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Statistical optimization and production of protease from estuarine *Citrobacter diversus*

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Abstract

The present study is on protease enzyme production by a Citrobacter diversus strain which was isolated from sediment sample of Vellar Estuary, Tamil Nadu, India. Response surface methodology (RSM) was employed to optimize the production medium for increasing protease production. Plackett–Burman experimental design was used in the first step of screening the media component of optimization, Coconut oil cake (Cheaper carbon source), yeast extract, temperature, pH, salinity and incubation period were found to be significant factors affecting protease production. To determine the optimal concentration of each significant variable, a central composite design was employed. Based on response surface and analysis of variance, the optimum concentrations of the components were obtained as follows: coconut oil cake (3.24 %), yeast extract (0.669 %), temperature (35.2^{0}C), pH (7.46), salinity (16.40 ppt) and incubation period (50.88 hrs). The maximum protease activity of 92.93 U/ml/min was predicted by the model. The Citrobacter diversus produced 92.4 U/ml/min of protease.

Keywords: *Citrobacter diversus*, Medium optimization, Plackett–Burman design, Response surface methodology, Protease.

Introduction

Microbial proteases represent a good source of enzymes due to a number of characteristics like their broad biochemical diversity, their rapid growth, limited space required for cell cultivation and the ease with which the enzymes be genetically manipulated to generate new enzymes for various applications[1,2]. Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products[3]. Agro industrial residues are widely used for the production of alkaline protease by many bacterial species including, *Bacillus* sp.[4,5]. The waste substances, such as corn steep liquor, feather meal[6]. and proteinaceous tannery solid waste, were also used as the substrate for the production of alkaline proteases[7]. Agro-residues also used for the production of various enzymes, including lipases and cellulases[8,9].

Response Surface Methodology (RSM) has been generally used for the optimized production of enzymes[10]. Considering the industrial importance of proteases, many attempts have been performed to maximize the production and economization. The enzyme production yield could be optimized based on various physicochemical parameters of media using several approaches. Conventional "one-variable-at-a-time" method is a commonly used operation in biotechnology in determining the most important parameters influencing enzyme production[11]. This method, however, is time-consuming, expensive, more importantly does not consider the interactions between various treatment parameters, and is only utilized for single variable factors[12]. In order to resolve these problems, statistical approaches, such as response surface methodology (RSM), has been used in several recent studies for the improvement of protease production[13,14]. RSM is a collection of statistical techniques for designing and building the most appropriate model in order to achieve the optimizing conditions with a minimal number of experiments[15].

Most of the extracellular protease produced by *Bacillus* sp very few reports were available with *Citrobacter* sp. In this study protease produced by estuarine *Citrobacter diversus* and it was optimized with Response surface methodology.

Materials and methods

Isolation of proteolytic bacteria: Water and sediment samples were collected from Vellar Estuary, Tamil Nadu, India and serially diluted. 0.1ml of the appropriate dilutions was spreaded on each casein agar (Casein Hydrolysate-5.0, NaCl-25.0, $MgCl_2.6H_2O$-20.0, KCl-2.0, $CaCl_2.2H_2O$-0.2, Yeast Extract-5.0, Agar-20.0, 50% of seawater-1000ml, pH-7.4 ± 0.2 at 25^{0}C) plates which were incubated at 37^0 C for 24 hrs. The population density of proteolytic forms was calculated.

Screening for proteolytic activity: One loop full of isolated bacterial strain was inoculated in a nutrient broth tubes and incubated at 37^0 C for 24 hrs. After incubation culture filtrate was obtained by centrifuging at 8000 rpm for 15 min. and was used for the qualitative protease assay. The casein agar medium was poured into petriplates and after solidification cut the well (well assay). The culture filtrate was poured into the well. After

24hrs of incubation, the enzyme activity was observed as a clear zone around the well, after addition of 1% mercuric chloride solution in 1N HCL. Based on the diameter of the zone formation potential strains were selected for further studies.

