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STATISTICAL OPTIMIZATION OF PROCESS PARAMETERS FOR PRODUCTION OF ALKALINE PROTEASE FROM *VIBRIO METSCHNIKOVII* NG155 HAVING APPLICATION IN LEATHER INDUSTRY

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ABSTRACT

Alkaline protease producing microorganism *Vibrio metschnikovii* NG155, isolated from the soil samples of leather industry, having potential application in dehairing of animal skin was used in this study. To enhance the production of protease by this strain, various physical and nutritional factors were optimized using statistical methods like Plackett Burman and Response Surface Methodology. In accordance with the Plackett Burman design three nutritional variables soyabean meal, wheat gluten meal and cotton seed flour having a significant positive effect on protease production were selected and optimized by Response Surface Methodology. Production of protease was increased considerably (200 Uml^{-1}) in the optimized medium containing 2.46 (%) wheat gluten meal, 2.45 (%) soyabean meal and 1.46 (%) cotton seed flour, leading to an overall 13 fold increase from the unoptimised medium.

KEYWORDS: **Alkaline protease; *Vibrio metschnikovii*; Optimization; Plackett Burman design; Response Surface Methodology**

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INTRODUCTION

Proteases are a complex group of enzymes that are responsible for hydrolysis of peptide bonds in a protein molecule. Due to their broad substrate specificity, proteases make an important group of industrial enzymes, leading to an approximately 60% of all enzyme sales. In this regard, alkaline thermostable proteases which can withstand high temperature, high pH and chemical denaturation, are industrially more important than neutral or acidic proteases[1]. The use of proteases as detergent additives encouraged their commercial development resulting in significant expansion of fundamental research on this enzyme[2]. In addition to detergents, proteases have substantial application in other industrial sectors such as leather processing, food industry, peptide synthesis, pharmaceutical industry, waste treatment, silk industry, organic synthesis, and recovery of silver from waste photographic film[3, 4, 5]. Hence search is on, for new microorganisms which can produce proteases having properties beneficial for their application in various industrial processes. In this regard, previously we have isolated an alkaline protease producing bacterium *Vibrio metschnikovii* NG155 having potential application in dehairing of animal skin, making the process more ecofriendly, which otherwise generates large amount of pollution causing chemicals. In industrial production processes, where even small improvements in the yield can be crucial for commercial success, process optimization becomes an issue of central importance. In general, improvement in the productivity of any microbial metabolite is obtained by the manipulation of physiochemical parameters and mutation selection. In this context, the conventional methods based on the classical method of 'one variable-at-a-time' bioprocess design in which, variable is studied while fixing all others at a particular level, may fail to consider the combined effects of all involved factors leading to inaccurate conclusions. Hence statistical methodologies, in which interactive effect of different variables can be studied, are generally preferred[6, 7]. Among the statistical methods, the Plackett Burman (PB) factorial designs allow screening of significant factors from a large number of variables and are quite useful in preliminary studies in which the aim is to select variables that can be fixed or eliminated in further optimization processes. In addition, response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system may be determined. The aim is to obtain mathematical models showing the dependence of the enzyme activity on independent variables. The mathematical dependences are used for the prediction of the optimum values of the independent variables, ensuring optimal yield[7]. Statistical optimization has been used for increasing the yield of various industrially important enzymes[8, 9, 10, 11, 12]. The principle objective of this study was to optimize the production of extracellular alkaline protease from *Vibrio metschnikovii* NG155 using statistical methods to achieve maximum yield.

MATERIALS AND METHODS

(i) Chemicals

The chemicals used in the study were of analytical grade purchased from Hi Media, Mumbai, India.

(ii) Bacterial strain and culture conditions

Alkaline protease producing bacterium *Vibrio metschnikovii* NG 155(MTCC No.11401, GenBank Accession No. JN837684) isolated in our laboratory, was used in this study. The culture was grown and maintained in Horikoshi medium[13] agar slants (pH 10) containing 1% dextrose, 0.5% yeast extract, 0.5% peptone, 0.02% $MgSO_4.7H_2O$ and 0.1% KH_2PO_4) at 4°C and subcultured at regular intervals. For inoculum preparation, a single colony was inoculated in 20 ml broth of Horikoshi medium (pH10) and incubated overnight at 37°C with shaking (150 rpm).

