Statistical optimization of alkaline-protease production from *Nesterenkonia* sp. K-15-9-6 isolated from the Gulf of Khambhat, Gujarat, India

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Abstract
Alkaline proteases are by and large recognized as best-in-class industrially important enzymes. This study explored the one variable at a time (OVAT), Plackett–Burman (PB) and Response surface methodology (RSM)-central composite design (CCD) techniques for optimization of the protease biosynthesis. The bacterium was previously isolated from saline soil samples near the Gulf of Khambhat, Gujarat, India and identified as Nesterenkonia sp. K-15-9-6. The independent process parameters (temperature, pH, incubation time, inoculum volume, NaCl concentration, carbon and nitrogen sources) for protease biosynthesis were optimized via OVAT to maximize the yields.

While statistically the protease biosynthesis was optimized using PB and RSM. The maximum protease production (253.45 U/ml) was obtained at temperature 37 °C, pH 10.0, incubation time 48 h, gelatin 2% w/v, NaCl 2.5% w/v, peptone 1% w/v and inoculum volume 3% v/v. Alkaline protease production showed a significant improvement (2.11-fold) in yield, using statistical tools.

Keywords: Alkaline protease, *Nesterenkonia*, Production-Optimization, Plackett-Burman, Response surface methodology.

Introduction

Industrially, microbial alkaline proteases (EC: 3.4.21–24) are the most significant class of enzymes, largely used as protein-degrading enzymes and employed widely as additives in detergents, leather, food, cosmetics, pharmaceuticals, meat tenderization, bakery and brewery sectors[2,25]. Proteases are produced by plants, animals and microbes. Among the microbial proteases, bacterial proteases are considered the most significant hydrolytic enzymes that have been extensively explored due to their rapid growth, low cost and ability to be genetically modified[5].

Specificity, stability and activity under a wide variety of physicochemical variables are a few peculiar properties of bacterial proteases for which large-scale synthesis and use in industries are obvious[27]. Recent reports on the global enzyme market tip off proteolytic enzymes to account for more than 60% of worldwide commercial enzyme manufacturing, with alkaline proteases dominating others with a share of 40%[8]. The isolation of an alkaline protease from the alkaliphilic bacteria *Nesterenkonia* has been reported by several studies[6,19]. *Nesterenkonia* is secreting protease in an alkaline medium using various organic and inorganic elements as carbon and nitrogen sources. The proteases from *Nesterenkonia* are reported to be most active at temperatures over 50 °C and a pH range of 7.0–11.0[6,16,19].

With the rising industrial demands and need for better strategies for bioprocess industries, an innovation in existing methods via novel isolate would render an advantage over traditional ones. Submerged fermentation is widely used for the determination of effects of medium and growth components viz. carbon source, nitrogen source, temperature, pH, inoculum volume, incubation time and NaCl concentration. Few reports on these variables, optimization via one variable at a time (OVAT), Plackett–Burman (PB) and Response surface methodology (RSM) are currently preferred[22,23]. RSM is a tried-and-true progressive analytical method for studying process design and optimization.

Expending an acceptable number of tests provides enough information to examine the interactions and their effects on the process parameters. The above statistical methods are employed as mathematical and analytical tools to assess interrelationships between numerical inputs, variables best affecting the process parameters and their values that best suit the process[18]. The current work emphasized the use of the statistical approaches PB and RSM to optimize process parameters for alkaline protease production from the halotolerant alkaliphilic *Nesterenkonia* sp. K-15-9-6.

Material and Methods

Microorganism: *Nesterenkonia* sp. K-15-9-6, originally isolated from the Gulf of Khambhat, Gujarat, India was previously identified by morphological and molecular methodologies (16S rRNA sequencing) as *Nesterenkonia aethiopica* strain K-15-9-6 (GenBank ON331909). The microorganism used in the present study was maintained on a nutrient agar plate supplemented with 2.5% w/v NaCl, pH 9.0 medium for 2 days at 37 °C. While the genome sequence submission to NCBI is in progress, the isolate being novel has already been deposited with Microbial Type Culture Collection (MTCC), IMTECH Chandigarh, India with accession number (MTCC13247). The genome-wide comparison with reported species of *Nesterenkonia* species suggests that the isolate is novel, hence the proposed name

is being designated. Moreover, as per the standard procedure, it is also under consideration to submit it to other culture collection centers as per directives and guidelines.

