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Enhanced fibrinolytic protease production by Serratia marcescens RSPB11 through Plackett-Burman and response surface methodological approaches

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ABSTRACT

A well characterized alkaline metalloprotease enzyme called serralysin with fibrinolytic activity has been reported in the newly isolated *Serratia marcescens* RSPB11. In view of its potential application in thrombolytic therapy this study has been made for understanding the nutritional parameters requirement needed for production. Therefore, medium components required for the production of serralysin were optimized using a two step statistical approach. Fermentation variables were selected in accordance with the Plackett-Burman design and were further optimized via response surface methodological approach. A total of seven parameters *viz.*, casein, dextrose, KH_2PO_4, $MgSO_4$, NaCl, $CaCl_2$ and inoculum have been considered for the optimization studies. The statistical model was constructed via central composite design (CCD) using five screened variables (casein, dextrose, KH_2PO_4, $CaCl_2$ and inoculum size). An overall 51.8% increase in protease production was achieved in the optimized medium as compared with the unoptimized casein medium. With the application of statistical design methodology serralysin production increased significantly with optimized casein medium (23910 U/ml) when compared to yeast extract-peptone medium (5363 U/ml).

1. INTRODUCTION

Serralysin is a proteolytic enzyme initially isolated from *Serratia marcescens*, a potentially pathogenic bacterium, found in the gut of the Japanese silkworm. The isolated protein belongs to alkaline metalloprotease and known to activate the Hageman factor-kallikrein-kinin systems of mammals and directly involve in degradation or inhibition of IgG and IgA immune factors as well as regulatory proteins such as 2-macroglobulin, 2-anplasmin and anti-thrombin III [1]. Because of these functionalities, it is administered in dietary supplements for the treatment of assorted inflammatory disorders in Asia and Europe, it gained wide clinical usage as cardiovascular, anti-inflammatory, respiratory, or immune support agent and as an adjunct to antibiotic therapy and to treat other chronic inflammatory diseases, like atherosclerosis, arthritis, bronchitis, carpel tunnel syndrome, fibrocystic breast disease, and sinusitis [2]. Interestingly, this enzyme production is mostly reported from clinical isolates [3, 4] however, reports from marine microbial strain, *Pseudomonas* sp., also noticed in the literature [5, 6]. Much attention has been focused on serralysin production by *Serratia marcescens* due to its potential for higher enzyme yields compared to literature reports [7, 8]. Fibrin and fibrinogen degradation activity of serralysin which acts like plasmin in human body (directly degrades the fibrin unlike plasminogen activator) produced by *Serratia marcescens* RSPB11 has been reported earlier [9]. Chromatographically purified enzyme has been characterized in terms of pH and temperature, where the serralysin produced by this strain was alkaline thermostable metalloprotease with effective application as thrombolytic therapeutic agent. The conventional method of optimization involves variation of one parameter at a time and keeping the others constant. This is a extremely time consuming and expensive method when a large number of variables are considered and also does not often bring up the effect of interaction of various parameters as compared to factorial design [10]. Besides this, it is a tedious, cumbersome, and time-consuming process especially when a large number of parameters are taken into account. An alternative and more efficient approach is the use of statistical method [11].

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The Plackett-Burman factorial designs facilitates the screening of main factors from a large number of process variables, and these designs are thus quite useful in preliminary studies in which the principal objective 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 [12]. A combination of Plackett-Burman and RSM was applied successfully for optimizing process parameters for the production of different biomolecules from different microbes like protease, asparginase from *Bacillus sp*., *Serratia rubidea* [13-15], laccase from *Coriolus versicolor* [16], fibrinolytic protease from gram negative *Bacillus sp*., [17], lipase from *Rhodotorula sp.,* MTCC 8737 [18], tannase from *Aspergillus niger* [19], polysaccharide from *Neisseria meningitides* [20], epothilone-B from *Sorangium cellulosum* [21]. A hybrid system of feed-forward neural network (FFNN) and genetic algorithm (GA) was used to optimize the fermentation conditions to enhance the alkaline protease production by *Bacillus circulans* [22].

Research activities related to serralysin production optimization techniques were not much available where as the mathematical modeling via evolutionary operation (EVOP) based statistical optimization technique was applied to optimize the media composition in shake-flask culture of *Serratia marcescens* NRRL B-23112 [23]. Therefore the present work has been carried out for optimizing the serralysin production from the newly isolated *Serratia marcescens RSPB11,* using a combination of Placket-Burman and response surface methodology.

2. MATERIALS & METHODS

2.1 Organism & Inoculum preparation

Previously isolated *Serratia marcescens* RSPB11 [24] was used in this study. This isolated strain was grown on nutrient agar slants at 30^{o}C and sub-cultured regularly in the same media. For all experiments, inoculum developed by growing the isolate in nutrient broth at 30^{o}C for 18 h was used after adjusting the optical density to 0.8 (OD_{600nm}).

