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Research Article

Alkaline Protease Bioprocess Optimization through Response Surface Methodology for Alkaliphilic Bacillus subtilis SHmIIIa Mutant Strain from Warangal-Telangana

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Abstract

The present investigations were carried out to enhance the alkaline protease production by a mutant strain Bacillus subtilis SH2 isolated from slaughter house soils of Warangal and improved through two-tier mutagenesis first by UV and then HNO_2. Initially three efficient mutants with over production of alkaline protease were identified and among them only one stable mutant SHmIIIa was selected for further improvement through popular Response Surface Methodology of the FFCCD. Only X2 agitation, X6 KH_2PO_4 and interactive effects of X3*X3 inoculum, X4*X5 glucose and peptone have shown a significant improvement. The maximum alkaline protease production was achieved with the medium containing of X1 pH 9.8; X2 agitation 237.5 rpm; X3, inoculum size 4%; X4, glucose 6 g/L; X5, peptone 4g/L and X6, KH_2PO_4 2 g/L; under batch fermentative conditions with 33.33 fold increase.

Keywords: Bacillus subtilis SHmIIIa; Alkaline protease; Response Surface Methodology; Bioprocess optimization

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Introduction

Alkaline proteases are the most important industrial enzymes with 60% of the total global enzyme market [1]. The microbial extracellular protease production is significantly influenced by media components, especially carbon and nitrogen sources and also by physical factors such as temperature, pH, incubation time, agitation and inoculum density. Development of the cost effective fermentation process is very challenging. Some approaches have been made to enhance the yield and at the same time reduce the cost of production approximately to 30–40% making the industrial process economically sustainable. Response surface methodology (RSM) is an efficient mathematical approach widely applied to evaluate and understand the interactions between different parameters [2, 3]. It is relevant to select optimum conditions of variables and at the same time verifies a predicted model and desirable response or multiple responses [4, 5]. In the present investigations, an effort was made to optimize the levels of selected bioprocess parameters for the maximum production of alkaline protease production by a mutant strain of *Bacillus subtilis* SH2 using RSM of Designs of Experiments (DoE). To improve the enzyme production Plackett-Burman Design and RSM statistical approaches were made in submerged shake culture condition. Finally, an appropriate explanation was derived by polynomial model.

Material and methods

Microorganism source

The test organism employed in the present study, designated as *Bacillus subtilis* SHmIIIa , a mutant of *Bacillus subtilis* SH2 strain that was isolated from the soil of a slaughter house located in Warangal Telangana State, India. It was evaluated for prospective production of alkaline protease. Cultural and biochemical profile of the isolate *Bacillus subtilis* SH2 was carried out as per the guidelines of the Bergy's Manual of Determinative Bacteriology. Further confirmation of the isolate was made based on molecular typing of 16S rDNA ITS region homology [6].

Starter culture

The pure culture of the organism was maintained on Horokoshi basal medium (HBM) with 10% glycerol at -20 ℃, and activated twice before for the development of seed inoculation. The starter culture for alkaline protease production was developed by raising a cell suspension in a 500 ml baffled flask containing 100 ml of HBM, with the pH adjusted to 11.5±0.5 and incubation for 24 h on a shaker at 200 rpm. The starter culture for inoculation was adjusted to an absorbance of 0.3 (600 nm).

Enzyme production and assay

Enzyme production was carried out in 100 ml of modified HBM with 1% skimmed milk. Varying concentrations of carbon, nitrogen, and inducer substances were employed in different experimental conditions. Incubation was carried out under the conditions as defined by the statistical designs for 24 h. The cell-free

supernatant was recovered by centrifugation (10000g/10 min) and used for determining extracellular alkaline protease activity.

