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Original Article

Optimization of Chitinase Production by *Bacillus pumilus* Using Plackett-Burman Design and Response Surface Methodology

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Abstract

A soil bacterium capable of degrading chitin on chitin agar plates was isolated and identified as *Bacillus pumilus* isolate U_5 on the basis of 16S rDNA sequence analysis. In order to optimize culture conditions for chitinase production by this bacterium, a two step approach was employed. First, the effects of several medium components were studied using the Plackett-Burman design. Among various components tested, chitin and yeast extract showed positive effect on enzyme production while $MgSO_4$ and $FeSO_4$ had negative effect. However, the linear model proved to be insufficient for determining the optimum levels for these components due to a highly significant curvature effect. In the second step, Box-Behnken response surface methodology was used to determine the optimum values. It was noticed that a quadratic polynomial equation fitted he experimental data appropriately. The optimum concentrations for chitin, yeast extract, $MgSO_4$ and $FeSO_4$ were found to be 4.76, 0.439, 0.0055 and 0.019 g/L, respectively, with a predicted value of chitinase production of 97.67 U/100 mL. Using this statistically optimized medium, the practical chitinase production reached 96.1 U/100 mL.

Keywords: Optimization; Bacillus pumilus; Chitinase; Box-Behnken; Plackett-Burman.

Introduction

Chitinases are the enzymes responsible for the biological degradation of chitin (1). These enzymes are produced by a wide range of organisms including bacteria, fungi, plants, insects, crustaceans and vertebrates (2-5). Bacteria produce chitinolytic enzymes to meet nutritional needs. In fungi, insects and crustaceans, these enzymes are involved in morphogenesis. In plants and probably vertebrates, they play a role in defense mechanism against pathogens. Chitinases have found many industrial and pharmaceutical applications including biocontrol of plant pathogenic fungi and insects, production of chitooligosaccharides, and management of chitinous wastes (6, 7).

In microorganisms, chitinase production is controlled by a receptor-inducer system; therefore, the composition of the culture medium can affect chitinase production (7). The

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conventional method for medium optimization involves changing one parameter at a time while keeping all others constant. This method may be very expensive and time consuming. In addition, it fails to determine the combined effect of different factors. A number of statistical experimental designs have been used to address these problems (8). Among theses, full factorial designs provide more complete information, but they require lots of experiments (L^k, where k is the number of variables and L is the number of levels for each variable) which makes them impractical when a large number of variables are to be studied. The Plackett-Burman design (9), as a two level fractional factorial design, is especially useful in screening studies by estimating the main effects of k variables in just k + 1 experiments according to a linear model. However, this design does not consider the interaction between variables. The variables screened by Plackett-Burman design may be optimized by using statistical and mathematical optimization tools such as Response Surface Methodology (RSM) (10). This empirical technique enables to evaluate the relationship between independent variables and to predict the response in an effective experimental design. In recent years, the use of statistical experimental design in medium optimization has gained considerable attention and a number of publications describing the application of such methods in chitinase production have appeared in the literature (11-19). The aim of this study was to screen and optimize the most important factors affecting the production of chitinase by *Bacillus pumilus* using Plackett-Burman design and RSM. To our knowledge, this approach has not been used in medium optimization for the production of chitinase by this microorganism.

Experimental

Media and Culture condition

The basal medium used for fermentation contained the following constituents (g/L): powder chitin (2.5), yeast extract (0.25), $(NH_4)_2SO_4$ (0.1), $MgSO_4.7H_2O$ (0.05), $CaCl_2.2H_2O$ (0.025), NaCl (0.5), KBr (0.05), $MnCl_2.4H_2O$ (0.0005), $ZnSO_4.7H_2O$ (0.0005), and $FeSO_4.7H_2O$ (0.01), pH 6.5. Chitin agar medium containing (g/L): Na_2HPO_4 (0.65), KH_2PO_4 (1.5), NaCl (0.5), NH_4Cl (0.25), $MgSO_4.7H_2O$ (0.12), $CaCl_2.2H_2O$ (0.005), colloidal chitin (5.0), and agar (20), pH 6.5 was used for screening the bacterial strains in plates (20).