Inoculum preparation, protease production and protease assay: A single isolated colony was inoculated into a 100 ml Erlenmeyer flask containing 25 ml nutrient broth medium (pH 9.0) (supplemented with 2.5 % w/v NaCl) and incubated at 37 °C for 24 h under shaking conditions at 150 rpm for inoculum preparation. Growth was monitored till the optical density reached 1.0 values at 600 nm. Further 3.0% v/v inoculum was added into 50 mL of production medium with the following composition (g/L): gelatine, 30.0; peptone, 10.0; NaCl, 25.0 (pH 9.0) and the flask was incubated for 5 days at 37 °C and 150 rpm. After the incubation period, the broth was centrifuged for 10 minutes at 10,000 rpm at 4 °C using a Remi CPR-30 plus centrifuge. The alkaline protease production was determined using the cell-free supernatant. Enzyme activity for protease was assessed by the Anson-Hagihara method using Hammerstein casein as a substrate[10].

Optimization of the medium component - One variable at a time (OVAT): To study the effect of various process parameters on the production of alkaline protease and the range in which they affect the process, various parameters like incubation time (24–120 h), temperature (28–60 °C), pH (8.0–13.0), inoculum size (1.0–5.0% v/v), nitrogen sources (1.0% w/v) (casein, tryptone, yeast extract, urea, peptone, skim milk, gelatin, beef extract and L-asparagine) and carbon sources (1.0% w/v) (glucose, fructose, sucrose, lactose and starch) were studied. OVAT approach was employed for K-15-9-6 protease production, which included changing one specific parameter while the other components maintained constant at the time. Furthermore, observing the impact of various constituents' viz. gelatin, peptone, yeast extract, glucose and lactose their concentrations were also investigated. At the end of the experiment, protease production was quantified as per above method.

Statistical optimization of production medium: Determining key media components that enhance alkaline protease production, is the most significant phase in optimizing process parameters. The significant variable(s) were used in the first phase of PB design followed by CCD (Central composite design)-Response surface methodology (RSM) for maximum alkaline protease production by the isolate K-15-9-6.

Evaluation of significant variables using Plackett–Burman (PB) experimental design: Plackett–Burman (PB) experimental design was used to screen factors that had a significant impact on protease production using Minitab 17 software[12,26]. Six variables (Gelatin (A), peptone (B), NaCl (C), pH (D), inoculum volume (E) and incubation time (F)) were selected in PBD to study their significance based on a two-multi-factorial design that helps to determine if the variables have a substantial impact on enzyme synthesis. Each independent variable was investigated at two levels: low (-1) and high (+1) (Table 1). The PB design had arranged distinct levels of six variables in 23 separate sets (Table 2).

The analysis was done by an Analysis of Variance (ANOVA), main effect plots and a Pareto chart. <95 % of significant variables were selected from other independent variables based on the p-value ($p<0.05$) and the highest confidence level. The first-order polynomial model is used in the experimental design:

$$Y=\beta 0 + \Sigma \beta i Xi$$

where the response (protease production in U/ml) is denoted by Y, the intercept is denoted by β0, the linear coefficient is denoted by βi and the level of the independent variable is denoted by Xi.

The disadvantage of this approach is that it does not account for factor interaction and is only used to assess and screen the most critical elements that impact the response (Table 2). The most important determinants for protease production were identified by regression analysis of the variables which were then further improved using the CCD-RSM.

Central composite design (CCD): CCD-RSM aids in determining the optimal concentration of each of the medium's components, as well as their combined influence on the response. For each run, a protease assay was performed to identify which series has the best combination of the factors to support high yields.