The quantification of extracellular alkaline protease activity was determined by adopting the standard method described by Yang *et al.* (1994) [8]. Culture filtrate containing the enzyme (0.1 ml) mixed with 0.9 ml buffered casein (5 mg casein dissolved in glycine-NaOH buffer 0.1M, pH 9 was incubated at 55°C for 10 min, then added 2 ml of 5% trichloro acetic acid (TCA) to terminate the reaction. The reaction mixture was passed through Whatman No.2 filter paper to remove denatured proteins. The absorbency of the fraction was read at 275 nm. One unit of protease activity was defined as the amount of enzyme required to produce an increase 0.001 in the absorbency at 275 nm per min under assay conditions. Specific activity was expressed as enzyme units per mg protein.

Identification of crucial bioprocess parameters

Application of OVAT method followed by Plackett-Burman Design has revealed that a total of six independent variables, X1 pH; X2 agitation; X3 inoculum size; X4 glucose; X5 peptone and X6 KH_2PO_4 are the most important . Hence they were chosen to optimize enzyme production by the test organism.

Central Composite Design (CCD) to optimize the bioprocess parameters

A central composite design (CCD) was simulated and implemented to optimize the major variables in 12 experiments. The variables were coded according to the model. The Table 1 indicates the design layout of the simulated and responses produced.

Validation of the experimental model

The optimized medium obtained from the CCD was tested for its accuracy. The statistical model was validated with respect to all variables within the design space. A random set of experimental combinations was used to study the protease production under the given experimental conditions. The roles of the variables, their interactions on yield were analyzed by using the following equation

$$X_i = X_i - X_0/\Delta X \text{ --------- } (1)$$

Where x_i is the dimensionless coded level of the variable, X_i is the actual value of that variable, X_0 is the average of the high and low level values of that variable, and ΔX is the high value minus the low value of that variable. Predicted response is calculated by using the following second order polynomial equation

$$Y= \beta_0+\sum\beta x_i+\sum\beta x_i^2+ \sum \beta_{ij}x_ix_j \text{ ----------- } (2)$$

Where Y is the predicted response, β are the coefficients of the equation, and x_i and x_j are the coded levels of variables i and j, respectively. This equation can be used to evaluate the linear, quadratic, and interactive effects of independent variables on the chosen response. The statistical significance of the coefficients and predicted protease production were evaluated using linear regression analysis.