(iii)Protease Activity

Protease activity was determined by casienolytic method[14] in which 100µl of appropriately diluted protease enzyme (50 mM, carbonate bicarbonate buffer, pH10) was

mixed with 400µl of casein (6 mg ml^{-1}). After 5 min of incubation at 60°C, reaction was stopped with 5%TCA. Then the mixture was centrifuged (7826 x g) for 10 min at 4°C and supernatant was used to measure the tyrosine released by Lowry method[15]. One unit of protease activity was defined as the amount of enzyme that releases 1µmol tyrosine per minute under the assay conditions.

(iv) One variable at a time method

Optimization of protease production was done by varying different nutritional and physical parameters one at a time keeping the other constant *viz* different carbon sources, nitrogen sources, metal ions, incubation time, % inoculum , % Na_2CO_3, incubation time, agitation rate and incubation temperature.

(v) Selection of significant variables by Plackett Burman design

On the basis of results of one variable at a time studies and literature, the variables that significantly influence the protease production were screened using PB design[16] of Design Expert 8.0.7.1 (Stat-Ease, Inc., Minneapolis, USA). A variety of carbon sources (glucose and starch), inorganic salts ($(NH4)_2SO_4$), $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, NaCl and KH_2PO_4), nitrogen sources (yeast extract, peptone, soyabean meal, wheat gluten meal, cotton seed flour, beef extract, $NaNO_3$, glycine and tryptone) and physical parameters (inoculum size, %Na_2CO_3 (to maintain the pH) and temperature) were selected. The experimental design with the name of the factor, their corresponding units and actual level of the variables is shown in Table 1. 19 independent medium compositions were evaluated at two levels (high and low). The significant variables were screened in 20 combinations in accordance with the design matrix and the responses were measured. All the experiments were carried out in triplicates and average of protease activity was taken as response. The factors showing highest positive effects were selected for optimization using RSM.

Table 1
Plackett Burman design for screening variables at different level for the production of alkaline protease

Factor	Unit	Low	High
Temperature	°C	25	30
$MnSO_4.7H_2O$	mM	1	2
Tryptone	%	0	1
SoyabeanMeal	%	0	1
Beef Extract	%	0	1
Inoculum	%	0.1	1
$MgSO_4.7H_2O$	mM	0	2
Cotton seed flour	%	0	2
$(NH4)_2SO_4$	mM	0	1
Wheat gluten meal	%	0	1
Yeast Extract	%	0	1
Na_2CO_3	%	0.25	1
Peptone	%	0	1
$NaNO_3$	%	0	1
Glucose	%	0	2
NaCl	mM	0	1
Starch	%	0	1
KH_2PO_4	mM	0	2
Glycine	%	1	2

(vi) Response Surface Methodology

Response Surface Methodology was employed to optimize the nutritional factors having significant positive influence on protease production as obtained from PB design i.e. Wheat gluten meal (WGM), Soybean meal (SBM) and Cotton seed flour (CSF) for enhancing the protease production. It was studied at five different levels (Table 2). A 2^3-factorial central composite design (CCD), with eight axial points (α = 1.68) and six replications at the center points (n_0 = 6) led to a total number of 20 experiments. The statistical software Design Expert Version 8.0.7.1 was

used to analyze the results of the experimental design. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} Xi^2 + \Sigma\beta_{ij} X_i X_j \quad (1)$$

Where Y represents response variable (protease U ml^{-1}), β_0 is the interception coefficient, β_i is the coefficient of the linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of interaction effect. The significance of each coefficient was determined by p values. The variability in the dependent variable was explained by R^2. The statistical software was used for multiple regression analysis and to construct the plots of the obtained data. The coded variables of SBM, WGM and CSF were constructed into their actual values to find out the optimum range of the variables for the production of protease.

Table 2
Experimental range and levels of independent factors of RSM

Codes	Factors	Units	Level				
			-1.682	-1	0	+1	+1.68
A	Soyabean meal	(%)	1	2	2.5	3	4
B	Cotton seed flour	(%)	0	1	1.5	2	3
C	Wheat gluten meal	(%)	1	2	2.5	3	4

Validation of the experimental model: To validate the statistical model, the fermentation was carried out using the optimal conditions predicted by the model and response (enzyme yield) was measured by protease assay as described earlier.

RESULTS

Alkaline protease producing bacterium *Vibrio metschnikovii* NG155 isolated in our laboratory was used in this study. The alkaline protease produced by this strain could efficiently remove hair from animal skin and possessed all other essential properties for its application in leather industry[17] Hence studies were done to optimize the physiochemical factors effecting its production for improving the yield to make its industrial application economically viable.