Table 1
Experimental Plackett-Burman Design (PBD) data analysis using Analysis of Variance (ANOVA)

Factors	Units	Coded levels	
		Low	High
A-Gelatin	% (w/v)	1	3
B-Peptone	% (w/v)	0.5	1.5
C-NaCl	% (w/v)	2	3
D-pH	-	8	10
E-Inoculum volume	% (v/v)	2	4
F-Incubation Time	h	24	72

Table 2
Plackett–Burman Design (PBD) matrix for optimizing protease production

Run Order	Gelatin % (w/v)	Peptone % (w/v)	NaCl % (w/v)	pH	Inoculum volume % (v/v)	Incubation Time (h)	Obtained protease (U/ml)	Predicted protease (U/ml)
1	1	0.5	2.0	8	4	24	38.539	39.45
2	1	0.5	2.0	8	2	24	60.371	67.80
3	3	0.5	3.0	10	2	24	100.995	104.33
4	1	1.5	3.0	8	4	72	168.621	167.15
5	3	1.5	2.0	8	4	72	208.795	218.92
6	1	1.5	2.0	10	4	72	149.715	146.56
7	3	0.5	3.0	10	4	72	159.271	151.85
8	3	1.5	3.0	8	2	72	237.079	238.90
9	1	0.5	2.0	10	2	72	128.025	126.50
10	1	1.5	3.0	10	4	24	76.059	72.50
11	1	0.5	3.0	8	4	24	44.991	42.10
12	2	1.0	2.5	9	3	48	241.410	240.15
13	1	0.5	3.0	10	2	72	134.477	126.70
14	3	1.5	2.0	8	2	24	150.519	117.82
15	2	1.0	2.5	9	3	48	241.410	241.30
16	1	1.5	3.0	8	2	24	110.345	120.05
17	3	0.5	2.0	10	4	24	72.711	73.58
18	3	1.5	2.0	10	4	24	116.233	123.85
19	3	0.5	2.0	8	2	72	187.105	195.20
20	3	1.5	3.0	10	2	24	144.517	153.80
21	2	1.0	2.5	9	3	48	241.410	242.78
22	3	0.5	3.0	8	4	72	171.725	170.70
23	1	1.5	2.0	10	2	72	171.547	173.88

Table 3
Individual Variables and their Coded Values for Central Composite

Factors	Units	Coded levels				
		-α	**-1**	**0**	**+1**	**+α**
A-Gelatin	% (w/v)	1	1.5	2	2.5	3
B-Peptone	% (w/v)	0.5	0.75	1	1.25	1.5
C- Incubation Time	h	24	36	48	60	72
D- Inoculum volume	% (v/v)	2	2.5	3	3.5	4

The experimental data was designed and analyzed using the statistical program Design Expert version 13.0.1.0. The CCD method was applied to improve the fermentation conditions by evaluating four key independent variables viz. gelatin (A), peptone (B), incubation time (C) and inoculum volume (D). Each of these components had five values (−α, −1, 0, +1, +α) (Table 3) composed of 30 experimental runs (Table 4).

The data was analyzed using a second-order polynomial regression equation that took into account both the individual and cross effects of each variable.

$$Y=\beta0+\beta1A+\beta2B+\beta3C+\beta4D+\beta11A^2+\beta22B^2+\beta33C^2+\beta44D^2 +\beta12AB+\beta13AC+\beta14AD+\beta23BC +\beta24BD+ \beta34CD$$

where Y represents the response (total enzyme activity (U/ml)), β0 represents the intercept term, βi represents the linear effect, βii represents the square effect, βij represents the interaction effect and βi, βj, βk and βl represent the variables.

Fisher's test was used to determine the statistical significance of the model equation and model components. The coefficient of determination-R^2 and the corrected-R^2 represent the quality of the second-order polynomial model equation. The fitted polynomial equation was shown as a three-dimensional surface plot to demonstrate the relationship between the responses and the experimental levels of each of the variables studied. Next, to validate the model, 100 ml of optimal production medium was prepared in 250 ml Erlenmeyer flask. Following, 3.0% (v/v) of bacterial suspension was added from the freshly prepared inoculum and then incubated at 37 °C for 48 h at 150 rpm. At every 24 h interval, growth was monitored at regular intervals by measuring turbidity at 600 nm. Protease assay

was used to assess the protease production at various time intervals.

Results

Production and Optimization of the medium component - One variable at a time (OVAT): The quantitative and qualitative analysis for the isolates revealed that isolate K-15-9-6 had a much greater proteolytic activity than the others, hence was chosen for future study. Figure 1 describes the protease production by K-15-9-6 in a gelatin broth medium. The OVAT approach was used in the preliminary optimization of process parameters. The results were displayed in figure 2 and figure 3 respectively for physical and nutritional factors that affect protease production. Temperature changes the physical condition of the cell membrane, which can impact both substrate uptake and utilization. Temperature studies revealed that considerable protease synthesis was observed between 25 to 50 °C, with the highest activity at 37 °C (159 U/ml) and the lowest activity at 50 °C (0.59 U/ml).

