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Full Length Research Paper

Optimization of production of subtilisin in solid substrate fermentation using response surface methodology

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Subtilisin (EC 3.4.21.62) is a type of serine protease that is of high commercial importance. It is mainly produced by *Bacillus* species as an extra cellular enzyme. Subtilisin being stable over a wide range of pH and temperature is exploited as a detergent enzyme and also favoured by its non-specificity. The yield of this enzyme needs to be optimized for cost effective production of subtilisin for commercialization. Here, various solid substrates were screened for the production of subtilisin using *Bacillus subtilis* MTCC 441 and the maximum yield was found with green gram husk. Response surface methodology (RSM) was used to optimize pH, temperature, and moisture content. The optimal conditions were obtained after solving the polynomial equation using inverse matrix. The optimum pH, temperature and moisture content obtained under the conditions of study were 7.00, 30.04°C, and 73.38% respectively. The predicted response under these conditions was to be 571.73 U/mg protein whereas the actual response was found to be 571.32 U/mg protein.

Key words: *Bacillus subtilis*, subtilisin, solid substrate fermentation, response surface methodology, Box Benkhn Design**.**

INTRODUCTION

Subtilisin (EC 3.4.21.62) is a type of serine protease that is of high commercial importance. Proteases (serine protease (EC. 3.4.21), cysteine (thiol) protease (EC.3.4.22), aspartic proteases (EC. 3.4.23) and metallo-protease (EC. 3.4.24)) constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales (Beg and Gupta, 2003; Ellaiah et al., 2003; Shang and Yang, 1999). Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases and among bacteria, *Bacillus* species are specific producers of extra cellular proteases. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries (Pastor et al., 2001). Over the past 20 years, the development of subtilisins as typical detergent proteases has employed all the tools of enzyme technology, resulting in a constant flow of new and improved enzymes. Apart from their importance in physiology e.g. in the activation of zymogenic preforms of enzymes, blood clotting, the lysis of blood clots, the processing and transport of secretory proteins across membranes and as pathogenic factors, proteases are highly relevant in technical enzyme applications too (Egmont, 1997). Enzymes, and particularly proteases, have become an important and indispensable part of industrial processes such as laundry detergents, pharmaceuticals and food products. Of these, their use in detergents is the most prominent with respect to market volume and tonnage (Mart, 2004). From the beginning these enzymes were produced using Bacillus species like *Bacillus amyloliquefaciens* and *Bacillus licheniformis* (Christiansen and Nielsen 2002; Karl-Heinz, 2004). The alkaline proteases from these species represent the lead molecules for the subtilisins. Members of the subtilisin super family of proteases have now been identified, with different functions, practically in all-living organisms (Karl-Heinz, 2004). Nevertheless, the Subtilisins from *Bacillus*

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species still provide all the proteases used in the detergent industry. In the mid-1980s the subtilisins were shown to be an excellent model for testing genetic engineering approaches, and by the end of the decade they were amongst the first technical enzymes to be manufactured using recombinant strains. Subtilisins are defined by their catalytic mechanism as serine proteases. Although the size of subtilisins varies from 18 to 90 kDa, all the subtilisins used in detergents have a size of approximately 27 kDa (John and Branden, 1991). The success of subtilisins is based on several factors, including their high stability and relatively low substrate specificity features common in extra cellular proteases. Their production as extra cellular enzymes is of course an important factor in itself, as this greatly simplifies the separation of the enzyme from the biomass and facilitates other down-stream processing steps. Another important point is the ability of *Bacillus* strains to secrete enzymes over a very short period of time into the fermentation broth (Ellaiah et al., 2002; Jeong et al., 2002; Karl-Heinz, 2004; Mart, 2004). Subtilisins are used in all types of laundry detergents and in automatic dishwashing detergents. Their function is to degrade proteinaceous stains (Karl-Heinz, 2004) typical stains include blood, milk, egg, grass and sauces. An aspect that has to be considered when screening candidate enzymes for better performance is that they are not acting on soluble substrates in solution, but on substrates bound to the solid surface, water-insoluble substrate. In contrast to more biochemical environments, where the denaturation of protein substrates normally leads to improved enzyme activity, the denaturation of proteinaceous stains by aging, heating and oxidization makes them less accessible to enzymatic degradation (Mart, 2004). At present, less than 15 different enzyme molecules are used in detergents worldwide (Karl-Heinz, 2004). These enzymes originate from *B. amyloliquefaciens*, *B. licheniformis*, *Bacillus clausii*, *Bacillus lentus*, *Bacillus alkaloophilus*, and *Bacillus halodurans* (Egmont, 1997). The production of this enzyme even involves some fungi for example *Aspergillus oryzae* (Ellaiah et al., 2002), *Teredinobacter turnirae*, etc.