1. One Variable at a time method

Optimization of different nutrient and physical parameters for protease production were studied by varying one factor at a time keeping the other constant. Starting from the 15 Uml^{-1}, protease production was increased to 110 Uml^{-1} under optimized conditions viz. incubation time (24 hrs), temperature (25°C), pH (10.0), inoculum size (1%), inoculum age (18 h), soybean meal (1%) starch (1%) and KH_2PO_4 (0.1%).

2. Selection of influential factors by Plackett Burman design

On the basis of results of one variable at a time studies and literature, nineteen factors *viz*, temperature, $MnSO_4.7H_2O$, tryptone, soybean meal, beef extract, inoculum, $MgSO_4.7H_2O$, starch, $(NH_4)_2SO_4$, wheat gluten meal, yeast extract, Na_2CO_3, peptone, $NaNO_3$, glucose, NaCl, cotton seed flour, KH_2PO_4 and glycine were selected to investigate their influence on protease production by *Vibrio metschnikovii* NG155 using PB design. The design matrix for the screening of significant variables for protease production and the corresponding responses are shown in Table 3. In 20 runs, variation ranging from 1 Uml^{-1} to 70 Uml^{-1} in the yield of protease was observed. Factors evidencing p values less than 0.05 were considered to have significant effects on the response. Out of the 11 factors showing positive effects, three nutritional factors viz. WGM, SBM and CSF with p values 0.0008, 0.0014 and 0.0019 respectively having the most significant positive effect as shown in the pareto chart (Fig 1) were selected for further optimization using CCD. All other insignificant variables and variables with a negative effect were neglected.

Table 3

Parameters, Experimental runs and responses of Placket Burman design used for the selection of significant parameters for production of protease from Vibrio metschnikovii NG155

Run	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	T	Response
1	30	2	0	1	0	1	0	0	0	0	1	1	0	1	2	0	0	2	2	1.22
2	25	1	1	1	1	1	0	2	0	1	0	0.25	0	0	2	1	0	2	2	70
3	25	2	1	0	0	1	2	2	1	0	1	0.25	1	0	0	0	0	2	2	1
4	25	2	1	0	1	1	0	0	1	1	1	1	0	1	0	1	0	0	1	1.5
5	25	2	0	0	0	0.1	2	2	0	1	1	0.25	0	1	2	1	1	0	2	2.5
6	30	1	1	1	0	0.1	2	2	1	1	0	1	0	1	0	0	0	0	2	12
7	25	1	1	1	0	1	2	0	0	1	1	1	1	0	2	0	1	0	1	40
8	30	2	0	1	1	0.1	0	2	1	1	1	0.25	1	0	2	0	0	0	1	19
9	25	2	1	1	1	0.1	2	0	1	0	0	0.25	0	1	2	0	1	2	1	1.2
10	25	2	0	1	0	0.1	0	0	1	1	0	1	1	0	0	1	1	2	2	45
11	25	1	0	1	1	0.1	2	2	0	0	1	1	1	1	0	1	0	2	1	1.1
12	30	2	0	0	1	1	2	2	0	1	0	1	0	0	0	0	1	2	1	41
13	30	1	0	0	0	1	2	0	1	1	0	0.25	1	1	2	1	0	2	1	1.32
14	30	2	1	0	1	0.1	2	0	0	0	0	1	1	0	2	1	0	0	2	1.11
15	30	1	0	0	1	1	0	2	1	0	0	1	1	1	2	0	1	0	2	34.5
16	30	1	1	0	0	0.1	0	2	1	0	1	1	0	0	2	1	1	2	1	5.6
17	30	1	0	1	1	1	2	0	1	0	1	0.25	0	0	0	1	1	0	2	5.6
18	30	1	1	0	1	0.1	0	0	0	1	1	0.25	1	1	0	0	1	2	2	2.44
19	30	2	1	1	0	1	0	2	0	0	0	0.25	1	1	0	1	1	0	1	15
20	25	1	0	0	0	0.1	0	0	0	0	0	0.25	0	0	0	0	0	0	1	4

A:Temperature (° C), B: $MnSO_4.7H_2O$ (mM) , C:Tryptone (%) , D:SoyabeanMeal (%) , E:Beef Extract(%) , F:Inoculum (%), G: $MgSO_4.7H_2O$ (mM), H:Cotton Seed Flour(%), J: $(NH_4)_2SO_4$ (%) , K:WheatglutenMeal (%) ,L:Yeast Extract(%), M:% Na_2CO_3,N:Peptone (%) O: $NaNO_3$ (%), P:Glucose (%), Q:NaCl (%), R:Starch (% , S: KH_2PO_4 (mM) , T:Glycine (%), Response (units/ml)