At 60 °C temperature, isolate was unable to produce protease. The effect of pH values shows that protease was produced in alkaline pH ranges 8.0-13.0, with the highest protease production of 201.55 U/ml reported at pH 10.0. The inoculum volume reflects the number of cells present that have a direct impact on the enzyme's synthesis, cell development and nutrient depletion. The influence of inoculum volume on protease production was revealed as maximum protease production (194.43 U/ml) with 3.0% (v/v) inoculum. As the organism is halophilic, we need to optimize NaCl concentration. K-15-9-6 was able to grow and synthesize protease in the presence of NaCl in the range of 2-10% (w/v) concentration. 2.5% (w/v) NaCl is optimum for growth as well as for protease biosynthesis (193.38 U/ml).

Table 4
Experimental design used in RSM studies by using four independent variables each at five levels showing experiential and predicted values of protease production

Std	A: Gelatin	B: Peptone	C: Incubation Time	D: Inoculum Volume	Predicted Protease	Obtained Protease
	% (w/v)	% (w/v)	h	% (v/v)	U/ml	U/ml
1	1.5	0.75	36	2.5	166.42	167.73
2	1.5	1.25	36	2.5	188.06	189.73
3	1.5	0.75	60	2.5	193.64	202.49
4	1.5	1.25	60	2.5	205.23	208.07
5	1.5	0.75	36	3.5	152.11	159.99
6	1.5	1.25	36	3.5	179.93	175.92
7	1.5	0.75	60	3.5	189.43	184.03
8	1.5	1.25	60	3.5	207.20	213.27
9	2.5	0.75	36	2.5	184.51	184.55
10	2.5	1.25	36	2.5	188.93	203.20
11	2.5	0.75	60	2.5	198.71	211.6
12	2.5	1.25	60	2.5	193.07	191.30
13	2.5	0.75	36	3.5	182.40	188.44
14	2.5	1.25	36	3.5	192.99	190.24
15	2.5	0.75	60	3.5	206.69	225.06
16	2.5	1.25	60	3.5	207.24	214.80
17	2	1	48	3	241.55	241.55
18	2	1	48	3	241.55	241.90
19	1	1	48	3	176.06	175.25
20	1	1	48	3	176.06	176.01
21	3	1	48	3	194.19	191.20
22	3	1	48	3	194.19	191.93
23	2	0.5	48	3	158.34	149.28
24	2	1.5	48	3	180.53	177.56
25	2	1	24	3	185.31	182.05
26	2	1	72	3	226.77	218.00
27	2	1	48	2	198.71	187.53
28	2	1	48	4	198.74	197.53
29	2	1	48	3	241.00	241.55
30	2	1	48	3	241.50	241.55

