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Research Article

ENHANCEMENT OF ALKALINE PROTEASE PRODUCTION ISOLOATED FROM STREPTOMYCES PULVERACEUS USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Response surface methodology (RSM) was conducted for optimization of maximum protease production under submerged fermentation using isolated *Streptomyces pulveraceus* (MTCC 8374). The preliminary studies revealed that Starch, Casein, NaCl, pH-9, Inoculum and temperature played a vital role in enhancing the protease production. The interactive behavior of each of these parameters along with their significance on enzyme yield was analyzed using Full Factorial Central Composite Design (FFCCD). The above results were analyzed using statistical program and the coefficient of determination (R^2) was calculated as 0.9691 for alkaline protease production. Alkaline protease yield improved from 1500U to 2298.16 U/ml which more than150% is using FFCCD as a means of optimizing conditions.

Keywords: Alkaline proteas; Full Factorial Central Composite design; *Streptomyces pulveraceus*; Optimization.

INTRODUCTION

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications in different industries viz., detergent, food, pharmaceutical, leather and for recovery of silver from used x-ray films etc. Proteases account for 30% of the total worldwide enzyme production. Their enormous diversity of function makes them one of the most fascinating groups of enzymes.

Alkaline proteases produced by *Streptomyces* are characterized by its activity at wide range of pH, Temperature and stability towards detergents. The possibility of using *Streptomyces* for protease production has been investigated because of their capacity to secrete the proteins into extra cellular media, which is generally regarded as safe (GRAS) with food and drug administration[1]. Optimization of alkaline proteases is generally done to obtain maximum yield from minimum possible inputs, efficient utilization of media components and cost-effective enzyme production. Since each organism or strain has its own special conditions for enzyme production, the present study deals to optimize the important media constituents which have been predicted to play a significant role in enhancing the production of protease.

Optimization of medium by classical methods is extremely time consuming and expensive, when large numbers of variables are evaluated. To overcome this difficulty, central composite design can be employed to optimize the medium components. Hence response surface methodology was applied to study group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected variables for protease production.

MATERIALS AND METHODS

Nutritional factors affecting growth and protease production

In the preliminary studies of optimization the effect of pH, temperature, inoculum, carbon sources, organic nitrogen sources and NaCl concentration on alkaline protease production produced by *S pulveraceus* was studied. The optimum conditions obtained after the preliminary experiments were taken as the central points for performing RSM.

Estimation of protease activity

Protease activity was determined using modified Auson – Hagihara method[2]. In this 1 ml of the enzyme solution was added to 1 ml casein solution (1%, w/v casein solution prepared in 50 mM Glycine- NaOH buffer, pH 11) and incubated at 70 °C for 20 min. The reaction was terminated by adding 4 ml of 10% trichloroacetic acid and the contents were filtered through a Whatman No. 1 filter paper. The filtrate absorbance was read at 280 nm using UV–Visible spectrophotometer and the protease activity was calculated using tyrosine standard curve. One unit of alkaline protease activity was defined as 1 µg of tyrosine liberated ml^{-1} under the assay conditions. All experiments were conducted in triplicates and results reported here were average values having 3% experimental error.

Experimental design and Optimization by RSM (Response Surface Methodology)

Based on the results obtained in preliminary experiments[1] Starch, casein from classical approach, other factors such as pH, incubation temperature, inoculum percentage and NaCl concentrations were selected for the study of RSM. Six critical components of the production medium were selected and further evaluated for their interactive behaviors using a statistical approach. The central values (zero level) chosen for experiment design were Starch 3 g/L; Casein 10 g/L; pH 9.0; Temperature 33^{0}C NaCl concentration 10 g/L and inoculums 3%, were selected and each of the variables were coded at five levels –2.38, –1, 0, 1, and 2.38 by using Eq. 1.

The regression equation for the test factors were coded according to the equation.

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

Where x_i is the dimension less coded value of the variable X_i, X_0 the value of the X_i at the centre point and ΔX_i is the step change. For statistical calculations, the variables Xi were coded as xi according to the following transformation according to Eq. 1. The range and levels of the variables in coded units for RSM studies were reported in Table 1.The behavior of the system was explained by the following quadratic model Eq. 2.

$$Y = \beta_0 + \sum \beta_i * x_i + \sum \beta_{ii} * x_i^2 + \sum \beta_{ij} * x_{ij} \quad \text{-------- (2)}$$

Where Y is the predicted response, β_0 is intercept term, β_i is linear effect, β_{ii} is the squared effect, and βij the interaction effect.