Table 1 Full details of FCCD design for *Bacillus subtilis* SHmIIIa

RUN	BLOCK	X1	X2	X3	X4	X5	X6	Y1
1	1	-0.420448208	-1	-0.420448208	-1	-0.420448208	-1	28451
2	1	-0.420448208	-1	-0.420448208	1	0.4204482076	-1	23455
3	1	-0.420448208	-1	0.4204482076	-1	0.4204482076	-1	24571
4	1	-0.420448208	-1	0.4204482076	1	-0.420448208	-1	29741
5	1	-0.420448208	1	-0.420448208	-1	-0.420448208	1	24811
6	1	-0.420448208	1	-0.420448208	1	0.4204482076	1	25874
7	1	-0.420448208	1	0.4204482076	-1	0.4204482076	1	33456
8	1	-0.420448208	1	0.4204482076	1	-0.420448208	1	25343
9	1	0.4204482076	-1	-0.420448208	-1	-0.420448208	1	23421
10	1	0.4204482076	-1	-0.420448208	1	0.4204482076	1	22346
11	1	0.4204482076	-1	0.4204482076	-1	0.4204482076	1	23457
12	1	0.4204482076	-1	0.4204482076	1	-0.420448208	1	25811
13	1	0.4204482076	1	-0.420448208	-1	-0.420448208	-1	25674
14	1	0.4204482076	1	-0.420448208	1	0.4204482076	-1	37534
15	1	0.4204482076	1	0.4204482076	-1	0.4204482076	-1	32946
16	1	0.4204482076	1	0.4204482076	1	-0.420448208	-1	29456
17	1	0	0	0	0	0	0	29456
18	1	0	0	0	0	0	0	31457
19	1	0	0	0	0	0	0	32457
20	1	0	0	0	0	0	0	33457
21	2	-0.420448208	-1	-0.420448208	-1	0.4204482076	1	24751
22	2	-0.420448208	-1	-0.420448208	1	-0.420448208	1	26457
23	2	-0.420448208	-1	0.4204482076	-1	-0.420448208	1	23457
24	2	-0.420448208	-1	0.4204482076	1	0.4204482076	1	21477
25	2	-0.420448208	1	-0.420448208	-1	0.4204482076	-1	28453
26	2	-0.420448208	1	-0.420448208	1	-0.420448208	-1	32547
27	2	-0.420448208	1	0.4204482076	-1	-0.420448208	-1	23271
28	2	-0.420448208	1	0.4204482076	1	0.4204482076	-1	32784
29	2	0.4204482076	-1	-0.420448208	-1	0.4204482076	-1	32247
30	2	0.4204482076	-1	-0.420448208	1	-0.420448208	-1	28457
31	2	0.4204482076	-1	0.4204482076	-1	-0.420448208	-1	21246
32	2	0.4204482076	-1	0.4204482076	1	0.4204482076	-1	23457
33	2	0.4204482076	1	-0.420448208	-1	0.4204482076	1	22342
34	2	0.4204482076	1	-0.420448208	1	-0.420448208	1	24715
35	2	0.4204482076	1	0.4204482076	-1	-0.420448208	1	25465
36	2	0.4204482076	1	0.4204482076	1	0.4204482076	1	25647
37	2	0	0	0	0	0	0	28456
38	2	0	0	0	0	0	0	31247
39	2	0	0	0	0	0	0	28456
40	2	0	0	0	0	0	0	34572
41	3	-1	0	0	0	0	0	23543
42	3	1	0	0	0	0	0	34127
43	3	0	-1	0	0	0	0	23378
44	3	0	1	0	0	0	0	31457
45	3	0	0	-1	0	0	0	24577
46	3	0	0	1	0	0	0	22457
47	3	0	0	0	-1	0	0	25678
48	3	0	0	0	1	0	0	32123
49	3	0	0	0	0	-1	0	28451
50	3	0	0	0	0	1	0	23457
51	3	0	0	0	0	0	-1	31457
52	3	0	0	0	0	0	1	34257
53	3	0	0	0	0	0	0	33421
54	3	0	0	0	0	0	0	33841

Results and discussion

Identification based on homology of ITS of 16S rDNA, the test isolate was identified as *Bacillus* sp. subspecies *subtilis* DSM10.

Fractional factorial central composite design

Table 1 depicts the FCCD design with 54 experimental matrixes simulated and the responses observed. The experimental data obtained was scrutinized through ANOVA of statistical analysis. Process variables which showed a greater confidence level > 95% (prob > [t] ≤ 0.05) were taken as significant for effective process execution [8]. The confidence limit of the RSM revealed that the agitation and KH_2PO_4 showed a linear effect i.e. X2 ($p < 0.007288$), X6 ($p< 0.011214$). Hence they are the most crucial out of six parameters and the interactive effects of X3*X3 ($p<0.005307$), X4*X5 (p<0.043381) are the best interactive effects (Table 2). Linear effect of X2 (agitation) was observed to be very crucial because it solely controlled the oxygen transfer and maintenance of the dissolved oxygen throughout the process under shake culture conditions. Tang *et al.* (2000) reported that the regulation of oxygen transfer plays a major role in the submerged fermentation condition [9]. Romasa *et al*. (2010) also reported a similar response with 250 rpm of the agitation speed [10]. EL-Enshapy *et al.,* (2008) reported that the high demand of oxygen rate is very important for protease production by *Bacillus*. sp under the submerged fermentation condition [11]. The second parameter X6 ($KH2PO_4$) was noted to influence significantly the entire process, because the buffering capacity of the dibasic potassium ions is a major contributing factor for the maintenance of the pH. This finding also confirms the alkaline nature of the native strain.