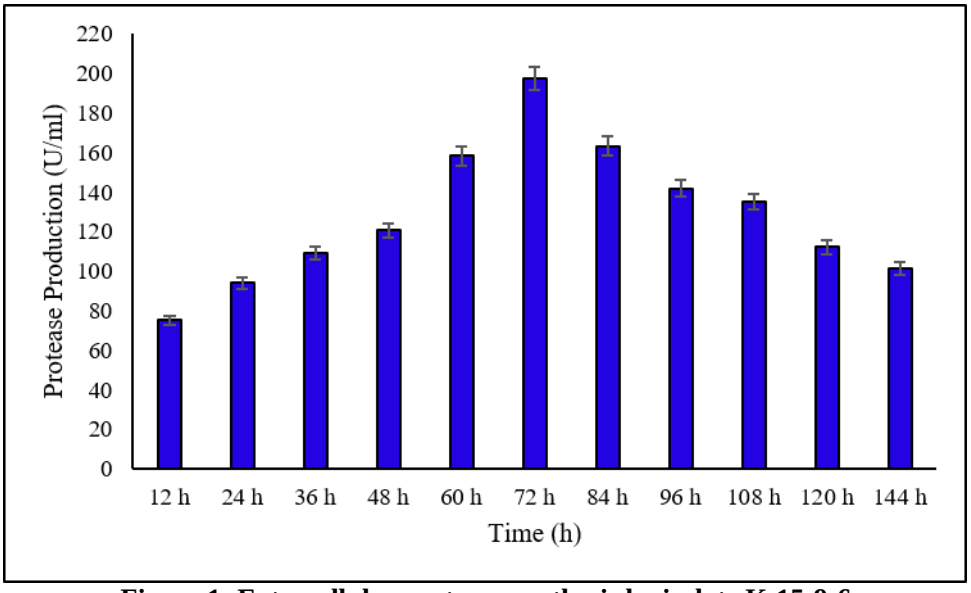

Figure 1: Extracellular protease synthesis by isolate K-15-9-6

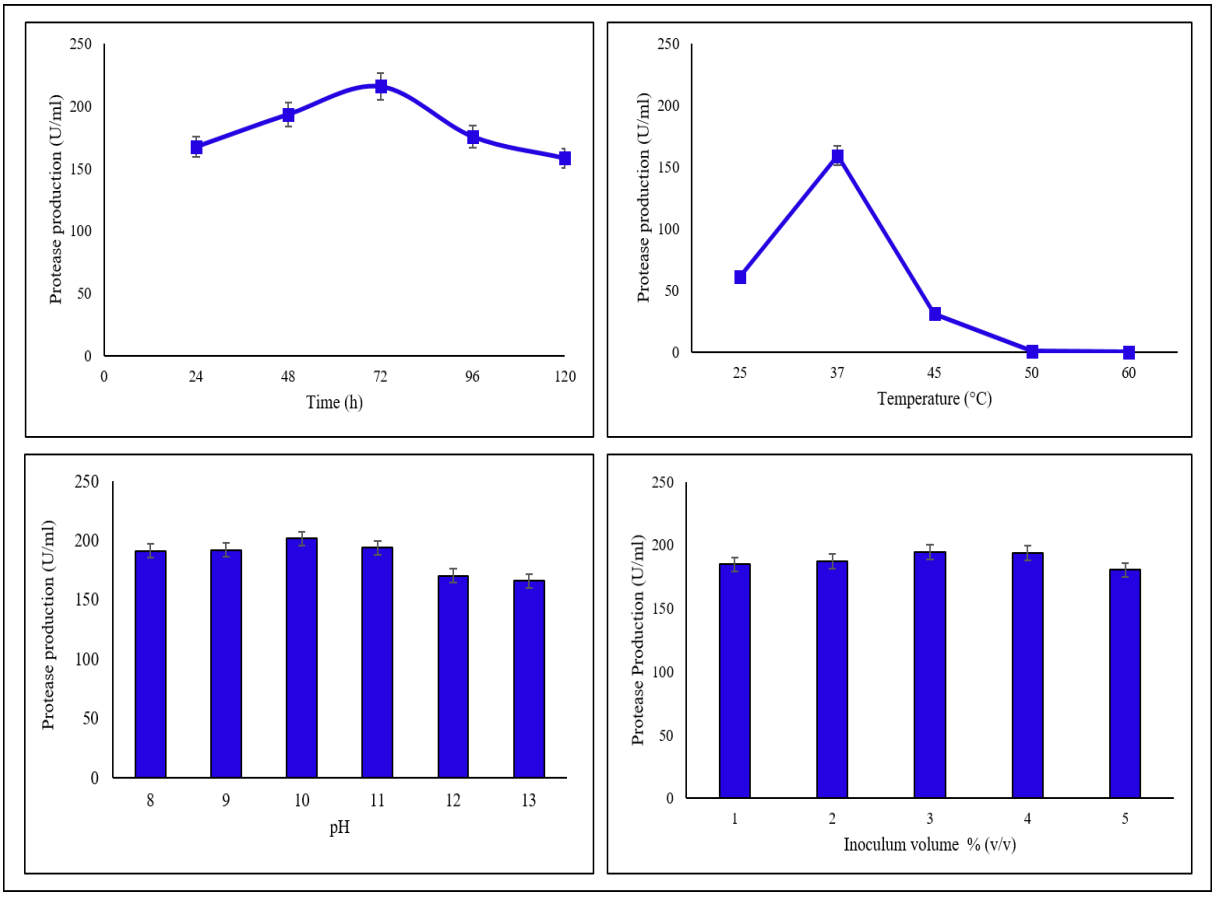

Figure 2: The effects of physical factors on protease production

Carbon and nitrogen sources change the pH and offer precursors for cellular components, therefore they play a significant role in the stability and generation of enzymes. Among the various carbon and nitrogen sources employed in the protease production, 1% (w/v) of peptone and 2% (w/v) of gelatin produced maximum protease 212.42 and 231.78 U/ml respectively. However, yeast extract, glucose and lactose are produced lower protease. After optimizing all variables, the effect of incubation time was assessed. K-15-9-6 releases a maximum of 215.93 U/ml proteases with 72 h incubation time.

Evaluation of significant variables using Plackett–Burman experimental design: The lack of ability of OVAT to leverage the impact of medium constituent interaction becomes a critical factor in attaining maximum yield during scale-up. The PBD and RSM have been identified as the finest developments in the field of media optimization for

overcoming this problem. Based on the developed design matrix, the significant variables necessary for protease synthesis were chosen.

