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OPTIMIZATION OF MEDIA CONSTITUENTS FOR THE PRODUCTION OF SUBTILISIN FROM *BACILLUS SUBTILIS* USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Optimization is used to maximize product yield in the pharmaceutical industry, increase high product quality in food production and amplify the degradation process in environmental biotechnology. Statistical analysis proved to be a useful and powerful tool in developing optimum fermentation conditions. Subtilisin is an extracellular alkaline serine protease secreted by members of the genus *Bacillus*. It catalyzes the hydrolysis of proteins, peptide amides and has a high substrate specificity to fibrin. It is mainly used in the development of quality detergents and also used household cleaning products to remove proteinaceous deposits and stains. A new strain –*Bacillus* spp that has ability for the production of subtilisin was screened. The production of subtilisin was dependent on the constituents influence on the medium. In this study, Response Surface methodology was designed to study the effect of compound and identified that at yeast extract, peptone and casein at a combination of 6.0 g/l, 5.0 g/l and 10.0 g/l respectively.TheR^2=0.991125, adjusted R^2=0.983138 and Predicted R^2=0.987229 shown in experimental results were in good understanding with the predicted value.

KEYWORDS : ***Subtilisin, Bacillus subtilis, RSM, Media optimization.***

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INTRODUCTION

Proteases are enzymes that catalyze the hydrolysis of peptide bonds of proteins and break them down into polypeptides or free amino acids[1]. They are classified into acid, neutral and alkaline based on their acid–base behaviour[2]. Proteases having optimum activity at pH 8 and classified as alkaline proteases[3]. Subtilisins (E.C.3.4.21.62) are extracellular alkaline serine monomeric proteases with molecular mass close to 27.5 kDa, secreted extracellularly by *Bacillus*strains[4]. Subtilisin has a high substrate specificity to fibrin and it is used as a stabilizer in improvement of superior laundry detergents. Subtilisin are generally produced by submerged fermentation. The component of media plays a major role in the productivity of subtilisin[5,6]. In the commercial practice, the optimization of medium composition is done in sustaining an equilibrium between the different medium components at the end of the reaction. The media optimization was performed using statistical methods such as RSM which result in a high production of subtilisin[7]. Box Behnken (BB) design and Central composite design (CCD) are some of the models used in RSM for optimization[8,9,10].

MATERIALS AND METHODS

Isolation and Identification of Bacteria

The soil sample was collected from the Sathyabama University campus and aseptically taken in plastic containers. Serial dilution was performed and isolates were identified using Gram staining and biochemical methods and molecular identification performed using 16s rRNA sequencing[11].

Identification of Subtilin production

Composition of medium

The organism was inoculated into medium containing casein (1%) in basal salts medium 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% NaCl for the determination of proteolytic activity. The pH of the culture media was adjusted to 8 and the cultures were incubated at 37°C and 100 rpm for a period of 2 days[12].

Subtilisin Assay

The subtilisin activity was determined by modified Anson method[13] 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 rpm for 15 minutes. The absorbance of the filtrate was determined at A280 nm using UV visible spectrophotometer (Varian 300). One unit of enzyme activity was defined as the amount of enzyme releasing one µM tyrosine/ml in one minute µM under assay condition. An estimation of protein present in the sample was done using Lowry's method[14,15,16,17].

Optimization of medium components

RSM is a collection of mathematical and statistical techniques for empirical model building[18,19]. The objective of RSM is to optimize a response that is influenced by several independent variables, by conducting each run of the experiment that are carefully designed process conditions[20]. The RSM was used to determine the optimal response of the cells for the synthesis of subtilisin under a wide range of nutrient conditions. CCD was chosen for the study of three independent variables. Design Expert software (Stat Ease, 7, trial version) was used for experimental design towards the construction of quadratic model. The second order polynomial equation was used for regression analysis to study the interaction of variables. The model evaluates the effect of each independent variable to a response, by means of polynomial equation.

Table 1
Coded Levels for Independent Factors Used in Experimental Design

Factor	Coded levels		
	-1	0	+1
A: Yeast extract	3	6	9
B: Peptone	2.5	5	7.5
C: Casein	5	10	15

Subtilisin production $=b_0 + b_1A + b_2B + b_3C + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{12}AB + b_{13}AC + b_{23}BC$ Where Response is the predicted value; A, B and C are independent variables; b_1,b_2 and b_3 are the coefficients of linear effects b_{11}, b_{22}, b_{33} are squared effect and b_{12}, b_{13}, b_{23} coefficients of interaction terms. The coefficients of the polynomial are estimated by ordinary least squares method applied to a number of observations of regression analysis. The significant variables of the medium components selected were yeast extract, peptone and casein, based on previous studies. The response surface method used here is Central composite Design. As per the design, 20 experimental runs with different concentrations of each component were made as shown in Table – 3.

