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Full Length Research Paper

Optimization of medium for the production of subtilisin from *Bacillus subtilis* MTCC 441

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Subtilisins (E.C.3.4.21.62) are alkaline proteases that are secreted by members of the genus *Bacillus*. They are serine proteases that exhibit high specific activity on proteinaceous substrates, function optimally at moderate temperatures, and are stable under alkaline conditions. Thus the use of subtilisin as an enzyme additive could help in development of quality laundry detergents. In this work the subtilisin production from *Bacillus subtilis* (MTCC 441) was improved by altering and optimizing the media components. This alteration was brought up by process development strategy. The effect of yeast extract, casein, peptone and sodium chloride on subtilisin production was studied and were optimized using Box Behnken Design. The optimal growth conditions for *B. subtilis* MTCC 441 were found to 37°C, and pH 7.5. The optimal media composition for subtilisin production was found to be yeast extract at 6.75 g/L, peptone at 4.41 g/L, sodium chloride at 6.08 g/L, casein at 10.75 g/L with glucose at 5 g/L. The predicted and observed response were 181.00 U/mg (with desirability =0.87) and 185.70 U/mg, respectively.

Key words: *Bacillus subtilis*, subtilisin, Box-Behnken design, media optimization.

INTRODUCTION

The major extracellular proteolytic enzymes secreted by microbes are neutral (metallo-) and alkaline serine proteases, such as subtilisin produced by *Bacillus subtilis*. Subtilisin is one of the most extensively studied of all bacterial proteins, due to its commercial importance (Ward, 1985). This protease is produced at the onset of sporulation and a complex network of genes and regulators, which regulates the transition state of protein (Kalisz, 1988), controls the expression. They are generally secreted extracellularly for the purpose of scavenging nutrients (Graycar, 1999). Proteases account for approximately 40% of the total enzyme sales in various industrial market sectors, such as detergent, food and pharmaceutical industries (Beg et al., 2003; Shang and Yang, 1999). Their importance is illustrated by the fact that the amount of subtilisin produced and used in the European Union in 2002 was 900 tons of pure enzyme Subtilisins comprise a group of serine endopeptidases (Mol.wt. = 27,500 Da) that are secreted in large amounts from a wide variety of *Bacillus* species. The industrial importance of subtilisins is very high, especially as components of household detergents. In 1988 the world market for industrial enzymes was approximately US $600 million, half of which corres-ponded to enzymes added to detergents (mainly subtilisins) (Ellaiah et al., 2002).

A wide range of microorganisms including bacteria, moulds, yeasts and also mammalian tissues produces alkaline proteases. Among bacteria, *Bacillus* sp. are specific producers of extra cellular proteases. These are used as cleansing additives in detergents to facilitate the release of proteinaceous materials in stains due to grime, blood, milk, etc. *Bacillus* sp. grows in a pH range of 7.0 - 11.0 and produces extra cellular alkaline proteases (Adinarayana and Ellaiah, 2003).

Alkaline proteases are generally produced by submerged fermentation. In addition, solid-state fermentation processes have been exploited to a lesser extent for production of these enzymes (Ward, 1985). In commer-

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cial practice, the optimisation of medium composition is done to maintain a balance between the various medium components, thus minimizing the amount of unutilised components at the end of fermentation. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimisation of environmental and fermentation parameters such as pH, temperature, aeration, and agitation.

Response surface methodology (RSM) is a useful tool for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. The use of experimental factorial design and response surface methodology, already successfully applied in other fields, is well suited to the study of the main effects and interaction effects of the factors on the production of alkaline protease. The conventional method of optimisation involves varying one parameter at a time and keeping the others constant. This often does not bring about the effect of interaction of various parameters as compared to factorial design (Griffin et al., 1992). In the present work, we have attempted to optimize the production of subtilisin from *B. subtilis* MTCC 441 using Box-Behnken design.

MATERIALS AND METHODS

Materials

Culture was obtained from IMTECH, Chandigarh, India. All other chemical were obtained from HiMedia, India.