2.2 Serralysin production & enzyme assay

Batch mode shake flask experiments were conducted at 30^{o}C and 150 rpm for 48 h in 250 ml Erlenmeyer flasks containing 50 ml of the medium. The unoptimized production medium consisting of (% w/v) casein - 2.0, dextrose - 1.0, $MgSO_4$ - 0.02, KH_2PO_4 - 0.02, NaCl - 0.02, $CaCl_2$ - 0.002 was prepared and kept as control. While performing optimization studies differing quantities of medium components were made according to the design and all the flasks were inoculated and incubated in a shaking incubator. After the cultivation specified for each set of experiments, the culture broth was centrifuged at 10,000g for 10 min in a bench-top centrifuge, and the total protease activity in the cell-free supernatant was determined. Protease activity was analyzed according to modified [24] method of Anson [25]. A suitable blank was run simultaneously, in which TCA was added to the enzyme solution, followed by substrate addition. One unit (U) of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1µg tyrosine per ml per minute from casein under specified assay conditions.

2.3 Selection of significant variables by Plackett-Burman design

For the selection of significant variables for serralysin production, a carbon source (dextrose), nitrogen source (casein), inorganic salts (potassium phosphate, magnesium sulfate, sodium chloride, calcium chloride), and size of inoculum were tested and identified via the Plackett-Burman design experiment. A total of seven parameters were included for selection, with each variable represented at two levels (lower and higher). The experimental design with the name, symbol code, and actual level of the variables is shown in Tables 1 and 2. The principal effects of each variable on protease activity were estimated as the difference between both averages of measurements made at the higher level and at the lower level. The significance of each variable was determined via Student's t-test.

Table 1: Experimental variables at different levels used for the production of serralysin using Plackett–Burman design.

Variables	Units	Symbol Code	Experimental values	
			Lower	Higher
Dextrose	% (w/v)	X_1	0.2	2
Casein	% (w/v)	X_2	1	5
$MgSO_4$	% (w/v)	X_3	0.02	0.1
KH_2PO_4	% (w/v)	X_4	0.02	0.08
NaCl	% (w/v)	X_5	0.1	0.4
$CaCl_2$	% (w/v)	X_6	0.001	0.003
Inoculum	% (v/v)	X_7	0.5	1.5

Table 2: Chart displaying Plackett-Burman design and serralysin production to the corresponding run.

Run order	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Enzyme activity (U/ml)	
								Observed	Predicted
1	1	-1	1	-1	-1	-1	1	2640	2988
2	1	1	-1	1	-1	-1	-1	7700	6398
3	-1	1	1	-1	1	-1	-1	9790	9020
4	1	-1	1	1	-1	1	-1	6050	5468
5	1	1	-1	1	1	-1	1	7590	8892
6	1	1	1	-1	1	1	-1	5885	6655
7	-1	1	1	1	-1	1	1	17900	19248
8	-1	-1	1	1	1	-1	1	11440	10326
9	-1	-1	-1	1	1	1	-1	8030	8378
10	1	-1	-1	-1	1	1	1	4070	3534
11	-1	1	-1	-1	-1	1	1	16170	14822
12	-1	-1	-1	-1	-1	-1	-1	1870	3405

Table 3: Estimated effects and coefficients for enzyme activity (U/ml).

Term	Effect	Coefficient	T	P
Constant		8261	13.03	0.000
Dextrose	-5211	-2605	-4.04	0.016 [c]
Casein	5156	2578	3.55	0.024 [b]
$MgSO_4$	1379	690	0.29	0.783 [a]
KH_2PO_4	3048	1524	2.72	0.053 [b]
NaCl	-921	-460	-1.54	0.198 [a]
$CaCl_2$	2846	1423	1.15	0.314 [b]
Inoculum	3414	1707	3.50	0.025 [b]

S = 1780.43; R-Sq= 95.29%, R-Sq (adj) = 87.05%

[a] Non-significant at P < 0.05., [b] Significant positive effect., [c] Significant negative effect.

Table 4: Experimental codes, ranges and levels of the independent variables for response surface methodological experiment.

Variables	Units	Symbol Code	Levels				
			-2	-1	0	1	2
Dextrose	% (w/v)	X_1	0.05	0.2	0.35	0.5	0.65
Casein	% (w/v)	X_2	1	2	3	4	5
KH_2PO_4	% (w/v)	X_4	0.04	0.06	0.08	0.1	0.12
$CaCl_2$	% (w/v)	X_6	0.002	0.004	0.006	0.008	0.01
Inoculum	% (v/v)	X_7	0.5	1	1.5	2	2.5

Table 5: CCD matrix with experimental values of protease production from *Serratia marcescens* RSPB11.