RESULTS AND DISCUSSION

Isolation and Identification of Bacteria

Appropriate dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were selected and replicates were maintained throughout the study. After 24hours of incubation the bacterial cultures were enumerated, isolated and inoculated in separate Petri plates and tubes and stored in the refrigerator for further analysis. The isolated organism was identified by Colony morphology, Gram staining, motility and biochemical test.

Colony morphology

Large, irregular, mucoid, flat tanned colonies were seen in Nutrient Agar.

Microscopic observation

Gram positive bacilli in chains were observed active motile organism were identified by Hanging drop method.

Biochemical characterization

In the biochemical tests (Table – 2) Indole, Urease, Nitrate showed negative results and Methyl red, Voges Proskaur, Citrate and Catalase showed positive results.

Table 2
Biochemical test

S.No	Name of the Biochemical test	Result
1	Indole	Negative
2	Methyl Red	Positive
3	Voges Proskaur	Positive
4	Citrate	Positive
5	Urease	Negative
6	Nitrate	Negative
7	Catalase	Positive
8	Gelatin liquefaction test	Positive

Molecular characterization (16s rRNA sequence)

CCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTA
A
CACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTT
G
AACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTA

GTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTG
GG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGT
TAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAA
C
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTC
G
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAA
C
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC
A
GTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA
G
GAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGG
C
AGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGT
C
TGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC
C
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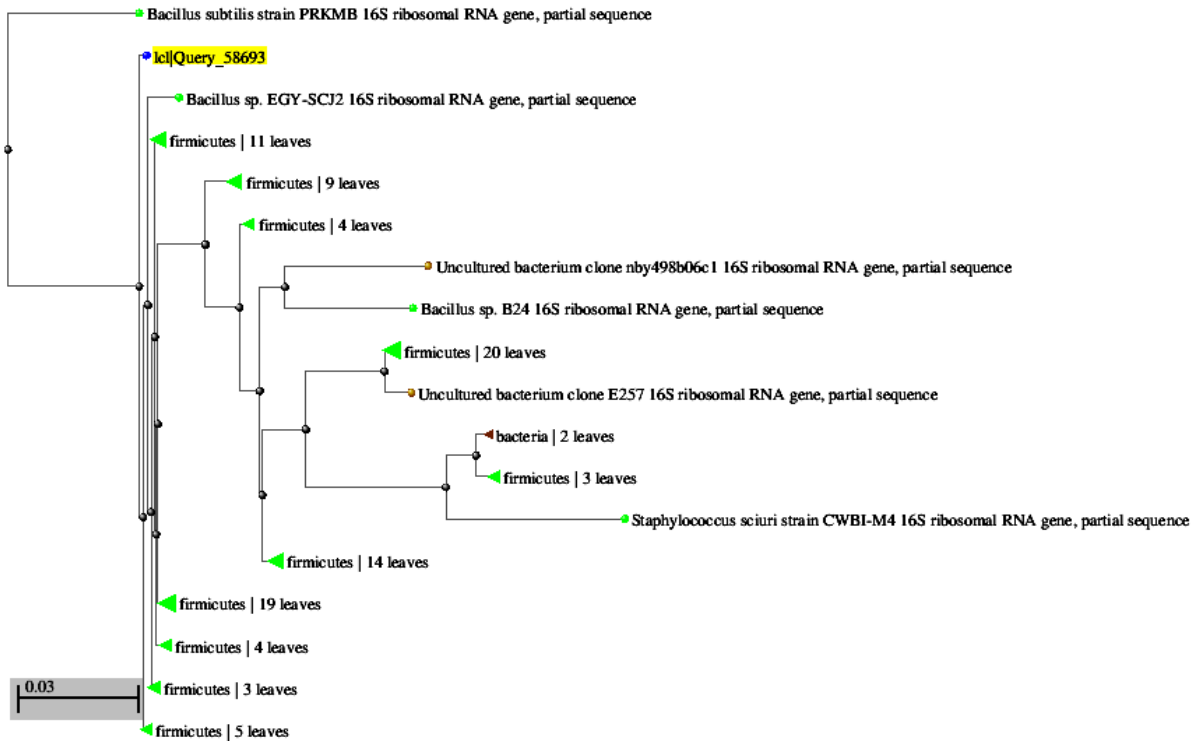

Figure 1
Blast results

The organism was also subjected to BLAST in NCBI (Figure - 1) showed 99% homology to *Bacillus* spp and confirmed and named as *Bacillus subtilis* SSN02. The sequence was submitted in GENBANK.