Table 2 Statistical evaluation of FCCD model for enhanced alkaline protease production by *Bacillus subtilis* SHmIIIa

Statistics	Master Model	Predictive Model
RMSE	0.117738	0.123022
R-square	73.25%	44.03%
Adjusted R-square	40.93%	35.51%
Coefficient of Variation	1.151616	1.203304

Relationship between the protease production (y) and test variables of the FFCCD model

The empirical relationship between protease production (*Y*) and the test variables in coded RSM approach was detected by multiple regression equation. The present model required small adjustment of R^2 value, which corrects the correlation value of the large sample size and number of terms in the predictive model [12]. In the present study, the adjusted R^2 value is lesser than the actual R^2 value. This is because, there are many terms in the model and the sample size is not very large [13]. As a result the smaller R^2 value appears to be less important [14]. In order to adapt the regression model the probability *p* values, i.e > F (0.022098) should be considered which specify the significant model terms. The F-value was found to be 2.26655 (Table 2). Therefore, the relationship between the selected parameters is confirmed.

Optimization of concentrations of the test variables

The yields of alkaline protease for different optimized concentrations of variables could also be fitted from the regression equation into design space 3D surface plots and 2D contour plots. The optimum conditions for the native strain for maximum protease production were evaluated from the following regression equation:

Y1 = 10.2952 - 0.018704*(BLOCK='1') - 0.061503*(BLOCK='2') + 0.059205*X2 - 0.055476*X6 + 0.102821*X2*X5 - 0.29479*X3*X3 - 0.105573*X4*X5.

The data obtained by regression equation was presented in Table 3. It is evident from the Table 3 that the above second order polynomial equation indicated that the linear effects of X2, X6 and quadratic influence of X3*X3 and interactive effects of X4*X5 are significant . Polynomial expression is also shown in Figure 1. The contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective 0 level. It is evident from the surface curves that the protease production is influenced by the selected fermentation parameters. The alkaline protease production at optimized conditions by the test organism is good enough and more than the targeted value.

The validation of experimental data

The present data indicates that interactions among the bioprocess parameters are the crucial phenomena in achieving the maximum enzyme production, which is possible with regulation of interactive power between selected fermentation parameters (Table 4). The simulated protease production by the current FFCCD model is capable of explaining 73.25% of variability in the response. On the other hand, the remaining 26.75% of variability is not explained by this model due to the randomized signal to noise ratio. However, a relatively lower value of the coefficient of variation (C_V= 1.151616) indicates a better precision and reproducibility of the experiments carried out (Table 4). Root mean square error was also found to be 0.123022 (RMSE) which indicates the 0.1 error from which the model was estimated. The present investigations revealed that the CCD of response surface model is fairly appropriate for the alkaline protease production under defined experimental conditions. Production of 27865.25 EU/mg/mL could be achieved with the medium consisting of (X1 pH 9.8, X2 agitation 237.5 rpm; X3, inoculum size 4%, X4, glucose 6 g/l; X5, peptone 4g/L and X6, KH2PO4 2 g/L; in 250 ml flask .

The data presented in Table 5 reveals that a substantial increase in the enzyme production when D (Y1) = 0, Y1 < 26000, D (Y1) = 0.5, Y1 = 30000D(Y1) = 1, Y1 > 30000. In contrast, the earlier investigations have not achieved more than 1890 EU/mL. Agrebi *et al.* (2009) reported that about 269.36 EU/mL of the protease enzyme production was achieved by *Bacillus mojavanensis* A26 using statistical methods. *Bacillus* sp was known to produce 410 EU/mL of the medium [15]. Oskoule *et al*. (2008) reported the 1520 EU/mL *enzyme production by B. clausii* with statistical experimental design [16]. Recently, *Bacillus mojavanensis* A21 improved about 1850 U/mL of the protease production by the same method [8]. The mutant strain has achieved 33.33 fold increase in the enzyme production.