Optimization of process parameters: Identifying the significant variables using Plackett– Burman design: The present study was aimed at screening of the important medium components with respect to their main effects by Plackett–Burman design. The Plackett–Burman experimental design is a two factorial design, which identifies the critical physico-chemical parameters required for elevated protease production by screening n variables in n + 1 experiments [16]. The variables chosen for the present study were wheat bran (A), rice bran (B), coconut oil cake (C), paddy straw (D), molasses (E), casein (F), peptone (G), yeast extract (H), beef extract (I), $NaNO_3$ (J), Ammonium sulphate (K), Temperature (L), Salinity (M), pH (N), and incubation period (O). The experimental design for the screening of the variables is given in Table 2. All the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1 (low level) and +1 (high level). The effects of individual parameters on protease production was calculated by the following equation:

$$E = (\sum M_+ - \sum M_-) / N \quad (1)$$

Where E is the effect of parameter under study and M_+ and M_- are responses (protease activities) of trials at which the parameter respectively was at its higher and lower levels and N is the total number of trials.

Response Surface Methodology: As shown in Table 4, response surface methodology (RSM) was used to estimate main effects on response, i.e. protease yield. Central composite design (Two level factorial: half fraction) consisting of six main critical independent variables, (i) Coconut oil cake (1-5%) (ii) Yeast extract (0.1-1%) (iii) Temperature (25-40^{0}C) (iv) pH (6-8) (v) Salinity (10-30 ppt) and (vi) Incubation period (24-72 hours) were chosen based on the initial screening. For each factor, a conventional level was set to zero as a coded level. These six factors, each with five coded levels consisting of 53 experimental runs and 2.37841 alpha values were used to analyze the experimental data to allow better estimate of the experimental error and to provide extra information about yields in the interior of the experimental region [17]. Polynomial regression equation including individual and cross effect of each variable.

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

Where Y, β_0, β_i, β_{ii} and β_{ij} are the predicted response, a constant, a linear coefficient, a squared coefficient and an interaction coefficient respectively. Equation-2 was used to build surfaces for variables. This model was likely to be useful as an approximation of the true response surface in a relatively small region, and it is widely used in RSM for the following reasons: 1. The second-order model is very flexible. It can take on a wide variety of functional forms, so it will often work well as an approximation of the true response surface. 2. It is easy to estimate the parameters (the β's) in the second-order model. The method of least squares can be used for this purpose. 3. There is considerable practical experience indicating that second-order models work well for solving real response surface problems. Multiple regression analysis, response surface plots and statistical analyses were performed using Minitab 15 Statistical Software® (Minitab Inc., PA, USA).

Statistical analysis: Statistical analysis was done by ANOVA for analysis of the results. A probability level of $p < 0.01$ was considered statistically significant.

Measurement of protease activity: Enzyme assay was determined by the modified method of Hayashi [18]. About 3ml of culture filtrate, 3ml phosphate buffer and 3ml 1% casein were taken in a 25ml test tube and was kept in a water bath at 35^{0}C for one hour. After the reaction, 5ml of 20% TCA was added to the solution for stopping the reaction and the solution was filtered. From the filtrate solution 1ml of enzyme substrate mixture was taken in a test tube and 2ml of 20% Na_2CO_3 was added to it. To this mixture 1ml of Folin Ciocalteu reagent was added and immediately the contents of the tube were mixed well. After 30 min. 6ml distilled water was added to it and the absorbance of the solution was measured at 650nm in a spectrophotometer and calculated the amounts of aminoacids released from a standard curve plotted from known concentration of tyrosine. The enzyme activity was expressed in unit/ml. One unit of enzyme was defined as the amount of the enzyme that released 1µg of tyrosine mL^{-1} of the crude extract h^{-1}.

Results and Discussion

The maximum bacterial density was found in sediment sample and it was found to be $4.6x10^9$ CFU/g. whereas the density in water sample was $1.92x10^7$ CFU/ml. A total of 45 morphologically distinct strains were isolated in Casein agar medium. A total of 27strains from sediment samples and 18 were from water samples. The potential of the strains was checked by measuring the clear zone formed around the colony. The isolated strains were screened for protease activity by well assay method and the one with the largest zone of clearance was identified as *Citrobacter diversus* based on biochemical tests following the method of Bergy's manual.