Subtilisin Assay

The absorbance of the filtrate was determined at A280 nm using UV visible spectrophotometer (Varian Cary UV -300).

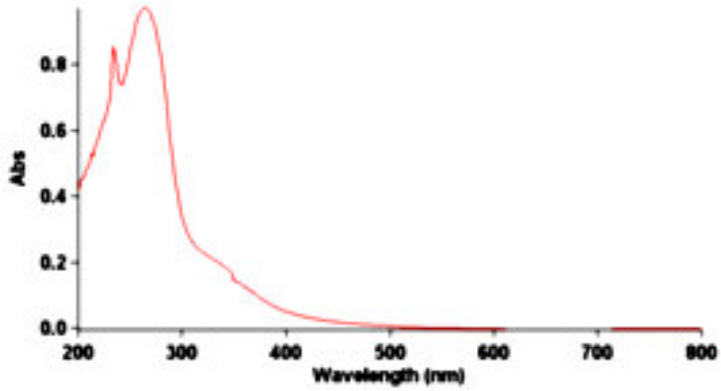

Figure 2
UV Vis - Presence of Subtilisin from the Filtrate

Optimization of media components

The experiment was designed using the minimal medium. 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 CCD. RSM was used to optimize the variables and to study the effects of individual variables and their interactions. The results of RSM are given in Table 3. Multiple regression analysis was used to evaluate the model and the effect of various factors on the yield of subtilisin was indicated by regression coefficients. The computation was carried out by multiple regression analysis making use of the least squares method at 99% 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 of the multiple linear regression model shows that the computed F value is much greater than the tabular F value. This demonstrates the model is highly significant. The 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 equivalent coefficient. The parameter estimate and the corresponding P values suggest that all the independent and interactive terms are highly significant. The value of R^2 = 0.991125 in Table 4 indicates good correlation between the experimental and predicted values. In this study, the lower value of coefficient of variation indicates the high reliability of the experiment. The polynomial equation was originated and was solved using inverse matrix method to obtain optimum concentration of media content and predicted response in terms of enzyme activity.

Table 3
RSM- CCD Showing Observed and Predicted Values.

Run	Factor 1	Factor 2	Factor 3	Response	
	A:Yeast extract	B:Peptone	C:Casein	Actual	Predicted
	g/L	g/L	g/L	IU/L	
1	6	0.79552	10	198.56	198.569
2	3	7.5	15	189.234	189.228
3	6	5	10	215.22	216.274
4	3	7.5	5	184.241	184.235
5	3	2.5	5	190.385	190.379
6	11.0454	5	10	195.59	195.598
7	0.95462	5	10	185.133	185.142
8	6	5	10	214.018	216.274
9	6	5	18.409	200.076	200.084
10	9	2.5	15	198.026	198.02
11	9	7.5	15	196.812	196.805
12	6	9.20448	10	192.373	192.381
13	3	2.5	15	195.258	195.252
14	6	5	10	214.17	216.274
15	9	2.5	5	195.243	195.237
16	6	5	10	217.12	216.274
17	**6**	**5**	**10**	**219.029**	**216.274**
18	9	7.5	5	193.909	193.903
19	6	5	10	218.09	216.274
20	6	5	1.59104	193.537	193.546

Table 4
Analysis of variance (ANOVA) for the factorial design

Source	Sum of Squares	Df	Mean Square	F Value	*p-value Prob> F	
Model	2482.81	9	275.8678	124.0881	< 0.0001	significant
A-Yeast extract	131.9845	1	131.9845	59.36794	< 0.0001	
B-Peptone	46.21593	1	46.21593	20.78838	0.0010	
C-Casein	51.60504	1	51.60504	23.21246	0.0007	
AB	11.56805	1	11.56805	5.203424	0.0457	
AC	2.18405	1	2.18405	0.982407	0.3450	
BC	0.0072	1	0.0072	0.003239	0.9557	
A^2	1208.77	1	1208.77	543.7166	< 0.0001	
B^2	779.2809	1	779.2809	350.5283	< 0.0001	
C^2	682.1029	1	682.1029	306.8167	< 0.0001	
Residual	22.23161	10	2.223161			
Lack of Fit	0.000747	5	0.000149	3.36E-05	1.0000	not significant
Pure Error	22.23086	5	4.446173			
Cor Total	2505.042	19				

Statistically significant at 95% of probability level.