Composition of nutrient medium

Media were prepared in distilled water and comprise of beef extract (1 g/l), yeast extract (2 g/l), peptone (5 g/l), sodium chloride (5 g/l). Initial pH of the medium was pH 7.2.

Culture conditions

Culture was incubated for 12 h at 37°C, pH 7.2 on a rotary shaker in a 250-mL Erlenmeyer flask containing 50 mL of medium. The crude enzyme was separated after 12 h by centrifugation at 10, 000 x g for 10 min and 4°C.

Subtilisin assay

The assay described by modified Anson method given by Yang and Huang (1994), was used for testing the activity of protease using casein as substrate. 0.5 mL of potassium phosphate buffer and 1 mL of casein solution (1% casein solution prepared in 10 mM potassium phosphate buffer pH 7.5) was taken in test tubes and incubated at 37°C in a water bath for 5 min. 0.5 mL of enzyme solution was added and incubated at 37°C for 30 min. The proteolytic reaction was stopped by addition of 3 mL 10% trichloroacetic acid and kept for 10 min at room temperature. Then the precipitate was centrifuged at 10000 g. The absorbance of the filtrate was determined at A280 nm using UV visible spectrophotometer (Shimadzu). One unit of enzyme activity was defined as the amount of enzyme releasing one µM tyrosine/ml in one minute µM under assay condition.

Protein estimation

Protein was estimated using Bradford's dye binding method (Bradford, 1976) with BSA as a standard protein.

Response surface methodology

Response surface methodology (RSM) is an empirical modelling technique used to evaluate the relationship between a set of controllable experiments factors and observed results. RSM was used to determine the optimum response of the cells for the synthesis of subtilisin under a wide range of nutrient conditions. The design of experiment chosen for study was the Box Behnken design for four independent variables to obtain the combination of values that optimizes the response within the region of three-dimensional observation space, which allows one to design a minimal number of experimental runs. The model evaluates the effect of each independent variable to a response. The mathematical relationship of the independent variables and the response can be calculated by the quadratic polynomial equation (Moreira et al., 2003).

$$y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X^2_1 + \beta_{22}X^2_2 + \beta_{33}X^2_3 + \beta_{44}X^2_4 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{34}X_3X_4$$

The computation was carried out by multiple regression analysis making use of the least squares method. The significant variables of the medium components selected were yeast extract, peptone, sodium chloride and casein, based on Plackett Burman studies (data not shown). The response surface method used here is Box-Behnken Design. As per the design, 27 experimental runs with different concentrations of each component were made as shown in Table 1.

RESULTS AND DISCUSSION

Growth studies

Initial growth studies on *B. subtilis* MTCC 441 were carried out using nutrient medium. The culture was grown at different temperature and pH to analyze the effect of temperature and pH on the growth and subtilisin production. The growth of organism has been measured by estimating optical density at 600 nm. Our studies indicate optimal pH and temperature at 7.5 and 37°C respectively for growth of *B. subtilis* MTCC 441 and production of subtilisin (data not shown). Hence For all future studies *B. subtilis* MTCC 441 was cultivated at pH 7.5 and 37°C

Response surface methodology (RSM)

Initially the experiment was designed using the nutrient medium. However, we observed that this strain exhibited a level of subtilisin in nutrient media the specific activity

Table 1. Design of experiment and response of the Box Behnken design for the production of subtisin using *B. subtilis* MTCC 441.