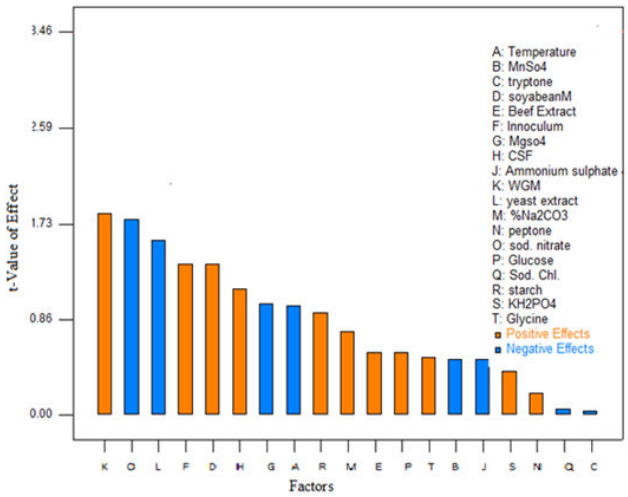

Figure 1

Pareto chart showing the effect of the selected nineteen factors on protease production.

2. Optimization with Response Surface Methodology

A CCD was employed to study the combined effect of variables WGM, SBM and CSF. According to the design, 20 runs were performed and experimental and predicted responses were obtained (Table 4). The relationships between factors were determined by fitting a second order polynomial equation to data obtained from 20 runs. The second order regression equation provided the levels of protease activity as the function of SBM, WGM and CSF which can be presented in terms of coded factors as in the following equation:

$$Y = +199.47 - 2.77 * A - 2.58 * B - 2.46 * C - 4.00 * A * B - 1.50 * A * C + 3.75 * B * C - 15.31 * A^2 - 13.26 * B^2 - 16.87 * C^2 \quad (2)$$

Where *Y* is the response value (activity [Uml^{-1}]) and A, B, and C are WGM (%), SBM (%), CSF (%) respectively.

Table 4
Central Composite Design matrix with experimental and predicted values of NG155 protease production

	Factors (%)			Protease activity (U/ml)		
Run	A	B	C	Actual	Predicted	Residual
1	0	0	-2	55.00	55.04	-0.037
2	-1	+1	+1	150.00	150.67	-0.67
3	+1	+1	-1	165.00	165.55	-0.55
4	+1	+1	-1	144.00	144.90	-0.90
5	0	0	0	200.00	199.47	0.53
6	+2	0	0	54.00	53.34	0.66
7	0	0	+2	41.00	40.27	0.73
8	0	0	0	201.00	199.47	1.53
9	0	-2	0	88.00	87.88	0.12
10	-1	-1	-1	160.00	161.01	-1.01
11	+1	+1	+1	143.00	144.47	-1.47
12	+1	-1	+1	149.00	150.13	-1.13
13	0	0	0	200.00	199.47	0.53
14	-2	0	0	70.00	69.96	0.04
15	0	0	0	200.00	199.47	0.53
16	0	-2	0	73.00	72.42	0.58
17	0	0	0	199.00	199.47	0.47
18	0	0	0	201.00	199.47	1.53
19	-1	+1	-1	155.00	155.44	-0.4
20	-1	-1	-1	160.00	160.09	-0.09

On Analysis of variance (ANOVA), a first order, second order and two level interactions were significant with 99.99% level of significance (Table 5). The value of the correlation coefficient, R^2 (0.9998) showed that the regression model provides an accurate description of the experimental data. A reasonable agreement between predicted (0.9986) and adjusted (0.9996) R^2 was also observed.

Table 5
Analysis of variance (ANOVA) for the model developed for protease yield after fermentation

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F	
Model	60077.53	9	6675.28	5030.13	<0.0001	significant
A-wheat gluten meal	199.38	1	199.38	150.25	< 0.0001	
B-soyabeanmeal	172.65	1	172.65	130.10	< 0.0001	
C-Cotton seed flour	157.54	1	157.54	118.71	< 0.0001	
AB	128.00	1	128.00	96.45	< 0.0001	
AC	18.00	1	18.00	13.56	0.0042	
BC	112.50	1	112.50	84.77	< 0.0001	
A^2	29110.55	1	29110.55	21936.13	< 0.0001	
B^2	21819.84	1	21819.84	16442.25	< 0.0001	
C^2	35325.18	1	35325.18	26619.14	< 0.0001	
Residua	13.27	10	1.33			
Pure Error	2.83	5	0.57			
Cor Total	60090.80	19				
Model fitting	C.V = 0.82	R-Sq = 99.98%	R-Sq (pred) = 99.82 %	R-Sq (adj) = 99.96%		

The three dimensional (3D) response surface graphs of protease production based on the final model are depicted in Fig. 2, which was generated for the pair-wise combination of the three factors while keeping the other one at its optimum level. The response at the central point corresponds to a maximum degree of achievable protease activity for the three factors.