Table 3 Analysis of variance for evaluation of significant model terms in FCCD model for alkaline protease production by *Bacillus subtilis* SHmIIIa

	Master Model					Predictive Model				
Source	**DF**	**SS**	**MS**	**F**	**Pr > F**	**DF**	**SS**	**MS**	**F**	**Pr > F**
BLOCK	2	0.019028	0.009514	0.68634	0.513025	2	0.034996	0.017498	1.156181	0.323661
X1	1	0.009673	0.009673	0.697787	0.411767					
X2	1	0.119178	0.119178	8.597359	0.007288	1	0.119178	0.119178	7.874622	0.007326
X3	1	0.008076	0.008076	0.582597	0.452734					
X4	1	0.020759	0.020759	1.497552	0.232927					
X5	1	2.017E-6	2.017E-6	0.000145	0.990476					
X6	1	0.104636	0.104636	7.54835	0.011214	1	0.104636	0.104636	6.913798	0.011592
X1*X1	1	0.012455	0.012455	0.898508	0.35263					
X1*X2	1	0.000022	0.000022	0.001613	0.968293					
X1*X3	1	0.001287	0.001287	0.092814	0.763256					
X1*X4	1	0.001021	0.001021	0.073658	0.788404					
X1*X5	1	0.007511	0.007511	0.541826	0.468811					
X1*X6	1	0.01395	0.01395	1.006361	0.325784					
X2*X2	1	0.033613	0.033613	2.424793	0.132519					
X2*X3	1	0.025061	0.025061	1.807852	0.191337					
X2*X4	1	0.011947	0.011947	0.86183	0.36247					
X2*X5	1	0.059805	0.059805	4.314268	0.048666	1	0.059805	0.059805	3.951589	0.052798
X2*X6	1	0.006497	0.006497	0.468721	0.500135					
X3*X3	1	0.130279	0.130279	9.398155	0.005307	1	0.166353	0.166353	10.9917	0.001792
X3*X4	1	0.000319	0.000319	0.02303	0.880648					
X3*X5	1	0.006588	0.006588	0.475237	0.497199					
X3*X6	1	0.033535	0.033535	2.419193	0.132946					
X4*X4	1	0.007532	0.007532	0.543316	0.468207					
X4*X5	1	0.06305	0.06305	4.548331	0.043381	1	0.06305	0.06305	4.165976	0.047005
X4*X6	1	0.020879	0.020879	1.506178	0.231626					
X5*X5	1	0.055141	0.055141	3.977773	0.057583					
X5*X6	1	0.01169	0.01169	0.84328	0.367598					
X6*X6	1	0.019059	0.019059	1.374916	0.252477					
Model	29	0.911161	0.031419	2.266555	0.022098	7	0.547669	0.078238	5.16956	0.000215
Error	24	0.332692	0.013862			46	0.696184	0.015134		
(Lack of	17	0.297816	0.017519	3.516183	0.048692	37	0.563161	0.015221	1.029778	0.520975
(Pure	7	0.034876	0.004982			9	0.133024	0.01478		
Total	53	1.243853				53	1.243853			

Table 4 Significant effects for alkaline protease production in FCCD model for *Bacillus subtilis* SHmIIIa

Term	Estimate	Std Err	t	Pr > |t|	Estimate	Std Err	T	Pr > |t|
(BLOCK=	0.032543	0.052619	0.61847	0.542091	-0.0187	0.042869	-0.43631	0.664652
(BLOCK=	-0.01026	0.052619	-0.1949	0.847109	-0.0615	0.042869	-1.43468	0.158144
X1	0.035543	0.042549	0.835336	0.411767				
X2	0.059205	0.020192	2.932125	0.007288	0.059205	0.021098	2.806176	0.007326
X3	-0.03248	0.042549	-0.76328	0.452734				
X4	0.02471	0.020192	1.223745	0.232927				
X5	0.000513	0.042549	0.012062	0.990476				
X6	-0.05548	0.020192	-2.74743	0.011214	-0.05548	0.021098	-2.62941	0.011592
X1*X1	-0.08404	0.088657	-0.9479	0.35263				
X1*X2	-0.00199	0.049503	-0.04017	0.968293				
X1*X3	-0.03587	0.117738	-0.30465	0.763256				
X1*X4	0.013435	0.049503	0.271401	0.788404				
X1*X5	0.086665	0.117738	0.736088	0.468811				
X1*X6	-0.04966	0.049503	-1.00318	0.325784				
X2*X2	-0.10898	0.069987	-1.55717	0.132519				
X2*X3	0.066559	0.049503	1.344564	0.191337				
X2*X4	0.019322	0.020813	0.928348	0.36247				
X2*X5	0.102821	0.049503	2.077082	0.048666	0.102821	0.051724	1.98786	0.052798
X2*X6	-0.01425	0.020813	-0.68463	0.500135				
X3*X3	-0.27179	0.088657	-3.06564	0.005307	-0.29479	0.088916	-3.31537	0.001792
X3*X4	-0.00751	0.049503	-0.15176	0.880648				
X3*X5	0.081165	0.117738	0.689375	0.497199				
X3*X6	0.076995	0.049503	1.555376	0.132946				
X4*X4	-0.05159	0.069987	-0.7371	0.468207				
X4*X5	-0.10557	0.049503	-2.13268	0.043381	-0.10557	0.051724	-2.04107	0.047005
X4*X6	-0.02554	0.020813	-1.22726	0.231626				
X5*X5	-0.17682	0.088657	-1.99444	0.057583				
X5*X6	-0.04546	0.049503	-0.9183	0.367598				
X6*X6	0.082065	0.069987	1.172568	0.252477				