Std order	Dextrose (%)	Casein (%)	KH_2PO_4 (%)	$CaCl_2$ (%)	Inoculum (%)	Enzyme activity (U/ml)	
						Observed	Predicted
1	0.2	2	0.06	0.004	2	17250	17155
2	0.5	2	0.06	0.004	1	18070	17710
3	0.2	4	0.06	0.004	1	13860	12591
4	0.5	4	0.06	0.004	2	16300	16626
5	0.2	2	0.1	0.004	1	17270	16806
6	0.5	2	0.1	0.004	2	19800	20931
7	0.2	4	0.1	0.004	2	10560	10782
8	0.5	4	0.1	0.004	1	19800	19757
9	0.2	2	0.06	0.008	1	19360	18401
10	0.5	2	0.06	0.008	2	21230	21866
11	0.2	4	0.06	0.008	2	16390	16177
12	0.5	4	0.06	0.008	1	12570	12032
13	0.2	2	0.1	0.008	2	16830	17361
14	0.5	2	0.1	0.008	1	8030	8296
15	0.2	4	0.1	0.008	1	6600	5958
16	0.5	4	0.1	0.008	2	19800	20753
17	0.05	3	0.08	0.006	1.5	10230	11632
18	0.65	3	0.08	0.006	1.5	18590	17332
19	0.35	1	0.08	0.006	1.5	18950	18536
20	0.35	5	0.08	0.006	1.5	12000	12559
21	0.35	3	0.04	0.006	1.5	22220	23414
22	0.35	3	0.12	0.006	1.5	21500	20541
23	0.35	3	0.08	0.002	1.5	20780	20984
24	0.35	3	0.08	0.01	1.5	18150	18091
25	0.35	3	0.08	0.006	0.5	10000	11932
26	0.35	3	0.08	0.006	2.5	21230	19442
27	0.35	3	0.08	0.006	1.5	24200	23901
28	0.35	3	0.08	0.006	1.5	23650	23901
29	0.35	3	0.08	0.006	1.5	24530	23901
30	0.35	3	0.08	0.006	1.5	23540	23901
31	0.35	3	0.08	0.006	1.5	23760	23901
32	0.35	3	0.08	0.006	1.5	23870	23901

2.4 Optimization by response surface methodology

The next step in the formulation of the medium was to determine the optimum levels of significant variables for serralysin production. For this purpose, the Response Surface Methodology (RSM), using a central composite design (CCD), was adopted for the augmentation of total protease production. The significant variables utilized were as follows: dextrose, casein, potassium phosphate, calcium chloride and inoculum size, each of which was assessed at five coded levels [-2,-1, 0, +1, and +2], as is shown in Table 4. A total of 32 experiments were conducted. All variables were taken at a central coded value, which was considered as zero. The minimum and maximum ranges of the variables were used, and the full experimental plan with regard to their values in actual and coded form is provided in Table 5. The response values (Y) in each trial were the average of the triplicates.

2.5 Statistical analysis and modeling

The data obtained from RSM on serralysin production were subjected analysis of variance (ANOVA). The experimental results of RSM were fitted via the response surface regression procedure, using the following second order polynomial equation:

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j$$

Here Y is the response (serralysin production, U/ml), X_i and X_j are independent variables, β_0 is the intercept; β_i, and β_j are linear coefficients; β_{ii} and β_{jj} are squared coefficients; β_{ij} is interaction coefficients. However, in this study, the independent variables were coded as X_1, X_2, X_4, X_6 and X_7. Thus, the second order polynomial equation can be presented as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_4 X_4 + \beta_6 X_6 + \beta_7 X_7 + \beta_{11} X_1 X_1 + \beta_{12} X_1 X_2 + \beta_{14} X_1 X_4 + \beta_{16} X_1 X_6 + \beta_{17} X_1 X_7 + \beta_{22} X_2 X_2 + \beta_{24} X_2 X_4 + \beta_{26} X_2 X_6 + \beta_{27} X_2 X_7 + \beta_{44} X_4 X_4 + \beta_{46} X_4 X_6 + \beta_{47} X_4 X_7 + \beta_{66} X_6 X_6 + \beta_{67} X_6 X_7 + \beta_{77} X_7 X_7$$

The statistical software package, Minitab 15 was used for the regression analysis of the experimental data, and also to plot the response surface graphs. The statistical significance of the model equation and the model terms was evaluated via the Fisher's test. The quality of fit of the second-order polynomial model equation was expressed via the coefficient of determination (R^2)

and the adjusted R^2. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables utilized in this study. The point optimization method was employed in order to optimize the level of each variable for maximum response. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