Using the PBD, a total of six variables were analyzed to determine their effects on protease production (Table 2). The t-test for ANOVA was used to identify which factors had the most statistically significant influence. It also exhibits that except NaCl, remaining all factors were most important with a p-value of 0.000. The more significant the effects for protease production are, the lower the likelihood values are. The F-value of 109.49 for the model indicates that it is significant. An F-value of this level has a 2.57% chance due to noise. If the probability > F value is less than 0.05, the model terms are significant.

As seen in the figure 4 Pareto chart, factors A, B, E and F are major model terms that affect this state. Thus, out of the six factors tested, four (gelatin, peptone, incubation time and inoculum volume) were determined to have the most impact on K-15-9-6 protease production. These were then chosen for further RSM optimization. The regression equation for the K-15-9-6 protease was as follows in terms of the coded factors:

Protease production (U/ml) = 34.0 + 23.31 Gelatin + 43.52 Peptone + 6.45 NaCl - 6.23 pH - 10.92 Inoculum volume + 1.6689 Incubation Time + 109.83 Ct Pt

The predicted R^2 value of 98.08% of the model is near to the Adj R^2 value of 97.18%, however, the difference is not more than 0.2 indicating a significant model. Adeq precision evaluates the overall signal-to-noise level. It was better to have a four-to-one ratio. In our experiment, the ratio generated a value of 6.466, suggesting a sufficient signal. Moreover, the design space may be navigated using this concept.