A single colony of U_5 was inoculated into 25 mL nutrient broth medium and incubated at 30˚C until optical density at 600 nm reached 1. This pre-culture was used to inoculate 100 mL of the production medium in a ratio of 5% (v/v). Cultures were then incubated at 30˚C, 150 rpm for 8 days using 500 mL Erlenmeyer flasks. All experiments were carried out in triplicates.

Screening for chitinase producing microorganisms

Soil samples (1 g) were shaken in sterile distilled water (100 mL) containing Tween 80 (0.5%, v/v) for 30 min, and 0.1 mL of the suspension was inoculated onto chitin agar plates, followed by incubation at 30˚C for 10 days. After this period, plates were examined for formation of clearing zones (CZ). Both the size of the CZ and the colony size (CS) were determined.

Identification of the isolate

The isolate used in this study was identified as *B. pumilus* based on 16S rDNA sequence analysis. In order to obtain genomic DNA bacterial cells were harvested from an overnight culture, washed three times with apyrogen sterile distilled water, and boiled for 10 min to release DNA. The suspension was centrifuged at 14000 rpm for 5 min and the supernatant was used as DNA template for PCR amplification of the 16s rDNA gene. A large fragment of the 16s rDNA gene was amplified using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3›) and 1492R (5›-TACGGTTACCTTGTTACGACTT-3›), and the first 500 bp were sequenced with primers 27F and 517R (5›-GTATTACCGCGGCTGCTGGC-3›) (21). The PCR amplification program included an initial denaturation at 94˚C for 180s , 30 cycles of [denaturation 94˚C for 60 s, annealing 60˚C for 45 s, and extension 72˚C for 90 s], and a final extension at 72˚C for 90 s. The obtained sequence was subjected to an NCBI BLAST search.

Enzyme assay

The culture broth was centrifuged at 14000

Table 1. Values for the Plackett-Burman design.

Variable	Component	-1 Value (g/L)	+1 Value (g/L)
X_1	Powder chitin	0.500	5.00
X_2	Yeast extract	0.020	0.50
X_3	$(NH_4)_2SO_4$	0.020	0.20
X_4	$MgSO_4.7H_2O$	0.010	0.10
X_5	$CaCl_2.2H_2O$	0.005	0.05
X_6	NaCl	0.010	1.00
X_7	KBr	0.010	0.10
X_8	$MnCl_2.4H_2O$	0.0001	0.001
X_9	$ZnSO_4.7H_2O$	0.0001	0.001
X_{10}	$FeSO_4.7H_2O$	0.0020	0.02

rpm for 5 min and the supernatant was used as enzyme solution for determining enzyme activity. Chitinolytic activity was determined by estimating the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl-N-acetyl-*β*-D-glucosaminide (*p*NP-GlcNAc) according to the method of Roberts and Selitrennikoff (22) with minor modifications. The reaction mixture consisted of 0.5 mL enzyme solution, 0.5 mL 10 mM *p*NP-GlcNAc solution, and 0.5 mL 0.1 M citrate-phosphate buffer pH 5.5. The mixture was incubated at 60°C for 30 min. The reaction was stopped by adding 0.5 mL 1 M Na_2CO_3 to the mixture. The release of *p*NP was spectrophotometrically measured at 400 nm, and enzyme activity was calculated using a standard curve for known concentrations of *p*NP. One enzyme unit was defined as the amount of enzyme that can release 1 µmole *p*NP per hour under assay conditions.

Experimental design and statistical analysis for optimization

Optimization of medium components for chitinase production by *B. pumilus* isolate U_5 was performed in two stages. At the first stage, the components that have significant effect on enzyme production were identified. At the second stage, the optimum values of these components for chitinase production were determined.