3. RESULTS & DISCUSSION

3.1 Screening of significant variables using Plackett–Burman design

For the rapid evaluation of the effects of the various medium components, Plackett–Burman experimental design proved to be a valuable tool. Since this design is a preliminary optimization technique, which tests only two levels of each factor, it cannot provide the optimal quantity of each factor required for the optimum enzyme production. This technique, however, provides indications of how each factor tends to effect bacterial growth and enzyme production [26]. In the present study a total of seven factors were analyzed with regard to their effects on protease production using a Plackett-Burman design (Table 1). The design matrix selected for the screening of significant factors for serralysin production and the corresponding responses are shown in Table 2. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via Student's t-test for ANOVA (Table 3). Factors evidencing P-values of less than 0.05 were considered to have significant effects on the response, and were therefore selected for further optimization studies. Dextrose and casein, with a probability value of 0.007, were determined to be the most significant factors, followed by inoculum (0.029), KH_2PO_4 (0.041), and $CaCl_2$ (0.05). The lower probability values indicate the more significant factors on the production of serralysin. One of the five significant variables screened, dextrose, exerted a negative effect, whereas the other variables, casein, KH_2PO_4 and $CaCl_2$ exerted positive effects on protease production. All other insignificant variables including NaCl and $MgSO_4$ were neglected, and the optimum levels of the five variables, were further determined by an RSM design. Standardized effect of all the seven factors with respect to serralysin enzyme activity also showed the significant factors importance in pareto chart (Figure 1).