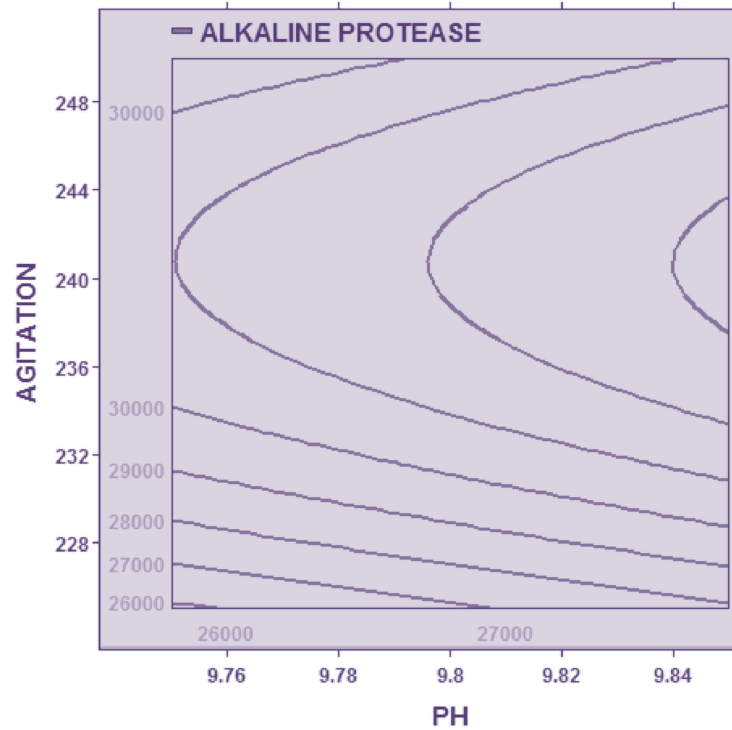

Figure 1 Contour plot at optimized FCCD model terms of *Bacillus subtilis* SHmIIIa

Table 5 Optimal levels of model terms for enhanced alkaline protease by *Bacillus subtilis* SHmIIIa

Factor	Label	Optimal Setting
BLOCK		1
X1	PH	9.8
X2	AGITATION	237.5
X3	INOCULUM	4
X4	GLUCOSE	6
X5	PEPTONE	4
X6	KH2PO4	2

Response	Label	Units	Estimated Value
Y1	Alkaline Protease	EU/mL	27865.25 [27863.17,27867.33]
Desirability			23.32%

Conclusions

The present investigations demonstrated that it is possible to develop an over producing strains with mutagenesis coupled with DoE. The DoE methodology is proved to be ideal for optimization of the bioprocess parameters and became an indispensible tool for media formulation in rational fermentation technology. Studies on further characterization and stability of the traits of the strain for industrial condition are under progress.

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