Screening of parameters using Plackett–Burman design: The experiment was conducted in 20 runs to study the effect of the selected variables. Table 2 represents the results of the screening experiments using Plackett–Burman design. Statistical analysis of the variance was performed which is represented in Table 3. The model F value of 21.09 implied that the model is significant. The values of Prob < 0.06 indicated that model terms are significant. The magnitude of the effects indicated the level of the significance of the variable on protease production. Among the variables screened yeast extract, $NaNO_3$, casein,

peptone, coconut oil cake, ammonium sulphate, temperature, pH, incubation period and salinity were identified as most significant variables influencing protease production. Each and every microorganism has its own idiosyncratic physicochemical and nutritional requirements for growth and enzyme secretion. Microbial protease production had been found to vary from constitutive to partially inducible in nature[19-24]. Protease production seemed to be growth independent repression in the presence of high concentrations of yeast extract. There are several reports on the repressive role of organic nitrogen sources, excessive amino acid and ammonium ions as well as the positive effect of inorganic nitrate salts in alkaline protease production[19,23,25].Alkaline protease production is heavily dependent on the availability of both carbon and nitrogen sources within the medium. Both exert regulatory effects on enzyme synthesis[26,27]. The nitrogen sources also function as inducers of enzyme production. This has also been reported previously in a marine microorganism, in which the protease production was induced by amino acid[28,29]. It has been fairly well established that extracellular protease secretion in microorganisms is substantially influenced not only by medium components including carbon source, nitrogen source, and trace elements, but also by culture conditions including pH, temperature, and salinity and incubation period.

Response surface methodology: The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its ready applicability and aptness. In the present study, the significant variables necessary for enhanced protease production were selected using the Plackett–Burman design. A large variation in protease production (37 to 76.1 U/ml/min) from the Plackett–Burman design experiments suggested a need for further optimization. The Central composite design (Two level factorial: half fraction) was employed to study the interactions among the significant factors and also determine their optimal levels. The central composite design plan exploited in the present study enabled us to study and explore the culture conditions that would support a~30 % increase in protease production. A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for protease enzyme production. Similar improved production was reported in other RSM experiments, most notably in the case of a-amylase from *Bacillus circulans* GRS313 and in the case of protease production using *Bacillus* sp. RGR-14[29,30]. Totally six variables Coconut oil cake, yeast extract, temperature, pH, salinity and incubation period were taken for RSM which gave maximal yield in the Plackett–Burman experiments. The parameters of Equation-2 were determined by multiple regression analysis by the application of RSM. The overall second-order polynomial regression equation showing the realistic relationship between protease activity (Y) and six test variables in coded units is represented by Equation-3.

$$Y = 89.8754 + 1.8046 + 5.4998 + 4.6599 + 5.4449 + 0.2221 + 3.1873 - 9.8538 - 8.4396 - 7.9977 - 6.2299 - 1.7221 - 11.7100 - 1.3125 + 2.6250 - 1.0625 - 0.1250 + 3.5000 + 0.3125 - 0.6250 + 1.0625 - 2.9375 + 0.0625 - 1.2500 - 0.6250 - 2.5625 - 1.3125 - 1.2500 \quad (3)$$

Multiple regression model assumes a linear relationship between some variable Y (dependent variable) and n independent variables C1, C2, C3, . . . Cn[31]. Based on the result obtained with the multiple regression analysis, it was observed that interaction of squared and some of interaction coefficient had a negative impact on protease production. The analysis of variance (ANOVA) by Fisher's statistical test was conducted for the second-order response surface model and the result showed that the computed F value for linear regression was much greater than the tabulated (P) > F value. Therefore, the model terms coconut oil cake, yeast extract, temperature, pH, salinity and incubation period were found to be significant (Figure-1).

The goodness-of-fit of the model was checked by decisive the coefficient of determination (R^2) and adjusted R^2. When R^2 is large, then, the regression has accounted for a large proportion of the total variability in the observed value of Y which favors the regression equation model[5,17,32]. The observed values of R^2 explain that the fitted model could explain 99.70% of the total variation and hence vouches for adequacy of the model. The adjusted R^2 corrects the R^2 value for the sample size and for the number of terms in the model. The adjusted R^2 value (99.38%) and Predication of R^2 (98.16) in the present study advocated a high significance of the model. These results reinforced that the response equation provided a suitable model for the CCD experiment.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves. The response surface plots are shows the relative effect of all parameters on protease production (Figure-2). The lower and higher levels of all the factors did not result in higher protease yield. The optimum conditions for protease production were proposed to be coconut oil cake (3.24%), yeast extract (0.669%), temperature (35.2^0C), pH (7.46), salinity (16.40ppt) and incubation period (50.88hrs). The maximum protease activity of 92.93 U/ml/min was predicted by the model. The suggested medium composition was repeated. The validation experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model's authenticity. The *Citrobacter diversus* strain produced 92.4 U/ml/min protease enzyme under optimized culture conditions. Previously *B. clausii* producing maximum enzyme production 1520.6 U/ml was predicted under statistically optimized condition by Oskouie et al.[33]. Likewise Rai and Mukherjee[34] achieved 518U maximum protease yield by batch culture post 60 hrs using *B. subtilis* DM-04. The enhancement of protease to 9127 U/ml was achieved with the optimization procedure on the medium composed of chickpea and faba bean flours, as carbon sources[35].