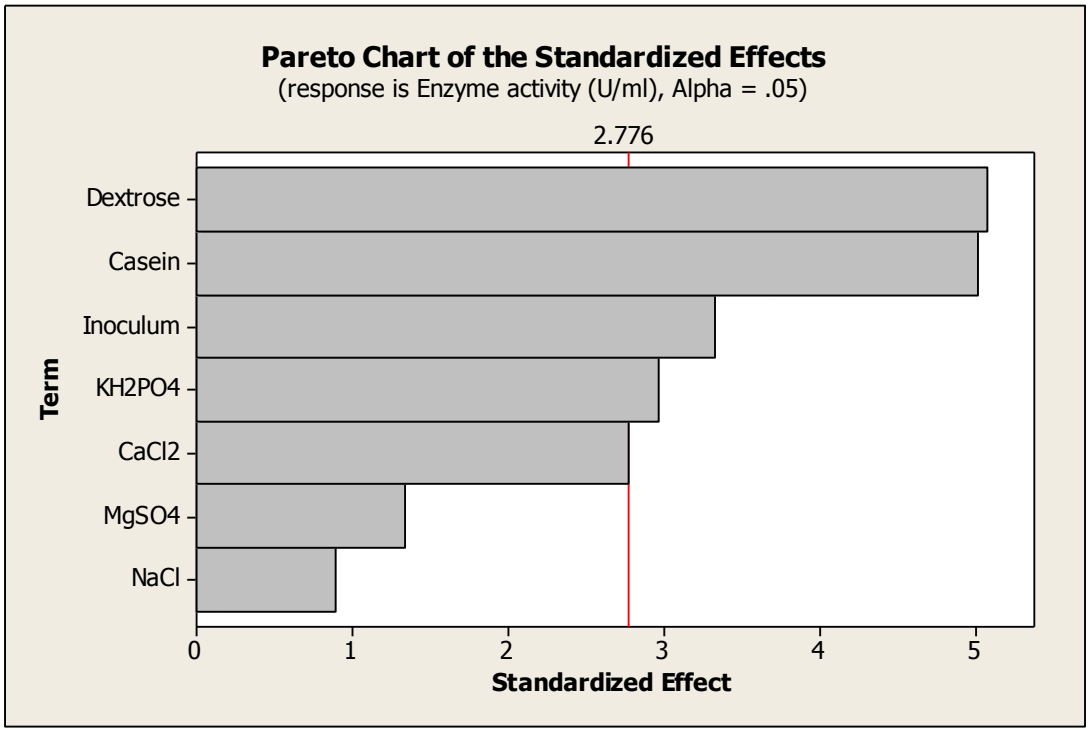


Fig. 1: Pareto chart for Serralysin enzyme activity (U/ml) X1-X7

Table 6: Model coefficient estimated by multiple linear regressions

Term	Coefficient	SE Coefficient	T-stat	P - value
Constant	23900.9	551.0	43.374	0.000
Dextrose *(X_1)*	2850.0	564.0	5.053	0.000
Casein *(X_2)*	-2988.3	564.0	-5.298	0.000
KH_2PO_4 *(X_4)*	-1418.7	564.0	-2.627	0.024
$CaCl_2$ *(X_6)*	-1446.7	564.0	-2.565	0.026
Inoculum *(X_7)*	3775.0	564.0	6.658	0.000
$X_1 * X_1$	-9418.6	1020.3	-9.231	0.000
$X_2 * X_2$	-8353.6	1020.3	-8.187	0.000
$X_4 * X_4$	-1968.6	1020.3	-1.929	0.080
$X_6 * X_6$	-4363.6	1020.3	-4.277	0.001
$X_7 * X_7$	-8213.6	1020.3	-8.050	0.000
$X_1 * X_2$	6160.0	1381.5	4.459	0.001
$X_1 * X_4$	3715.0	1381.5	2.689	0.021
$X_1 * X_6$	-3145.0	1381.5	-2.276	0.044
$X_1 * X_7$	3680.0	1381.5	2.664	0.022
$X_2 * X_4$	2905.0	1381.5	2.103	0.059
$X_2 * X_6$	445.0	1381.5	0.322	0.753
$X_2 * X_7$	-540.0	1381.5	-0.391	0.703
$X_4 * X_6$	-5060.0	1381.5	-3.663	0.004
$X_4 * X_7$	1995.0	1381.5	1.444	0.177
$X_6 * X_7$	8195.0	1381.5	5.932	0.000

S = 1381.53 PRESS = 545157207
R-Sq = 97.31%, R-Sq(adj) = 92.43%

Table 7: Analysis of variance (ANOVA) for quadratic model

Source	Degree of freedom	Seq SS	Adjusted SS	Adjusted MS	F	p
Regression	20	760686741	760686741	38034337	19.93	0.000
Linear	5	212645050	212645050	42529010	22.28	0.000
Square	5	367191141	367191141	73438228	38.48	0.000
Interaction	10	180850550	180850550	18085055	9.48	0.000
Residual Error	11	20994809	20994809	1908619		
Lack of fit	6	20299059	20299059	3383177	24.31	0.002
Pure Error	5	695750	695750	139150		
Total	31	781681550				

3.2 Optimization of significant variables using response surface methodology

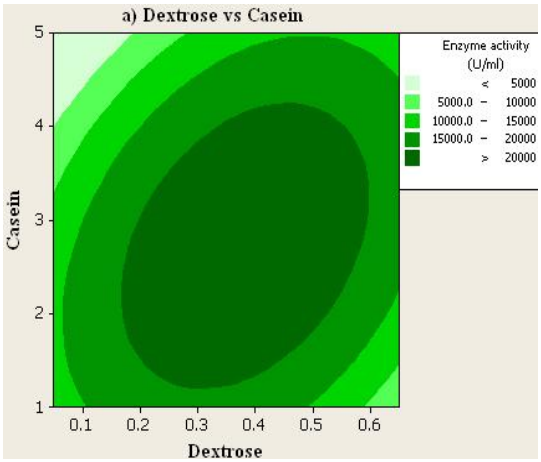
The experiments conducted in the present study were targeted toward the construction of a quadratic model consisting of 32 trials. The design matrix and the corresponding results of RSM experiments to determine the effects of five independent variables are shown in Table 5, along with the mean predicted values. The ANOVA analysis of the optimization study (Table 7) indicated that the most of the model terms, were significant ($P < 0.05$), except few insignificant terms like, $X_4 * X_4$, $X_2 * X_7$, $X_2 * X_6$, $X_2 * X_4$ *and* $X_4 * X_7$. The linear effects of dextrose, casein and inoculum ($P < 0.001$) were determined to be more significant than the effects of the other two variables. These results indicate that the concentration of the carbon and nitrogen source with respect to a particular amount of inoculum size bears a direct relationship to serralysin production. The high F-value and non-significant lack of fit indicate that the model is a good fit. The P-values for the model (<0.0001) and lack of fit (0.002) also suggested that the obtained experimental data was a good fit with the model (Table 6). The regression equation coefficients were calculated and the data was fitted to a second-order polynomial equation. The response, serralysin production (Y) by *Serratia marcescens* RSPB11 can be expressed in terms of the following regression equation:

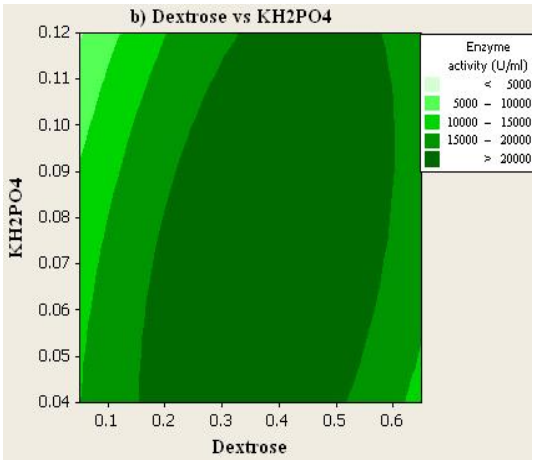
$$Y = 23900.9 + 2850.0\ X_1 - 2988.3\ X_2 - 1418.7\ X_4 - 1446.7\ X_6 + 3775.0\ X_7 - 9418.6\ X_1X_1 + 6160.0\ X_1X_2 + 3715.0\ X_1X_4 - 3145.0\ X_1X_6 + 3680.0\ X_1X_7 - 8353.6\ X_2X_2 - 5060.0\ X_4X_6 - 4363.6\ X_6X_6 + 8195.0\ X_6X_7 - 8213.6\ X_7X_7$$