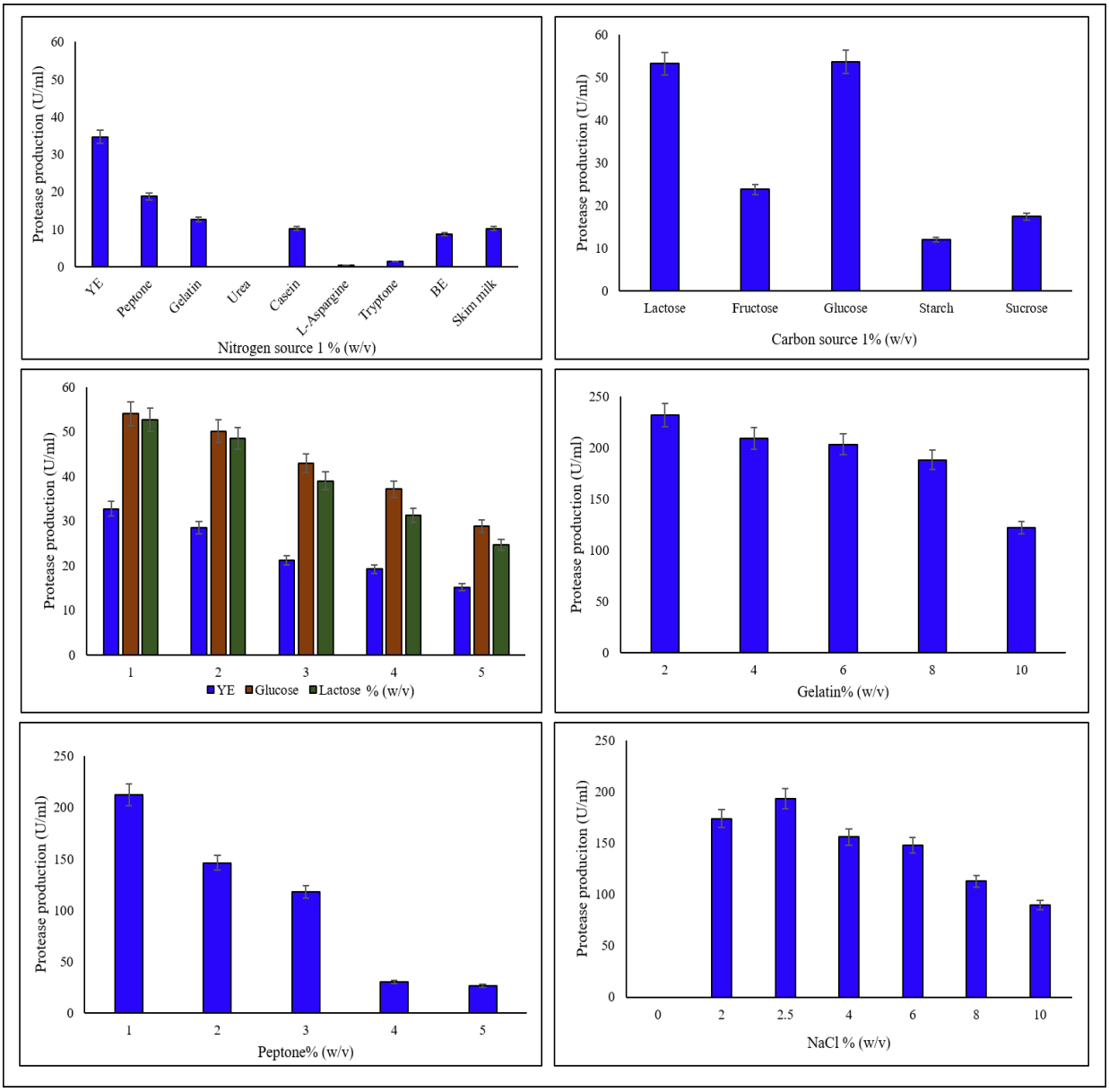

Figure 3: The effects of the nutritional factors on protease production

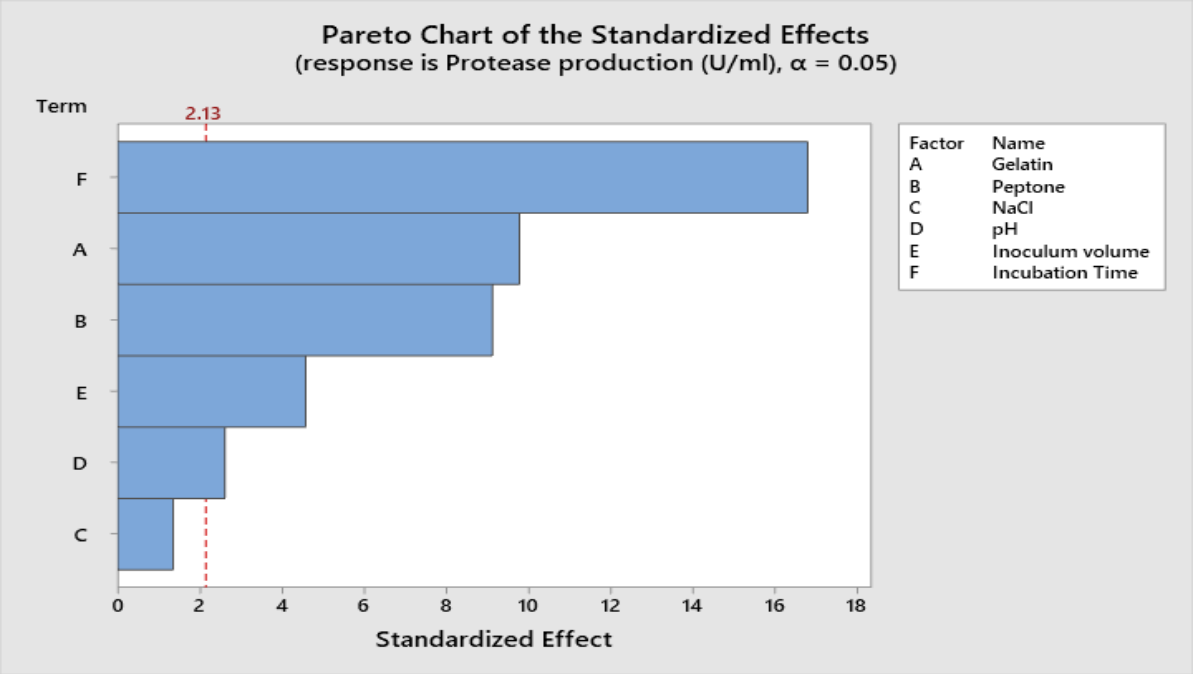

Figure 4: Perturbation curve showing the effect of independent variables on enzyme activity from center points (A) Gelatine, (B) Peptone, (C) Incubation Time, (D) Inoculum Volume.

Table 5
Response Surface Methodology (RSM) -Central Composite Design (CCD)-Analysis of Variance (ANOVA) for protease production

Source	Term DF	Error DF	F-value	p-value	
Whole-plot	2	5.33	39.36	0.0006	significant
A-Gelatine	1	4.70	4.99	0.0793	
A^2	1	6.12	73.82	0.0001	
Subplot	12	10.85	16.36	< 0.0001	significant
B-Peptone	1	12.66	16.31	0.0015	
C-Incubation Time	1	12.66	56.93	< 0.0001	
D-Inoculum Volume	1	12.66	0.0007	0.9796	
AB	1	12.66	6.55	0.0241	
AC	1	12.66	3.75	0.0756	
AD	1	12.66	3.28	0.0939	
BC	1	12.66	2.23	0.1601	
BD	1	12.66	0.8432	0.3757	
CD	1	12.66	2.25	0.1582	
B^2	1	7.92	102.35	< 0.0001	
C^2	1	7.92	24.82	0.0011	
D^2	1	7.92	36.40	0.0003	

*DF; Degree of freedom

Central composite design- Response surface methodology: The four major factors that had the greatest impact on protease production, as identified by PB, were further optimized using RSM-CCD, while the remaining parameters remained constant. The effective factors were examined using Design-Expert 13.0. The experimental design was subjected to an analysis of variance. At the end of the experiment, the model terms B, C, AB, A^2, B^2, C^2 and D^2 are important representatives of optimum model terms. The quadratic model's regression equation for protease production by K-15-9-6 was found to be:

$$\text{Protease Production} = +241.55 + 4.53A + 5.55B + 10.37C - 0.0358D - 4.31AB - 3.26AC + 3.05AD - 2.51BC + 1.55BD + 2.52CD - 14.11A^2 - 18.03B^2 - 8.88C^2 - 10.75D^2$$

The regression coefficients and ANOVA suggested it to be a statistically significant model (Table 5). The R^2 value should be near 1.0 and all of the components should be