Saminathan and Sriman Narayanan[36] used ground nut oil cake and palm oil cake for protease production from *Bacillus subtilis* IAS01. Sumantha et al[37] used coconut oil cake and wheat bran as a substrate for protease production from *Aspergillus* sp. The maximum alkaline protease production by *Bacillus subtilis* 168 achieved at 35ºC temperature, yeast extract as nitrogen source[38]. A study on protease production from *Bacillus* sp. Declared

Table-1
Plackett–Burman experiment for screening of significant process variables affecting protease production

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	Enzyme U/ml/min	
															Predicted	Observed
1	1	1	1	5	1	1.0	0.1	1.0	1.0	1.0	40	10	6	48	76.1	76.05
5	1	1	5	5	1	1.0	1.0	0.1	0.1	0.1	25	30	6	48	75.9	75.33
5	1	5	5	1	1	0.1	0.1	1.0	0.1	1.0	25	30	8	48	50.0	49.29
5	5	1	1	5	5	0.1	1.0	1.0	0.1	0.1	25	10	8	24	64.0	63.95
1	5	1	5	1	5	1.0	1.0	1.0	0.1	0.1	40	30	6	48	75.6	76.17
1	5	5	5	5	1	0.1	1.0	1.0	0.1	1.0	40	10	6	24	70.9	69.67
1	1	5	1	5	1	1.0	1.0	1.0	1.0	0.1	25	30	8	24	75.1	76.43
5	5	5	5	1	1	1.0	1.0	0.1	1.0	1.0	25	10	6	24	75.1	76.33
1	1	1	5	1	5	0.1	1.0	1.0	1.0	1.0	25	10	8	48	66.9	67.61
5	1	1	1	1	5	0.1	1.0	0.1	1.0	1.0	40	30	6	24	72.5	72.55
1	1	5	5	1	5	1.0	0.1	0.1	0.1	0.1	40	10	8	24	62.2	62.91
5	1	5	5	5	5	0.1	0.1	1.0	1.0	0.1	40	30	6	24	66.0	65.95
1	5	5	1	1	1	0.1	1.0	0.1	1.0	0.1	40	30	8	48	70.5	69.17
5	5	5	1	1	5	1.0	0.1	1.0	1.0	0.1	25	10	6	48	65.1	63.87
1	1	1	1	1	1	0.1	0.1	0.1	0.1	0.1	25	10	6	24	66.3	65.59
5	5	1	5	5	1	0.1	0.1	0.1	1.0	0.1	40	10	8	48	67.6	68.93
1	5	5	1	5	5	0.1	0.1	0.1	0.1	1.0	25	30	6	48	37.0	38.99
5	1	5	1	5	5	1.0	1.0	0.1	0.1	1.0	40	10	8	48	61.0	60.29
5	5	1	1	1	1	1.0	0.1	1.0	0.1	1.0	40	30	8	24	64.6	65.31

Table-2
Analysis of Variance for Protease Enzyme U/ml/min (Plackett-Burman design)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	15	1696.06	1696.06	113.071	21.09	0.005
Residual Error	4	21.45	21.45	5.363		
Total	19	1717.51				