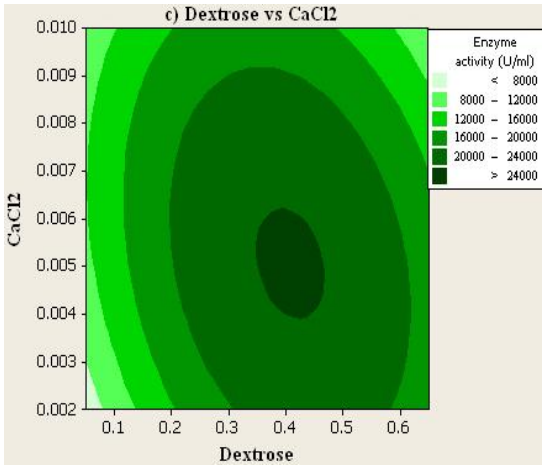
Where Y (serralysin production in U/ml) is the response and the coded values of the test variables X_1 is dextrose, X_2 is casein, X_4 is potassium phosphate, X_6 is calcium chloride and X_7 is inoculum size. The regression equation obtained from the ANOVA showed that the R^2 [multiple correlation coefficient] was 0.9731. Since this value is > 0.75, thus it indicates fitness of the model. This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the model is capable of explaining 97.31% of the variation in response where as the 'adjusted R^2' is 92.43%. This indicates that the model is good (For a good statistical model, the R^2 value should be in the range of 0-1.0), where the closer R^2 is to 1.0, the stronger the model and the better it predicts the response [11].

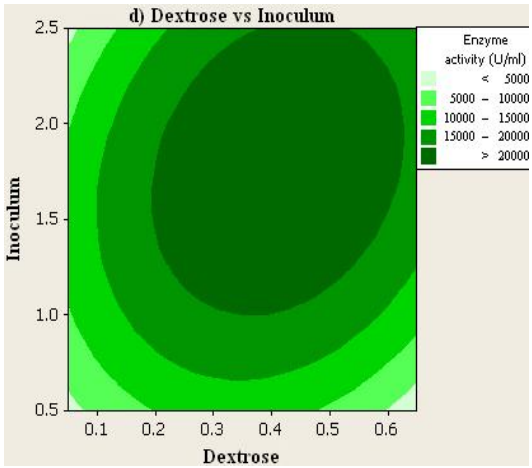
In order to determine the optimal levels of each variable for maximum protease production, three-dimensional response surface plots were constructed by plotting the response (protease production) on the Z-axis against any two independent variables, while maintaining other variables at their optimal levels. Figure 2a-d indicates the interaction of dextrose with other variables viz.,

casein, KH_2PO_4, $CaCl_2$ and inoculum and their effects on serralysin production. The Figure depicts the importance of glucose on maximizing protease production (> 20,000 U/ml) where the concentration of glucose should be in the range of 0.2-0.5% (w/v). Figure 2(a) clarifies that serralysin production is dependent on interaction of dextrose and casein and is also evidenced by their p-value of 0.001 (Table 6) which is highly significant. Similar effect between carbon and nitrogen source has been observed in case of protease production by *Serratia rubidea* [15]. Interaction of casein with other variables like KH_2PO_4, $CaCl_2$ and inoculum can be observed in Figure 2(e, f, g), where > 24,000 U/ml serralysin can be yielded if the casein concentration is around 3% (w/v) in the production media. Serralysin production in this microbial strain is inducible with the presence of casein hence it is an important and significant variable in serralysin production at individual level (p-value of 0.000) however, the presence of this nitrogen source doesn't signifies the interaction with other variables like (Table 6). The flat surface between KH_2PO_4 and dextrose in Figure 2 (b), KH_2PO_4 and casein in Figure 2 (e), KH_2PO_4 and inoculum in Figure 2 (i) indicates that KH_2PO_4 doesn't interact with any other media components except $CaCl_2$ where a significant p-value of 0.004 (Table 6) were observed between these two variables. The influence of physical and nutritional parameter variations on serralysin production using the microbe *Serratia marcescens* RSPB11 was depicted in Table 8. It is evident from the table that a step by step improvement of serralysin production is clearly visible, where the initial yeast extract-peptone-glucose medium composition has given 5,363 U/ml followed by the replacement of casein as nitrogen source and change in dextrose concentration yielded 15,290 U/ml. At this step a 2.85 fold rise in serralysin production exhibits the possible use of the isolated microbe *Serratia marcescens* RSPB11 for achieving higher yields of extracellular protease. Overall variation in nutritional parameters has resulted in 4.46 fold rise of serralysin yield, compared to yeast extract-peptone-glucose medium and a 1.56 fold rise in serralysin yield compared to unoptimized casein medium. This could be observed with the use of Plackett-Burman and response surface methodology, respectively.