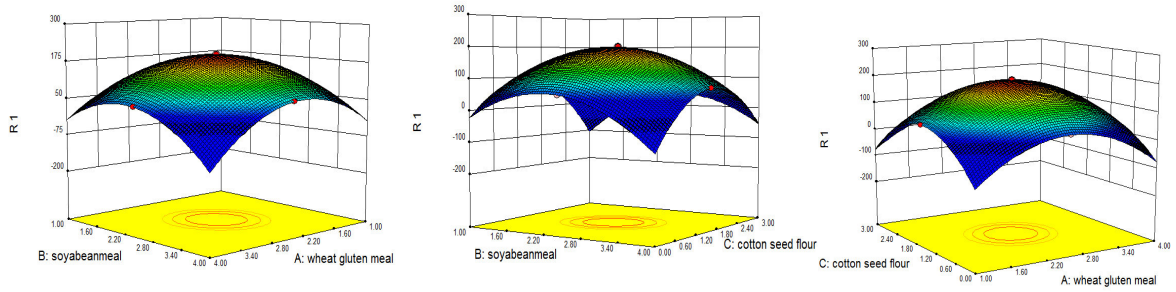

Figure 2

Three dimensional response surface plots for the effect of a) Wheat gluten meal and Soybean meal, b) Cotton seed flour and Wheat gluten meal, c) Cotton seed flour and Soyabean meal on the yield of NG155 protease.

The RSM model predicted that a medium containing 2.46 (%) WGM, 2.45 (%) SBM and 1.46 (%) CSF should give maximum protease yield of 199.79 Uml^{-1}. Validation experiment under these conditions produced 201.05 Uml^{-1} yield. The experimental value was found to be very close to the predicted value and hence, the model was successfully validated. The enzyme production under unoptimized conditions was 15.0 Uml^{-1} which after optimization increased to 200 Uml^{-1} resulting in an approximately 13 fold increase in yield.

DISCUSSION

Alkaline protease producing bacterium *Vibrio metschnikovii* NG155 used in this study was isolated from soil samples of leather industry. In the preliminary studies, the alkaline protease produced from this strain, was found to be active in a broad range of pH and temperature and it could efficiently remove hair from animal skin doing no damage to the collagen layer[17]. As this protease was satisfying all the important criteria for its application in leather industry, studies were done to for improving the enzyme yield to make its industrial application more economical. Some of the other *Vibrio* species like *V. metschnikovii* RH530, *V. metschnikovii* DL33-51 and *V. metschnikovii* J1 have been known to secrete proteases[18, 19, 20]. But their optimization using statistical approach has not been explored. In the present study, the physiochemical variables having a significant positive effect on protease production were screened using the PB design. A large variation in protease production (1-70 Uml^{-1}) from PB design experiments suggested a need for further optimization. Temperature showed a significant but negative effect on the enzyme production, hence its lower value was fixed for the rest of the experiment. Inoculum density positively influenced the protease production hence its upper value was fixed. Three nutritional factors WGM, SBM and CSF having significant positive influence on enzyme production were selected for further optimization using central composite design. The 3D surface graphs obtained were elliptical, which indicates that there may be significant interaction occurring among the independent variables corresponding to the response surfaces. The use of statistical methods for optimization led to an approximately 13 fold increase in protease production. In similar type of studies a 3.9 fold increase in the protease yield has been reported from *Bacillus amyloliquefaciens* [21]

and *Halobacterium* sp. SP1[22]. A 2.3 fold increase in the protease yield has been reported from *Bacillus* sp. RKY3[23] and 6.36 fold increase from *Bacillus* species BGS[24]. The close relationship between the predicted and experimental response values from the validation experiment demonstrated the validity and applicability of the statistical model for achieving maximum yield. Thus, the results of optimization studies allowed effective economization of alkaline protease production by *Vibrio metschnikovii* NG 155.

CONCLUSION

The central composite design and Response Surface Methodology enabled the determination of optimal conditions for obtaining greater alkaline protease production from *Vibrio metschnikovii* NG155. A significant improvement (13 fold) in the production of alkaline protease by was accomplished using cheaper sources. The optimized medium established in this work might result in a significant reduction in the cost of medium constituents making the process economically viable.

CONFLICT OF INTEREST

Conflict of interest declared none.

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