Screening design

Initial screening of the most important components affecting chitinase production by *B. pumilus* was performed by Plackett-Burman design. A total number of ten components were selected for this study, with each being represented at two levels, high (+1) and low (-1) as shown in Table 1. In this design, it is assumed that the main factors have no interactions and a first-order multiple regression model is appropriate:

$$Y = \beta_\circ + \sum \beta x_i \ (i = 1, \ldots, k) \qquad \text{Equation (1)}$$

Where Y is the response function (chitinase production) and β_i is the regression coefficient. In Table 2, the design matrix built by the statistical software package Design-Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA) for the evaluation of eleven variables in twelve experiments is presented. Variables X_1 through X_{10} represent the medium components and *D* represents a dummy variable. In order to detect the curvature that may exist in the model, five replicates in the center point were also performed (trials 13 to 17). The main effect of each variable was determined according to Equation 2.

$$E(xi) = \frac{2(\sum Pi + \sum Pi\ -)}{N} \qquad \text{Equation (2)}$$

Where E(xi) is the effect of tested variable, P_{i+} and P_{i-} represent chitinase production from the trials where the tested variable was present at high and low levels, respectively, and *N* is the number of trials (8). Data were analyzed through analysis of variance (ANOVA). Statistical significance was at p-value < 0.05.

Table 2. Plackett-Burman design matrix (trails No. 1 to 12) and center point experiments (trails No. 13 to 17) with corresponding results.

Trial No.	Variable											Chitinase activity (U/100 mL)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	D	
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	94.49
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	69.86
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	76.73
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	55.50
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	15.98
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	20.66
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	85.20
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	113.36
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	96.16
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	34.75
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	60.74
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	38.11
13	0	0	0	0	0	0	0	0	0	0	0	80.97
14	0	0	0	0	0	0	0	0	0	0	0	73.20
15	0	0	0	0	0	0	0	0	0	0	0	81.89
16	0	0	0	0	0	0	0	0	0	0	0	83.33
17	0	0	0	0	0	0	0	0	0	0	0	79.74

X_1-X_{10}: medium components; D: dummy variable; (+1): high level; (–1): low level; (0): basal level.

Optimization design

After selecting the most important components influencing chitinase production by *B. pumilus*, Box-Behnken response surface methodology (23) was used to determine the optimum levels of these variables. Selected variables (powder chitin, yeast extract, $MgSO_4$ and $FeSO_4$) were studied at three different concentrations coded as -1, 0, and 1. The coded and actual values of the variables are given in Table 3. According to the Box-Behnken design matrix generated by Design-Expert software, a total number of 29 experiments including 24 factorial points and 5 replicates at the center point were performed (Table 4).

Predicted chitinase activity was calculated using the following second order polynomial equation:

$$Y = \beta_\circ + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{24} X_2 X_4 \quad \text{Equation (3)}$$

Where Y is predicted response, $\beta_\circ$ is intercept, β_1, β_2, β_3, and β_4 are linear coefficients, β_{11},β_{22}, β_{33}, and β_{44} are squared coefficients, β_{12} ,β_{13}, β_{14}, β_{23}, β_{24} and, β_{34} are interaction coefficients, and X_1, X_2, X_3, and X_4 are independent variables. By using this equation, it is possible to evaluate the linear, quadratic and interactive effects of the independent variables on the response appropriately.

Statistical analysis and graph plotting was performed using Design-Expert software. ANOVA through Fisher's test was used to evaluate the effect of independent variables on the response and significant results were identified by a p-value of < 0.05. Multiple correlation coefficient (R^2) and adjusted R^2 were used as quality indicators to evaluate the fitness of the second order polynomial equation. Contour and three-dimensional surface plots were employed to demonstrate the relationship and interaction between the coded variables and the response. The optimal points were determined by solving the equation derived from the final quadratic

Table 3. Coded and real concentration values for the Box-Behnken design.

Variable	Component	Level of variable (g/L)		
		-1	0	1
X_1	Powder chitin	0.5	2.75	5.0
X_2	Yeast extract	0.02	0.26	0.5
X_4	$MgSO_4.7H_2O$	0.0010	0.0055	0.0100
X_{10}	$FeSO_4.7H_2O$	0.00150	0.01075	0.02000

model and grid search in RSM plots.

Results and Discussion

Identification

Eighteen bacteria that could produce chitinolytic enzymes were isolated from soil samples collected from various locations in Iran using chitin agar plates. Among these, isolate U_5 was selected for further study based on its high CZ/CS ratio (data not shown). In order to identify this isolate, the 16S rDNA analysis method was employed. A comparison of the partial (500 bp) 16S rDNA sequence with the NCBI Nucleotide database with BLAST software revealed 99% sequence identity with the published sequence of *B. pumilus.* The partial sequence was submitted to the NCBI Nucleotide database (accession number EU274636).