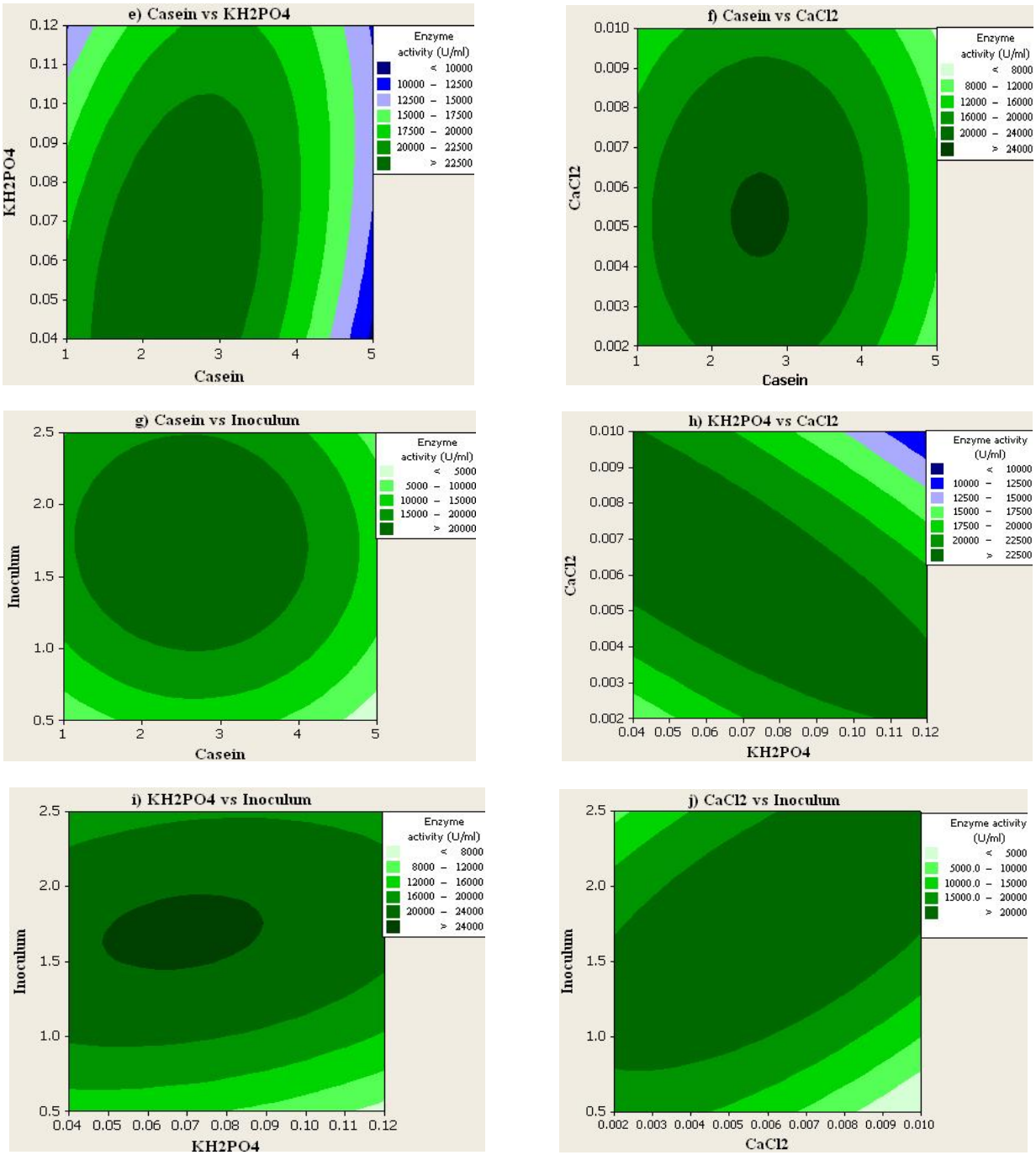


Fig. 2: Response surface plots : interactive effects of (A) dextrose and casein, (B) dextrose and KH_2PO_4, (C) dextrose and $CaCl_2$, (D) dextrose and inoculum, (E) casein and KH_2PO_4, (F) casein and $CaCl_2$, (G) casein and inoculum, (H) KH_2PO_4 and $CaCl_2$, (I) KH_2PO_4 and inoculum, (J) $CaCl_2$ and inoculum on serralysin production.