Table 5
Standard deviation and correlation coefficients

Std. Dev.	1.491027	R-Squared	0.991125
Mean	200.3012	Adj R-Squared	0.983138
C.V. %	0.744393	Pred R-Squared	0.987229
PRESS	31.99302	Adeq Precision	30.38838

The quadratic equation

Response = 216.274 + 3.108752A-1.83959B + 1.943886C + 1.2025AB - 0.5225AC + 0.03BC + $9.15841A^2$-$7.35352B^2$-$6.87976C^2$

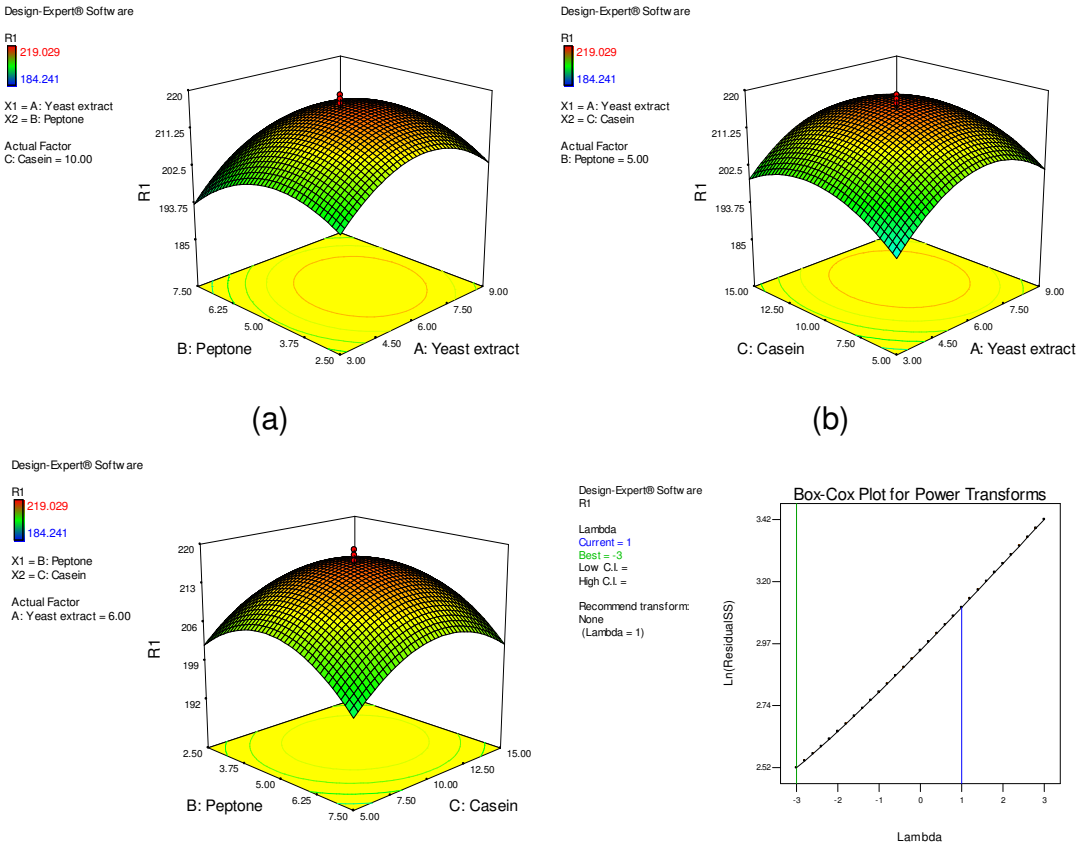

Figure 3
Response surface plots showing the effect of mutual effect of the factors on the production of subtilisin.

Graphics of the response surface function analysis of the effects between the parameters on subtilisin given in Figure 3. The interaction of the (A) Yeast extract (6.00 gm) and (B) Peptone (5.00) showed maximum production of subtilisin compared at lower and higher concentration of the above told parameters. Likewise, at 10.00 gm of Casein and 6.00 gm of Yeast extract the maximum effect was observed. A high accord was observed between the predicted and experimental results, which suggested the precision and applicability of RSM to optimize the production of Subtilisin. Varun Bhaskar et al., 2008[9] analyzed the production of subtilisin using solid state fermentation with

substrate such as Wheat bran, Rice bran, Corn flour, Pea flour, Soya meal and Green gram husk that exhibited a better results compared with submerged fermentation.

CONCLUSION

This study will serve as a base line of the initial studies in optimization production of subtilisin. Through these optimization experiments, the optimal conditions for maximum production of subtilisin were determined. The maximum activity of 219.029 U/mg was seen when a media composition of 6.0 g/l of Yeast extract, 5.0 g/l of Peptone and 10.0 g/l of casein were used.

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