Chitinase production through B. pumilus isolate U_5

After 8 days of incubation using basal medium, the chitinolytic activity in the culture supernatant of *B. pumilus* reached a maximum of 79.8 U/100 mL. The crude enzyme was thermally stable for more than 5 h at temperatures up to 60°C (data not shown).

Screening of important medium components

Plackett-Burman screening experimental design was employed to determine the influence of independent variables on the production of chitinase by *B. pumilus*. Among the variables under study, powder chitin (X_1), yeast extract (X_2), $MgSO_4$ (X_4), and $FeSO_4$ (X_{10}) concentrations were significant (Table 5). It is also apparent from Table 5 that this model is highly significant. In addition, the model›s lack of fit is not significant, implying that the model fits the data. However, the highly significant effect of curvature means that at least one variable is involved in an order higher than one. This indicates that a linear model would not be appropriate for determining the optimum concentrations of significant variables and a higher order model must be employed. For this reason, optimization was performed using Box-Behnken design as described later.

By using Design Expert, the equation obtained for Plackett-Burman design was as follows:

$$Y = 63.46 + 24.32(X_1) + 13.89(X_2) - 6.32(X_4) - 5.59(X_{10}) \quad \text{Equation (4)}$$

Where Y is the response (chitinase activity), and X_1, X_2, X_4, and X_{10} are chitin, yeast extract, $MgSO_4$, and $FeSO_4$ concentrations, respectively. It can be seen from Equation 4 that chitin and yeast extract exerted positive effect, while $MgSO_4$ and $FeSO_4$ had negative effect on chitinase production by *B. pumilus*. Further statistical analysis revealed that the difference between the means of center point and factorial trials in this design was not significant ($p > 0.05$). This indicated that the optimum levels for chitinase production would be near or within the experimental ranges chosen for Plackett-Burman design and there was no need to apply the steepest ascend method (23). Instead, minor modifications were made to the levels of the negative significant components, $MgSO_4$ and $FeSO_4$, using the one factor at a time approach (data not shown).

Optimization of medium components

The four significant variables selected based on the results of Plackett-Burman design (chitin, yeast extract, $MgSO_4$, and $FeSO_4$) were optimized using Box-Behnken response surface methodology, and obtained results were analyzed by ANOVA (Table 6). By using the residual

Table 4. Box-Behnken design matrix.

Trial No.	Variable				Chitinase activity (U/100 mL)
	Chitin	Yeast extract	$MgSO_4$	$FeSO_4$	
1	-1	-1	0	0	18.20
2	1	-1	0	0	57.36
3	-1	1	0	0	56.90
4	1	1	0	0	97.39
5	0	0	-1	-1	62.32
6	0	0	1	-1	41.24
7	0	0	-1	1	67.10
8	0	0	1	1	66.75
9	-1	0	0	-1	50.80
10	1	0	0	-1	77.17
11	-1	0	0	1	29.49
12	1	0	0	1	54.60
13	0	-1	-1	0	51.37
14	0	1	-1	0	87.31
15	0	-1	1	0	43.20
16	0	1	1	0	82.01
17	-1	0	-1	0	46.71
18	1	0	-1	0	69.51
19	-1	0	1	0	37.90
20	1	0	1	0	74.64
21	0	-1	0	-1	43.77
22	0	1	0	-1	91.11
23	0	-1	0	1	40.78
24	0	1	0	1	89.50
25	0	0	0	0	65.31
26	0	0	0	0	68.42
27	0	0	0	0	68.88
28	0	0	0	0	69.51
29	0	0	0	0	70.90

plot of predicted values, trials number 6 and 12 (Table 4) were detected as outliers. Therefore, they were ignored in further statistical analysis of the data. As can be seen from Table 6, only two linear (chitin and yeast extract), one quadratic (chitin), and one interaction (chitin-$FeSO_4$) terms were significant (p-value < 0.05). The mathematical model describing the relationship between variables (X_1, X_2, and X_{10}) and response (Y) could be reduced to:

$$Y = 67.06 + 18.3(X_1) + 20.79(X_2) + 6.91(X_1X_{10}) - 8.76\,(X_1^2) \quad \text{Equation (5)}$$

The positive coefficients of X_1 and X_2 indicate that chitinase activity increases with increasing levels of chitin and yeast extract. The squared correlation coefficient (R^2) and adjusted R^2 were calculated to be 0.98 and 0.95, respectively, indicating that this model can explain 98% variability in the response and only less than 2% of the variability is due to noise. Moreover, the similarity between R^2 and adjusted R^2-values shows the adequacy of the model to predict the response. The value of the coefficient of variation (CV% = 6.42) also indicates the precision and reliability of the model. Insignificant factors can

Table 5. ANOVA for Plackett-Burman design.