3.3 Validation of the model

The validation of the statistical model and regression equation were conducted by taking 0.35% dextrose, 3% casein, 0.08% KH_2PO_4, 0.005% $CaCl_2$ (w/v) and 1.5 % (v/v) inoculum size. Under these optimized conditions, the predicted response for protease production was 24001.5 U/ml, with composite desirability = 0.9993 and the observed experimental value was 23910 U/ml, which is an average of three replicates. These results confirmed the validity of the model, and the experimental values were determined to be quite close to the predicted values.

Table 8: An overview of serralysin production improvement under different conditions using the batch culture of *Serratia marcescens* RSPB11.

Method of optimization	Physical parameters	Nutritional parameters % (w/v)	Enzyme activity (U/ml)	Ref
One factor at a time	30°C , 150 rpm, 1% Inoculum (OD600nm~1.5), pH 7.0	Yeast extract-1.0, Peptone-1.0, Dextrose-0.2, $MgSO_4$-0.02, KH_2PO_4-0.02, NaCl-0.02	5,363	24
One factor at a time	-do-	Tryptone-2.0, Dextrose -1.0, $MgSO_4$ -0.02, KH_2PO_4-0.02, NaCl -0.02	9,845	8
Unoptimized conditions	-do-	Casein-2.0, Dextrose-1.0, $MgSO_4$-0.02, KH_2PO_4-0.02, NaCl -0.02,$CaCl_2$ -0.002	15,290	Current study
Plackett-Burman design	30°C ,150 rpm, 1.5 % (v/v) Inoculum (OD600nm~1.5), pH 7.0	Casein-2.0, Dextrose-0.2, $MgSO_4$-0.1, KH_2PO_4-0.08, NaCl-0.1,$CaCl_2$-0.003	17,900	-do-
Response Surface Methodology	-do-	Casein-3.0, Dextrose-0.35, $MgSO_4$-0.1, KH_2PO_4-0.08, NaCl-0.1, $CaCl_2$-0.005	23,910	-do-

Table 9: Serralysin production from different sources at various cultivation conditions.

Organism	Method of optimization	Physical parameters	Nutritional parameters % (w/v)	Enzyme activity (U/ml)	Ref
Serratia marcescens RSPB11	Plackett-Burman & RSM design	30°C , 150 rpm, 1.5 % (v/v) Inoculum, pH 7.0	Casein-3.0, Dextrose-0.35, $MgSO_4$-0.1, KH_2PO_4-0.08, NaCl-0.1, $CaCl_2$-0.005	23,910 at 48h	Current study
Serratia marcescens ATCC 25419	5l bioreactor	30°C, 30% of dissolved oxygen saturation, pH 7.6	2.6l Fresh sweet whey	8,800 at 36h	28
Serratia marcescens SB08	Plackett-Burman & RSM design	30°C, 100 rpm, 1% inoculum, pH 6.0	Peptone-0.5, Beef extract-0.3, Yeast extract-0.3, NaCl-0.5	281.23 at 51h	29
Serratia marcescens NRRL B-23112	EVOP design	25°C, 180rpm, 1% inoculum, pH 6.0	Maltose-4.5, Soybean meal-6.5, K_2HPO_4-0.8, NaCl-0.5	7,333 at 48h	30
Serratia marcescens NRRL B-23112	5l bioreactor	25°C, 400 rpm, 0.075 vvm, pH 7.0	Maltose-4.5, Soybean meal-6.5, K_2HPO_4-0.8, NaCl-0.5	11,580 at 42h	23

4. CONCLUSION

For the economization of the production process, enzyme over production is essential, and can be achieved both by genetic manipulation and media engineering. Using recombinants to increase the production of enzymes may not be able for certain metabolites.

Thus, media manipulation is the better alternative for the overproduction of enzymes, as the secretion of metabolism products is an important component of the survival strategies of some microbes occupying certain environments [27]. Every microorganism evidences its own idiosyncratic physicochemical and nutritional requirements for growth and protease secretion and their production yields differ considerably in microbial strains and mainly influenced by nutritional status of the growth medium or environmental nitch. *Serratia marcescens* RSPB11 with higher serralysin production potential to that of various strains has been compared in Table 9. The protease activity under unoptimized conditions was 15290 U/ml, which signifies that > 50% rise in serralysin production has been achieved with the current statistical optimization.

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