At present, the use of alkaline proteases has increased remarkably with large proportions of commercially available alkaline proteases derived from *Bacillus* strains (Shang and Yang, 1999; Siezen and Leunissen, 1997). Some of these species assignments have in recent years been changed; for example, Savinase1 and Esperase1 were assigned for several years to *Bacillus subtilis* or *B. lentus*. All major subtilisins for detergents are produced in *Bacillus*, because these species are able to secrete large amounts of extracellular enzymes (Mart, 2004). Detergents such as Tide®, Ariel® and Biz® contain proteolytic enzymes, most of them produced by members of the genus *Bacillus* (Sen and Satyanarayana, 1993). The control mechanisms involved in the production of proteases in *Bacillus* sp. is extremely complex and still not fully understood. An example is the two-component regulatory system that acts as a quorum sensing mechanism in *B. subtilis* that has been found to control the expression of the alkaline protease. This regulatory system is encoded on the chromosome and on endogenous plasmids (Mart, 2004). Industrial strain improvement programs using classical microbiological methods have been carried out over many years and have resulted in the development of several highly productive strains (Ganesh et al., 1998; Karl-Heinz, 2004; Mart, 2004).

In spite of high utility of subtilisin it is not being in use extensively due to high production cost. The starting material like cultivation medium contributes majority of the cost involved in the production of biomolecule in biotechnology industries.

The present study is aimed at optimization of fermentation condition in solid substrate fermentation. We have screened various solid substrates mostly the bran and husks for the production of subtilisin. Response surface methodology was applied for the optimization pH, temperature and moisture content for the production of subtilisin using *Bacillus subtilis* MTCC 441.

MATERIALS AND METHOD

Microorganism cultivation of *B. subtilis*: Inoculum development

B. subtilis MTCC-441 was obtained from MTCC Chandigarh, India and the culture was maintained on nutrient medium containing 1.5% agar.

Compsition of nutrient medium

Yeast extract 2 g/L, Beef extract 1 g/L, Peptone 5 g/L and Sodium Chloride 5 g/L pH 7.0 ± 0.1.

Cultivation condition

B. subtilis MTCC-441 was cultivated using the nutrient medium (composition mentioned above) at 37°C under constant agitation at 200 rpm on an orbital shaker.

Development of seed inoculum

Single colony of *B. subtilis* MTCC-441 grown on nutrient agar was inoculated in 10 mL nutrient medium in 100 mL Erlenmeyer flask and incubated for overnight (16 h) under conditions mentioned above. 1 mL from the overnight grown culture was then transferred to 500 mL Erlenmeyer flask containing 100 mL nutrient medium and incubated for 4 to 5 h. The seed inoculum was transferred to production flask when the optical density of the culture reached to 0.8 – 1.0 OD_{600nm}.

Selection of a suitable substrate

Various industries by-products like wheat bran, rice bran, soya meal, pea flour, corn flour and green gram husk were screened for

Table 1. Comparison of specific activity of subtilisin produced from various substrates and different time.

Days	Specific activity (U/mg)					
	Wheat bran	Rice bran	Corn flour	Pea flour	Soya meal	Green gram husk
1	26.01	30.19	17.58	10.34	26.15	66.16
2	51.66	100.31	70.77	50.28	59.17	354.64
3	104.05	114.33	127.24	64.55	122.16	423.55
4	192.08	121.39	132.36	42.65	121.13	459.47
5	109.67	53.35	90.18	10.81	74.28	115.83
6	106.86	30.88	46.09	10.09	19.56	91.25

subtilisin production. 5 g of sterilized bran were used as substrate. The substrate yielding the maximum subtilisin activity was identified and selected for further studies using RSM.

Estimation of moisture content

Moisture content of the substrate was estimated by drying 5 g of substrate to constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrate was soaked with the appropriate quantity of distilled water. The sample was then dried as described above and moisture content (%) was calculated as follows (Adinarayana et al., 2005):

Moisture content (initial) of solid medium (%) = [(wt .of substrate – dry wt.) / dry wt] x 100

Response surface methodology

The Response surface method used here is Box-Behnken Design. 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:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

The optimization of the temperature, pH, and moisture was done using the response surface methodology where Box Behnken design was used. The high and low values of the variables chosen were pH = 7 and 8, temperature = 30 and 45^{O}C, and moisture = 60 and 90%. Experiments were run according to design in a single block with 3 central points. The 3 central points represent the same value of the parameter. The specific activity of the subtilisin was used as the response to analyze the design. The computation was carried out by multiple regression analysis making use of the least squares method. From the optimal values the specific activity of subtilisin was predicted using the above mentioned polynomial model equation.

Extraction of enzyme

25 mL of 10 mM potassium phosphate buffer pH 7.5 was added and the suspension was filtered. The filtrate was centrifuged at 10000 g, 4°C for 15 min. The supernatant was collected and used as a source of subtilisin enzyme.

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 $A_{280\ nm}$ 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 under assay condition.

Estimation of protein

Protein concentration was measured using the method of Bradford (1976) with bovine serum albumin (BSA) as the standard protein.