In the present study 2^{6-1} fractional factorial design with 12 star points and 6 replicates at the central points was employed to fit the second order polynomial model, which indicated that 50 experimental tests (Table 1). Soft ware STATISTICA 6.0 (Stat Soft, Inc, Tulsa, OK) was used to find out the regression and graphical analysis of the data obtained.

RESULTS AND DISCUSSION

Optimization by RSM: Effect of medium variables on protease production

From classical approach, studies on the alkaline protease production by S. *pulveraceus* revealed that Starch, Casein and factors such as pH, incubation temperature, inoculum percentage and NaCl concentrations are the variables which supported maximum enzyme production. It was reported that effects of a specific carbon and nitrogen supplement on protease production differ from organism to organism although complex nitrogen sources are usually used for alkaline protease production[2-5]. The influences of different experimental variables were optimized by central composite design. The predicted value for each performed experiment was calculated and the correlation between experimental and predicted values is shown in Figure 1 and Table 1.

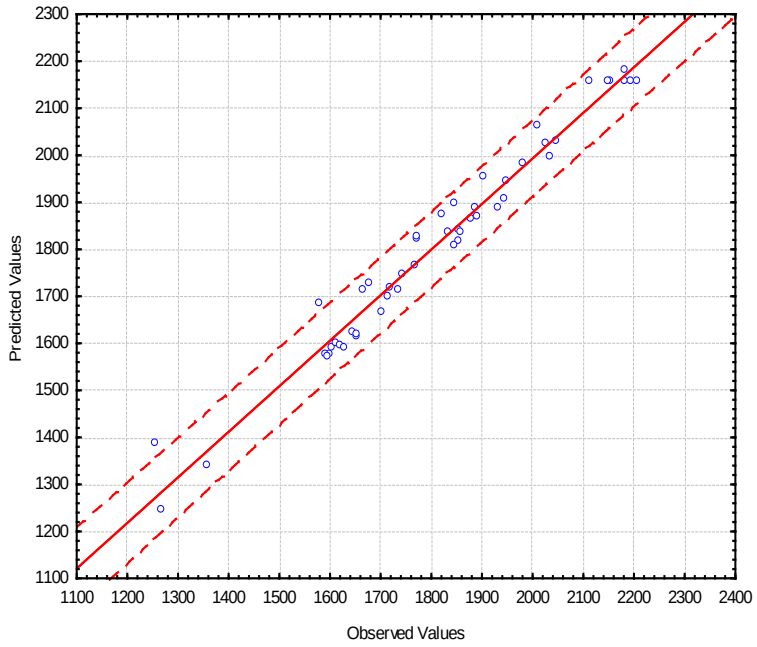

Fig. 1: Correlation between the observed and predicted values

Table 1: Experimental design along with observed and predicted protease activity

No	X1	x2	x3	X4	X5	x6	Protease activity (U/ml)		
							Observed	Predicted	Error
1	8.5	2	2	7.5	32	7.5	1654.844	1616.194	38.650
2	8.5	2	2	7.5	34	12.5	1745.112	1747.139	-2.027
3	8.5	2	2	12.5	32	12.5	1856.303	1842.041	14.262
4	8.5	2	2	12.5	34	7.5	1737.243	1714.354	22.890
5	8.5	2	4	7.5	32	12.5	1606.074	1592.367	13.707
6	8.5	2	4	7.5	34	7.5	1893.047	1870.712	22.335
7	8.5	2	4	12.5	32	7.5	1855.160	1816.536	38.624
8	8.5	2	4	12.5	34	12.5	2181.788	2183.841	-2.053
9	8.5	4	2	7.5	32	12.5	1357.874	1342.660	15.214
10	8.5	4	2	7.5	34	7.5	1858.687	1834.846	23.841
11	8.5	4	2	12.5	32	7.5	1931.360	1891.230	40.130
12	8.5	4	2	12.5	34	12.5	1980.694	1981.241	-0.547
13	8.5	4	4	7.5	32	7.5	1846.911	1807.335	39.575
14	8.5	4	4	7.5	34	12.5	1946.933	1948.035	-1.102
15	8.5	4	4	12.5	32	12.5	1716.442	1701.254	15.188
16	8.5	4	4	12.5	34	7.5	1599.994	1576.179	23.815
17	9.5	2	2	7.5	32	12.5	1590.838	1577.205	13.633
18	9.5	2	2	7.5	34	7.5	1595.964	1573.703	22.261
19	9.5	2	2	12.5	32	7.5	1945.531	1906.981	38.550
20	9.5	2	2	12.5	34	12.5	2025.485	2027.612	-2.127
21	9.5	2	4	7.5	32	7.5	1702.790	1664.795	37.995
22	9.5	2	4	7.5	34	12.5	1833.432	1836.114	-2.682
23	9.5	2	4	12.5	32	12.5	1879.835	1866.228	13.607
24	9.5	2	4	12.5	34	7.5	1267.700	1245.465	22.235
25	9.5	4	2	7.5	32	7.5	2035.374	1995.872	39.501
26	9.5	4	2	7.5	34	12.5	1888.721	1889.897	-1.176
27	9.5	4	2	12.5	32	12.5	2045.684	2030.571	15.114
28	9.5	4	2	12.5	34	7.5	1647.390	1623.649	23.741
29	9.5	4	4	7.5	32	12.5	1613.379	1598.820	14.559
30	9.5	4	4	7.5	34	7.5	1621.117	1597.931	23.186
31	9.5	4	4	12.5	32	7.5	1629.002	1589.527	39.476