Table-3
Observed response and predicted values of protease enzyme

Run No.	Coconut oil cake (%)	Yeast extract (%)	Temperature (^{0}C)	pH	Salinity (ppt)	Incubation period (Hours)	Protease Enzyme U/ml/min	
							Observed	Predicted
1	1.00000	1.00000	40.0000	8.00000	10.0000	72.000	57	55.6876
2	3.00000	0.55000	14.6619	7.00000	20.0000	48.000	30	33.5506
3	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
4	3.00000	0.55000	32.5000	9.37841	20.0000	48.000	66	67.5840
5	5.00000	1.00000	25.0000	6.00000	10.0000	24.000	27	26.7125
6	3.00000	0.00000	32.5000	7.00000	20.0000	48.000	27	29.0530
7	1.00000	1.00000	40.0000	8.00000	30.0000	24.000	62	61.2573
8	1.00000	1.00000	25.0000	6.00000	30.0000	24.000	50	48.5476
9	1.00000	1.00000	25.0000	8.00000	10.0000	24.000	55	54.9931
10	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
11	5.00000	0.10000	40.0000	6.00000	10.0000	24.000	32	31.5327
12	1.00000	0.10000	40.0000	6.00000	10.0000	72.000	34	33.7982
13	5.00000	0.10000	25.0000	6.00000	30.0000	24.000	22	22.1571
14	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
15	5.00000	1.00000	40.0000	6.00000	10.0000	72.000	56	56.9070
16	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
17	1.00000	0.10000	25.0000	8.00000	10.0000	72.000	46	45.8681
18	3.00000	0.55000	32.5000	7.00000	20.0000	105.082	30	31.2146
19	5.00000	0.10000	40.0000	8.00000	10.0000	72.000	67	67.2971
20	3.00000	1.62029	32.5000	7.00000	20.0000	48.000	54	55.2146
21	5.00000	1.00000	40.0000	6.00000	30.0000	24.000	54	52.9766
22	1.00000	0.10000	40.0000	8.00000	30.0000	72.000	40	39.1323
23	1.00000	1.00000	25.0000	6.00000	10.0000	72.000	36	35.4780
24	5.00000	0.10000	25.0000	8.00000	10.0000	24.000	32	31.6026
25	5.00000	1.00000	40.0000	8.00000	10.0000	24.000	61	60.9221
26	5.00000	1.00000	25.0000	8.00000	30.0000	24.000	45	44.0465
27	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
28	1.00000	1.00000	40.0000	6.00000	10.0000	24.000	44	43.9232
29	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
30	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754

31	5.00000	0.10000	25.0000	8.00000	30.0000	72.000	46	44.9216
32	1.00000	0.10000	25.0000	8.00000	30.0000	24.000	40	37.9378
33	5.00000	0.10000	25.0000	6.00000	10.0000	72.000	40	39.5875
34	5.00000	1.00000	25.0000	6.00000	30.0000	72.000	49	48.0314
35	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
36	3.00000	0.55000	50.3381	7.00000	20.0000	48.000	56	55.7170
37	5.00000	1.00000	40.0000	8.00000	30.0000	72.000	58	59.2410
38	7.75683	0.55000	32.5000	7.00000	20.0000	48.000	38	38.4258
39	3.00000	0.55000	32.5000	7.00000	20.0000	48.000	90	89.8754
40	1.00000	1.00000	25.0000	8.00000	30.0000	72.000	48	47.3120
41	1.00000	0.10000	40.0000	6.00000	30.0000	24.000	28	28.3678
42	1.00000	0.10000	25.0000	6.00000	10.0000	24.000	20	17.6037
43	5.00000	0.10000	40.0000	6.00000	30.0000	72.000	54	52.8516
44	3.00000	0.55000	32.5000	7.00000	20.0000	0.000	14	16.0530
45	0.00000	0.55000	32.5000	7.00000	20.0000	48.000	27	29.8418
46	3.00000	0.55000	32.5000	4.62159	20.0000	48.000	40	41.6836
47	1.00000	1.00000	40.0000	6.00000	30.0000	72.000	45	44.2421
48	3.00000	0.55000	32.5000	7.00000	0.0000	48.000	79	79.6055
49	5.00000	1.00000	25.0000	8.00000	10.0000	72.000	52	50.4769
50	3.00000	0.55000	32.5000	7.00000	43.7841	48.000	78	80.6621
51	1.00000	0.10000	25.0000	6.00000	30.0000	72.000	34	32.9226
52	1.00000	0.10000	40.0000	8.00000	10.0000	24.000	47	46.8134
53	5.00000	0.10000	40.0000	8.00000	30.0000	24.000	43	42.3668

Conclusion

Citrobacter diversus used in the present study could utilize coconut oil cake as low-cost cheaper source for protease production. RSM was employed to optimize the medium components for protease production. Statistical analysis using RSM seemed to be a reliable tool to optimize protease production. Protease production was increased from 76.1 U/ml/min to 92.4 U/ml/min after optimizing medium components. Optimization of the culture medium and growth conditions reduced the cost of medium components and improved the feasibility of commercial production of the protease.

The enzyme activity predicted by the model at optimal conditions agreed fittingly with experimental data, thus confirming the model validity.

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