S/N	Block	Yeast extract (g/L)	Peptone (g/L)	Sodium chloride (g/L)	Casein (g/L)	Specific activity (U/mg protein)
1	Block 1	3.00	2.50	5.00	10.00	93.24
2	Block 1	6.00	5.00	2.50	5.00	110.68
3	Block 1	6.00	5.00	7.50	5.00	114.84
4	Block 1	6.00	5.00	7.50	15.00	143.12
5	Block 1	9.00	2.50	5.00	10.00	135.84
6	Block 1	6.00	5.00	2.50	15.00	70.28
7	Block 1	6.00	5.00	5.00	10.00	178.00
8	Block 1	9.00	7.50	5.00	10.00	101.40
9	Block 1	3.00	7.50	5.00	10.00	132.60
10	Block 2	6.00	5.00	5.00	10.00	183.40
11	Block 2	6.00	2.50	7.50	10.00	156.84
12	Block 2	3.00	5.00	5.00	5.00	101.36
13	Block 2	6.00	7.50	2.50	10.00	148.20
14	Block 2	3.00	5.00	5.00	15.00	96.92
15	Block 2	6.00	7.50	7.50	10.00	141.80
16	Block 2	9.00	5.00	5.00	15.00	128.88
17	Block 2	9.00	5.00	5.00	5.00	123.32
18	Block 2	6.00	2.50	2.50	10.00	102.68
19	Block 3	9.00	5.00	2.50	10.00	52.36
20	Block 3	6.00	7.50	5.00	5.00	111.16
21	Block 3	6.00	2.50	5.00	5.00	79.60
22	Block 3	6.00	5.00	5.00	10.00	168.60
23	Block 3	6.00	2.50	5.00	15.00	117.36
24	Block 3	6.00	7.50	5.00	15.00	90.20
25	Block 3	3.00	5.00	2.50	10.00	75.40
26	Block 3	9.00	5.00	7.50	10.00	131.00
27	Block 3	3.00	5.00	7.50	10.00	75.48

was less. Hence, various components were substituted in the media to study the level of subtilisin production using *B. subtilis*. The effective variable that play a direct role in the production of subtilisin activity were chosen using Placket Burman method. The variables which show positive influence on the production of subtilisin were chosen for the further experiment using Box-Behnken design. Yeast extract, peptone, sodium chloride and casein were found to be good source for the enzyme production (data not shown). To study the individual and interactive effects of these variables and to optimize them, RSM was used.

The results of RSM are given in Table 1. The model was evaluated using multiple regression analysis and regression coefficients indicated the effect of various factors on the yield of subtilisin. The computation was carried out by multiple regression analysis making use of the leastsquares method at 95% significance level. Each of these regression coefficients represents the coefficients of the variables in the polynomial equation, which is then used to predict the specific activity of the subtilisin. The ANOVA analysis was done to investigate the effect of the various factors on the variation about the mean. Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and the results are shown in Table 2.

The analysis of variance of the quadratic regression model demonstrates that the model is highly significant, as the computed F value is much greater than the tabular F value. The Student t distribution and the corresponding P values, along with the parameter estimate, are given in Table 2. The smaller the magnitude of P, the more significant is the corresponding coefficient. The parameter estimate and the corresponding P values suggest that all the independent and interactive terms are highly significant.The closer the value of R (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. The value of R^2 = 0.9697 in Table 3 indicates good correlation between the experimental and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower is the reliability of experiment. In this

Table 2. ANOVA analysis for response surface quadratic model.

S/N	Source	Sum of Squares	df	Mean Squares	F Value	p-ValueProb > F	Significance
1	Block	4530.92	2	2565.46			
2	Model	24014.81	14	1715.34	22.84	<0.0001	significant
3	YE	797.07	1	797.07	10.61	0.0086	significant
4	Peptone	132.00	1	132.00	1.76	0.2144	
5	Sod. Chl	3450.34	1	3450.34	45.93	<0.0001	significant
6	Casein	2.80	1	2080	0.037	0.8507	
7	YE*Pep	1361.61	1	1361.61	18.13	0.0017	significant
8	YE*Sod.chl	1542.92	1	1542.92	20.54	0.0011	significant
9	YE*Casein	25	1	25	0.33	0.5768	
10	Pep*Sod.chl	916.88	1	916.88	12.21	0.0058	significant
11	Pep*Casein	862.01	1	862.01	11.48	0.0069	significant
12	Sod.chl*Casein	1179.24	1	1179.24	15.70	0.0027	significant
13	YE*YE	9467.08	1	9467.08	126.03	<0.0001	significant
14	Pep*Pep	2521.07	1	2521.07	33.56	0.0002	significant
15	Sod.Chl*Sod.Chl	5727.90	1	5727.90	76.25	<0.0001	Significant
16	Casein*Casein	7357.35	1	7357.35	97.95	<0.0001	Significant
17	Residual	751.14	10	75.12			
18	Correct Total	29296.90	26				

Table 3. Standard deviation and correlation coefficients.