32	9.5	4	4	12.5	34	12.5	1718.710	1719.911	-1.202
33	7.8	3	3	10	33	10	2009.778	2063.964	-54.18
34	10.1	3	3	10	33	10	1904.951	1956.685	-51.73
35	9	0.62	3	10	33	10	1771.836	1820.707	-48.87
36	9	5.37	3	10	33	10	1770.744	1827.793	-57.05
37	9	3	0.621	10	33	10	1820.769	1874.705	-53.93
38	9	3	5.378	10	33	10	1677.022	1729.006	-51.98
39	9	3	3	4.0	33	10	1664.640	1716.710	-52.07
40	9	3	3	15.9	33	10	1844.658	1898.508	-53.85
41	9	3	3	10	30.6	10	1581.221	1688.082	-106.8
42	9	3	3	10	35.3	10	1767.958	1767.017	0.941
43	9	3	3	10	33	4.05	1254.630	1390.510	-135.8
44	9	3	3	10	33	15.9	1652.310	1622.350	29.960
45	9	3	3	10	33	10	2153.002	2160.474	-7.472
46	9	3	3	10	33	10	2148.960	2160.474	-11.51
47	9	3	3	10	33	10	2195.340	2160.474	34.866
48	9	3	3	10	33	10	2112.640	2160.474	-47.83
49	9	3	3	10	33	10	2205.630	2160.474	45.156
50	9	3	3	10	33	10	2183.620	2160.474	23.146

The above results were analyzed and the calculated coefficient of determination (R^2) was 0.9691 for alkaline protease production by this bacterial strain indicating that the statistical model can explain 96.91% of variability in the response and only 3.19% of the total variations were not explained by the model. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj R^2 = 0.9316) was also very high suggesting a higher significance of the model used for analyzing the data[5- 6]. In this enzyme production study the adjusted R^2 value (0.9316) was lesser than the R^2 value (0.9691). The adjusted R^2 may be noticeably smaller than the R^2. At the same time, a relatively lower value of the coefficient of variation (CV= 3.28 %) indicated a better precision and reliability of the experiments carried out[7- 9].

The protease experimental data was analyzed by applying multiple regression and the results of the FFCCD design were fitted with a second order full polynomial equation. The empirical relationship between protease production (*Y*) and the 6 test variables in coded units obtained by the application of RSM is given by equation 4.

Y = 2160.47 -22.55 * x1 +1.48 * x2 -30.62* x3 +38.21 * x4 +16.59339 * x5 +48.73764 * x6 -26.54354 * x12 -59.43726 * x22 -63.39617 * x32 -62.37971 * x42 -76.53157 * x52 -115.6195 * x62+20.26438 * x1 * x2 -63.53937* x1 * x3 -20.99312 * x1 * x4 -61.32562 * x1 * x5 +35.53813 * x1 * x6 -35.05687 * x2 * x3 -32.08312 * x2 * x4 -3.19312 * x2 * x5 -30.24937 * x2 * x6 -51.79187 * x3 * x4 +4.73813 * x3 * x5 +31.1418* x3* x6 -52.35187 * x4 * x5 +75.559 * x4 * x6 +94.82188 * x5 * x6

Where *Y,* alkaline protease production in U/ml, was response and x1-x6 were the coded values of the test variables as per the Table. 1

The ANOVA was conducted for the second order response surface model. The significance of each coefficient was determined by Student's *t*-test and *p*-values, which were listed in Table 2 and 3. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient[9-12.]

