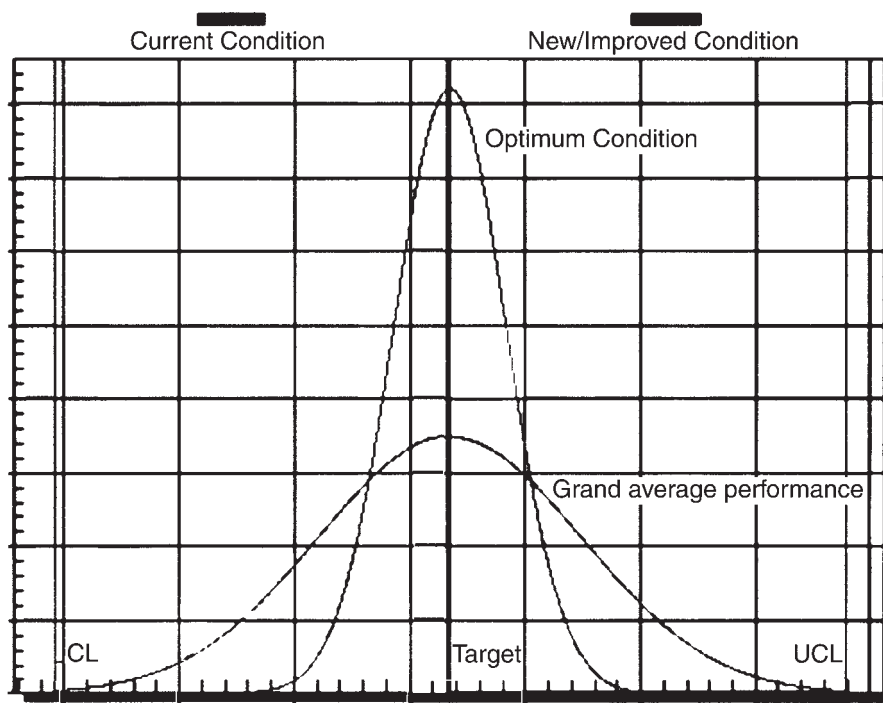


Fig. 3. Current vs improved production of alkaline protease by *Bacillus* sp.

improved alkaline protease production by more than 55% to that of presently studied conditions. The pH of the medium was found to be the most significant factor in maximum enzyme production.

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