Std. Dev.	8.67	**R-Squared**	0.9697
Mean	117.21	**Adj R-Squared**	0.9272
C.V. %	7.39	**Pred R-Squared**	0.7737
PRESS	5604.07	**Adeq Precision**	19.588

Table 4. Optimal media content concentration for subtilisin production using *B.subtilis* MTCC 441.

S/N	Media Composition	Concentration in g/L	Predicted activity of subtilisin (U/mg Protein)	Actual activity of subtilisin (U/mg Protein)
1	Yeast Extract	6.75	181.00	185.70
2	Peptone	4.41		
3	Sodium Chloride	6.08		
4	Casein	10.75		
5	Glucose	5.00		

experiment, a lower value (7.39) indicates high reliability. The "Pred R-Squared" of 0.7737 is in reasonable agreement with the"Adj R-Squared" of 0.9272. Adeq Precision ratio greater than 4 is desirable and in this model the ratio value is 19.588 that indicates an adequate signal. The CV of 7.39 is also in good agreement with the model.

The polynomial equation was derivative and was solved using inverse matrix method to obtain optimum concentration of media content and predicted response in terms of enzyme activity. The optimal concentrations of media content for subtilisin production using *B. subtilis* MTCC 441 has been given in Table 4. The predicted response using the regression coefficients in coded units (shown in Table 5) was found to be 181.00 U/mg (with desirability =0.87) whereas the observed response using optimized media was found to be 185.70 U/mg.

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Table 5. Estimate regression coefficients for specific activity using data in coded units.

Factor	Estimate coefficient
Intercept	176.67
Block 1	2.79
Block 2	14.28
Block 3	-17.08
A-YE	8.15
B-Pep	3.32
C-sod. chl	16.96
D-Casein	0.48
AB	-18.45
AC	19.64
AD	2.50
BC	-15.14
BD	-14.68
CD	17.17
A^2	42.13
B^2	-21.74
C^2	-32.77
D^2	-37.14

REFERENCES

Adinarayana K, Ellaiah P (2003). Production of alkaline protease by immobilized cells of alkalophilic *Bacillus sp*. J. Sci. Indian Res. (India). 62: 589-592.

Beg KB, Sahai V, Gupta R (2003). Statistical media optimisation and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem. 39: 203-209.

Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Ellaiah P, Adinarayana K, Pardasaradhi SV, Srinivasulu B (2002). Isolation of alkaline protease producing bacteria from Visakhapatnam soil. Indian J. Microbial. 42: 173-175.

Engineering Statistics Hand Book: Chapter-5; topics from 5.3.

Graycar TP (1999). Proteolytic cleavage, reaction mechanism. In: Flickinger MC, Drew SW (eds) Bioprocess technology: fermentation, biocatalysis and bioseparation. (Wiley, New York) pp. 2214-2222.

Griffin HL, Greene RV, Cotta MA (1992). Isolation and characterization of an alkaline protease from the marine shipworm bacterium. Curr Microbial. 24: 111-117.

Kalisz HM (1988). Microbial Proteinases. Adv Biochem Eng Biotechnol. 36:1-65.

Moreira KA, Porto TS, Teixeira MFS, Porto ALF, Lima Filho JL (2003). New alkaline protease from *Nocardiopsis sp*: partial purification and characterization. Process Biochem. 39(1): 67-72(6).

Shang SH, Yang JY (1999). Protease and Amylase production of *Streptomyces rimosus* in submerged and solid state cultivation. Bot. Bull. Acad. 40: 259-265.

Ward OP (1985). Proteolytic enzymes. In: Blanch HW, Drew S, Wang DI, eds. Comprehensive Biotechnol. 3: 789-818.

Yang SS, Huang CI (1994). Protease Production by Amylolytic fungi in Solid State Fermentation. J. Chin. Agric. Chem. Soc. 32: 589-601.