Table 2: Regression coefficients and effects

	Coefficients	**Effect**	**t-value**	**p-value**
Mean/Interc.	2160.474	2160.474	89.2804	0.000000
X1	-22.553	-45.105	-2.4979	0.020460
X2	1.490	2.979	0.1650	0.870448
X3	-30.630	-61.259	-3.3925	0.002618
X4	38.218	76.437	4.2331	0.000341
X5	16.594	33.188	1.8380	0.079611
X6	48.738	97.477	5.3982	0.000020
X1*x1	-26.543	-53.086	-3.2914	0.003330
X2*x2	-59.437	-118.873	-7.3702	0.000000
X3*x3	-63.395	-126.791	-7.8611	0.000000
X4*x4	-62.378	-124.757	-7.7350	0.000000
X5*x5	-76.531	-153.062	-9.4900	0.000000
X6*x6	-115.620	-231.240	-14.337	0.000000
X1*x2	20.265	40.530	1.9293	0.066698
X1*x3	-63.539	-127.078	-6.0490	0.000004
X1*x4	-20.993	-41.986	-1.9986	0.058160
X1*x5	-61.326	-122.653	-5.8384	0.000007
X1*x6	35.539	71.078	3.3834	0.002675
X2*x3	-35.056	-70.113	-3.3374	0.002985
X2*x4	-32.083	-64.166	-3.0544	0.005812
X2*x5	-3.193	-6.386	-0.3040	0.764004
X2*x6	-30.250	-60.499	-2.8798	0.008699
X3*x4	-51.792	-103.583	-4.9306	0.000062
X3*x5	4.739	9.477	0.4511	0.656310
X3*x6	31.142	62.285	2.9648	0.007154
X4*x5	-52.351	-104.703	-4.9839	0.000055
X4*x6	75.560	151.121	7.1935	0.000000
X5*x6	94.821	189.642	9.0271	0.000000

Table 3: Analysis of Variance (ANOVA)

	SS	Df	MS	F	P
X1	22030	1	22030.0	6.2395	0.020460
X2	96	1	96.1	0.0272	0.870448
X3	40636	1	40635.6	11.5092	0.002618
X4	63266	1	63266.1	17.9188	0.000341
X5	11927	1	11927.1	3.3781	0.079611
x6	102888	1	102888.3	29.1409	0.000020
x1*x1	38249	1	38248.7	10.8331	0.003330
x2*x2	191790	1	191789.8	54.3204	0.000000
x3*x3	218189	1	218189.3	61.7975	0.000000
x4*x4	211245	1	211244.5	59.8306	0.000000
x5*x5	317975	1	317974.9	90.0597	0.000000
x6*x6	725741	1	725741.4	205.5510	0.000000
x1*x2	13141	1	13141.4	3.7220	0.066698
x1*x3	129191	1	129191.0	36.5906	0.000004
x1*x4	14103	1	14102.8	3.9943	0.058160
x1*x5	120350	1	120349.5	34.0865	0.000007
x1*x6	40416	1	40416.4	11.4471	0.002675
x2*x3	39326	1	39326.3	11.1383	0.002985
x2*x4	32939	1	32938.7	9.3292	0.005812
x2*x5	326	1	326.2	0.0924	0.764004
x2*x6	29281	1	29281.1	8.2933	0.008699
x3*x4	85836	1	85835.6	24.3111	0.000062
x3*x5	719	1	718.6	0.2035	0.656310
x3*x6	31035	1	31035.1	8.7900	0.007154
x4*x5	87701	1	87701.3	24.8396	0.000055
x4*x6	182699	1	182699.5	51.7458	0.000000
x5*x6	287714	1	287713.6	81.4888	0.000000
Error	77676	22	3530.7		
Total SS	2520340	49			

It is observed that except linear terms of inoculum concentration and temperature all variables were significant at both linear and quadratic terms. Whereas the interaction terms of pH with inoculum concentration and casein concentration were insignificant, similarly the interactions of temperature with inoculum concentration and starch were also insignificant remaining all other interactions were significant (Table 3). The model F-value of 25.62, and values of probability > F (<0.05) indicated that the model terms were significant.

Protease production in most of the microbial strains was regulated by several fermentation factors[8-12]. The predicted value of Y protease activity at the above conditions is 2191.355 U/ml. The real values of the 6 test variables were obtained by substituting the respective coded values in equation 1 (Fig: 2 -5).