RESULTS AND DISCUSSION

Screening of various substrates and optimization of incubation time

The yield of subtilisin from *Bacillus subtilis* MTCC 441 in solid substrate was studied with different solid substrates supplemented with Nutrient medium. The studies on the production of subtilisin using various solid substrates were carried for six days. In the case of solid substrate wheat bran, rice bran, corn flour and green gram husk, the maximum production of subtilisin was observed on the fourth day (Table 1), whereas in case of pea flour and soya meal the maximum activity of subtilisin was noted on third day. Loss of enzyme activity was recorded from fifth day onward in case of solid substrate using wheat bran, rice bran, corn flour and green gram husk (Table 1). In case of cultivation on pea flour and soya flour the degradation of subtilisin activity was observed from fourth day onward (Table 1). This indicates autodegradation of subtilisin after the 4th day. Among all the solid substrate used for our study on the production of subtilisin, the

Table 2. Design of experiment and response of the box Behnken design for the production of subtisin in SSF using *B. subtilis* MTCC 441 tabulated as specific activity.

Run order	pH	Temp.	Moisture (%)	Specific activity (U/mg)
1	7.5	30	60	377.25
2	7	37.5	60	386.01
3	7.5	37.5	75	461.27
4	7.5	45	60	497.44
5	8	37.5	90	493.98
6	7	37.5	90	440.03
7	7	30	75	565.81
8	7.5	37.5	75	474.27
9	7	45	75	408.32
10	8	37.5	60	350.40
11	8	30	75	542.38
12	7.5	37.5	75	461.72
13	7.5	30	90	356.01
14	8	45	75	229.90
15	7.5	45	90	318.94

maximum subtilisin production was found using green gram husk on fourth day (Table 1). Since the difference in the activity of subtilisin in three-day-old culture (423.55 U/mg) and four day old culture (459.47 U/mg) is not very significant hence for all further studies *B. subtilis* MTCC 441 on solid substrate fermentation was incubated for three days.

RSM and regression analysis

The results of RSM are given in Table 2. 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 least-squares 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.

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 3. 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 3. 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 (Table 3 and 4). The closer the value of *R* (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values (Table 5). The value of R^2 (= 0.996) 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 (Table 5). Usually, the higher the value of CV, the lower is the reliability of experiment. In this experiment, a lower value (2.11) indicates high reliability (Table 5).

The optimal conditions were obtained using inverse matrix. The value of X1 (pH), X2 (Temperature), and X3 (Moisture content) were found to be 7.00, 30.04°C, and 73.38% respectively. The predicted response under these conditions was obtained by solving the polynomial equation and was found to be 571.73 U/mg protein. The actual response using the predicted optimal conditions was found to be 571.32, U/mg protein.

Conclusion

In the present experiment, we have found green gram husk which is feed material for animals as a good substrate for the production of subtilisin. RSM analysis indicated optimum pH, temperature and moisture as 7.0, 30.04°C and 73.3%, respectively, as an optimum condition for subtilisin production using *B. subtilis* MTCC 441.

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Table 3. ANOVA for response surface quadratic model.

	Analysis of variance table [Partial sum of squares - Type III]					
Source	**Sum of squares**	**df**	**Mean square**	**F-value**	**p-value Prob > F**	**Significance**
Model	141733.91	9	15748.21	194.89	< 0.0001	Significant
A- pH	815.31	1	815.31	10.09	0.0156	Significant
B-Temperature	27950.28	1	27950.28	345.90	< 0.0001	Significant
C- Moisture	12715.34	1	12715.34	157.36	< 0.0001	Significant
AB	2241.03	1	2241.03	27.73	0.0012	Significant
AC	1405.09	1	1405.09	17.39	0.0042	Significant
BC	7959.23	1	7959.23	98.50	< 0.0001	Significant
A^2	24556.20	1	24556.20	303.89	< 0.0001	Significant
B^2	7215.57	1	7215.57	89.29	< 0.0001	Significant
C^2	59786.04	1	59786.04	739.88	< 0.0001	Significant
Residual	565.63	7	80.80			
Lack of fit	434.86	3	144.95	4.434	0.0921	Not significant
Pure error	130.77	4	32.69			
Total	142299.54	16				

Table 4. Estimate regression coefficients for specific activity using data in coded units.

Factor	Coefficient estimate
Intercept	464.04992
A-pH	10.09525
B-Temperature	-59.10825
C-Moisture	39.8675
AB	23.66975
AC	18.74225
BC	44.60725
A^2	76.368165
B^2	-41.396835
C^2	-119.160335

Table 5. Standard deviation and correlation coefficients.

Standard deviation	**8.989126058**	***R*^{2}**	**0.996025**
Mean	424.4315647	Adjusted R^2	0.990914
C.V. (%)	2.117921193	Predicted R^2	0.949669
PRESS	7162.121468	Adeq Precision	59.72928

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