Source	Sum of Squares	df	Mean Square	f-Value	p-value (Prob > F)
Model	10265.35	4	2566.339	44.69918	< 0.0001[a]
Chitin	7096.252	1	7096.252	123.5989	< 0.0001
Yeast extract	2315.556	1	2315.556	40.33117	< 0.0001
MgSO4	478.7191	1	478.7191	8.338086	0.0148
FeSO4	374.8281	1	374.8281	6.528566	0.0268
Curvature	945.3387	1	945.3387	16.46543	0.0019
Residual	631.549	11	57.41355		
Lack of Fit	569.7909	7	81.3987	5.272097	0.0637
Pure Error	61.75812	4	15.43953		
Cor Total	11842.24	16			

affect the prediction ability of the model and consequently decrease its precision. By omitting these factors, the predicted R^2 increased from 0.86 to 0.93. Also, the adequate precision of the model, which is an indicator of signal to noise ratio, increased from 25.00 to 45.24.

The response surface from the interaction between chitin and $FeSO_4$ is illustrated in Figure 1. Chitinase activity increases as chitin concentration is increased; however, this relationship is not linear and, as expected, a curvature along the chitin axis is observed. The elliptical nature of the contour plot indicates that the interaction between chitin and $FeSO_4$ is significant.

Through solving Equation 5 and analyzing response surface plots, the optimum levels for chitin, yeast extract, and $FeSO_4$ were determined to be 4.76, 0.439, and 0.019 g/L, respectively. Since the effect of $MgSO_4$ on chitinase production was found to be insignificant, its middle level (0.0055 g/L) was considered as optimal. At these concentrations, predicted chitinase activity was calculated to be 97.67 U/100 mL.

Experimental validation

In order to validate the experimental model, five verification experiments were performed using the statistically optimized medium. The practical response was 96.1 ± 2.88 U/100 mL which is about 98% of the predicted value. The perfect agreement between the observed and predicted values confirms the validity and precision of the model.

In this study, the Plackett-Burman design was used to determine the most important factors influencing chitinase production by *B. pumilus*. There are many reports describing the use of this method in medium optimization with several microorganisms including *Alcaligenes xylosoxydans* (25), *Pantoea dispersa* (6), *Streptomyces* (14), *Azadirachta indica* (26), and *Bacillus circulans* (27). Based on Plackett-Burman design results it was found that among ten tested components, chitin, yeast extract, $MgSO_4$, and $FeSO_4$ exhibit statistically significant effect on enzyme production. It has been shown that chitin is a major inducer in chitinase production by many bacteria such as *A. xylosoxydans* (25), *Trichoderma hazarium* (17), *P. dispersa* (6), *Sterptomyces* (14, 28), *Moniliphthora perniciosa* (16), and *Bacillus laterosporous* (29). Microbial chitinases are inducible enzymes, the production of which is stimulated by chitin, chitooligosaccharides, chitobiose and/or GlcNAc (7, 30, 31). The molecular mechanism of chitinase induction in bacteria has been studied by cloning the chitinase gene from *Streptomyces lividans* in *Escherichia coli* (32). It has been suggested that for most microorganisms the optimum chitin concentration for chitinase induction is in the range of 10-20 g/L (33). Here, we demonstrated that the optimum chitin concentration for chitinase production by *B. pumilus* is 4.76 g/L, which is considerably lower than the range given above. Similarly, several reports have indicated the stimulatory effect of yeast extract on chitinase production by microorganisms (6, 16, 17, 25, 28, 34). According to Nampoothiri *et*

Table 6. ANOVA for Box-Behnken design.