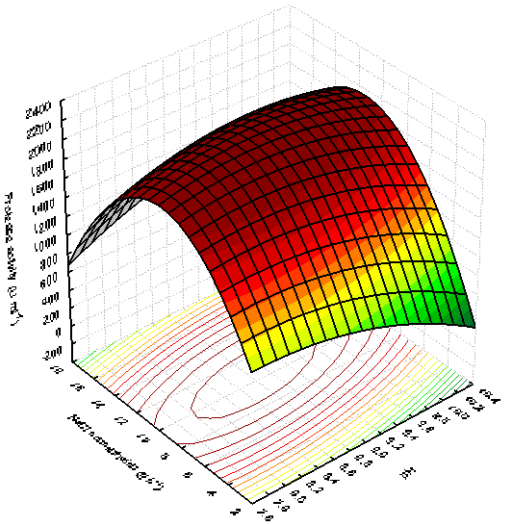

Fig. 2: Interaction influence of pH and NaCl concentration on protease production

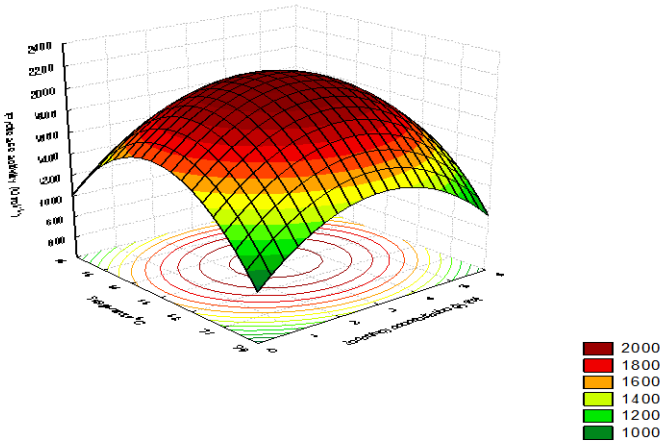

Fig. 3: Interaction influence of pH and NaCl concentration on protease production

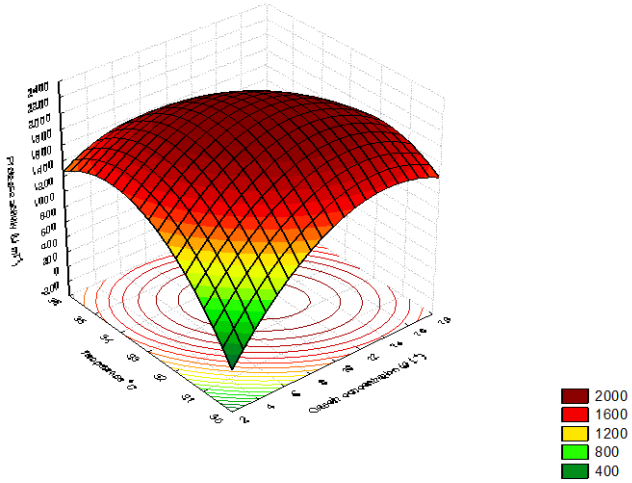

Fig. 4: Interaction influence of inoculum concentration and temperature on protease production

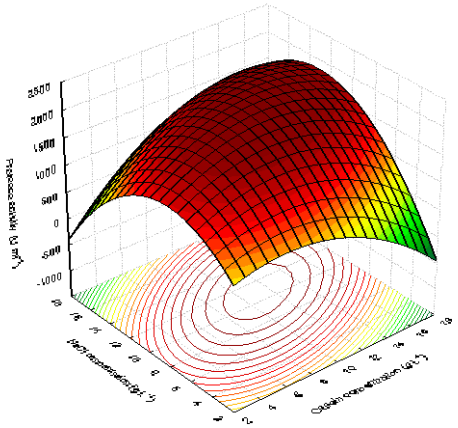

Fig. 5: Interaction influence of concentration of casein and temperature on protease production

CONCLUSION

The maximum predicted protease production (2191.355 U/ml) could be achieved with the medium consisting of starch 2.35 g/L; casein 12.03 g/L; pH-9.1 and NaCl concentration 11.06 g/L in 250 ml flask and 2.9 ml of initial inoculum concentration of 48 hours culture. The validation experimental protease production data revealed 2298.16 U/ml under optimized conditions. The

experimental value of the protease production was almost equal if we consider 95% of the confidence limits for the prediction of Y value at optimized conditions with shake flask results.

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