Source	Sum of squares	df	Mean square	f-Value	p-value Prob > F
Model	9644.93	14	688.92	39.34	< 0.0001
X_1- Chitin	3306.45	1	3306.45	188.83	< 0.0001
X_2- Yeast extract	5188.60	1	5188.60	296.31	< 0.0001
X_4- $MgSO_4$	18.85	1	18.85	1.08	0.3199
X_{10}- $FeSO_4$	27.79	1	27.79	1.59	0.2317
X_1X_2	0.44	1	0.44	0.03	0.8768
X_1X_4	48.56	1	48.56	2.77	0.1217
X_1X_{10}	108.90	1	108.90	6.22	0.0282
X_2X_4	2.07	1	2.07	0.12	0.7368
X_2X_{10}	0.48	1	0.48	0.03	0.8716
X_4X_{10}	0.29	1	0.29	0.02	0.8999
X_1^2	494.08	1	494.08	28.22	0.0002
X_2^2	19.71	1	19.71	1.13	0.3097
X_4^2	12.03	1	12.03	0.69	0.4234
X_{10}^2	0.03	1	0.03	0.00	0.9667
Residual	210.13	12	17.51		
Lack of Fit	193.09	8	24.14	5.67	0.0600
Pure Error	17.04	4	4.26		
Cor Total	9855.06	26			
R^2	0.98				
Adjusted R^2	0.95				
Predicted R^2	0.86				
C.V.%	6.42				
Adeq- Precision	25.00				

al. (17), this may be due to the presence of chitin or growth factors in yeast extract. However, such low concentrations of chitin fail to induce chitinase production in most micoorganims. In addition, some other nitrogen sources including peptone and urea are reported to enhance chitinase production (18), indicating that other mechanisms may be involved. The production of chitinolytic enzymes is also affected by minerals. Chitinase production by *P. dispersa* is enhanced by the addition of KBr and $MgSO_4$ to the production medium (6). $MgSO_4$ also influences chitinase production by *Streptomyces* (14). In this study, using statistical optimization methods we demonstrated that $MgSO_4$ and $FeSO_4$ can affect the production of chitinase by *B. pumilus*. It should be noted, however, that in optimization studies one should be very careful in selecting concentration ranges for such medium components as even very slight variations may result in quite different effects.

At the second step, chitin, yeast extract, $MgSO_4$ and $FeSO_4$, which exhibited significant effect on chitinase production, were selected for optimization of the production medium using Box-Behnken response surface methodology. This method has been used in similar studies including optimization of culture conditions for chitinase production by *Streptomyces* (14) and *A. xylosoxydans* (25). Using statistical experimental design, we were able to enhance chitinase production by *B. pumilus* to 96.1 U/100 mL. The good agreement between the predicted response (97.67 U/100 mL) and the experimental data obtained in the laboratory indicates the adequacy and applicability of experimental design methods for medium optimization.

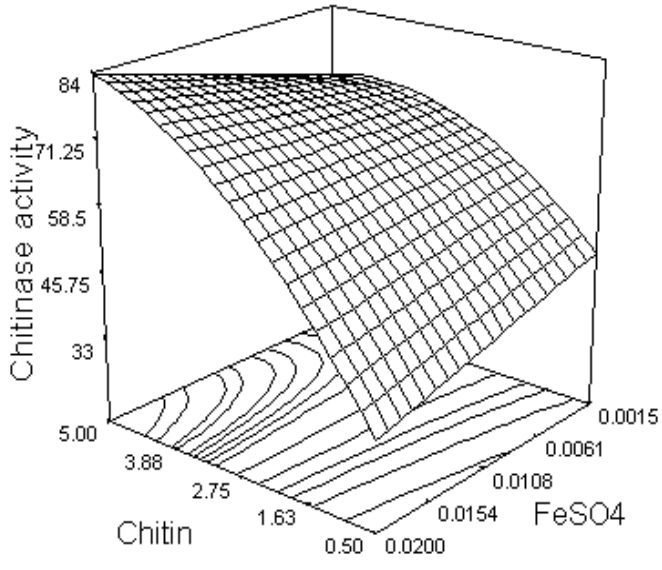

Figure 1. Response surface curve showing the effect of chitin and $FeSO_4$ on chitinase production by *B. pumilus*.

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