positive and close to each other for a successful statistical model. The strong R^2 value (0.988) indicated that the experimental results and the theoretical values predicted by the model were in good agreement[14]. The adjusted determination coefficient (adjusted R^2=0.89) and anticipated determination coefficient (predicted R^2=0.95) were both high, suggesting that this model is very significant[3]. Furthermore, the model has a precision value of 63.55, indicating that it may be utilized to explore the design space.

The accuracy value is a measure of the signal-to-noise ratio and a value of >4 is required for a model to fit well. The F-value of 39.36 for the model suggests that it is significant. An F-value of this magnitude has a 0.01 % probability of occurring due to the noise ratio. The finding that the coefficient of variation was reduced (CV=4.14) indicated that the trials were accurate and trustworthy[13]. If model has a lot of inconsequential terms, model reduction may help to improve it. Probability values greater than or equal to 0.05 suggest that the model terms are significant. A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2 and D^2 are important model terms in this situation. If the values are more than 0.1000, then the model terms are not important.

The three-dimensional (3-D) response surfaces shown in figure 5 are graphical representations of the regression equation, which can be used to estimate protease production for various concentrations of the variable. The responses were plotted on the Z-axis against two variables, with the remaining variables kept at zero. The model projected that at (g/L): gelatin, 30; peptone, 10; NaCl, 25 and pH 9.0, the maximal protease production of 241.90 U/ml will indeed manifest. The graph shows how enzyme activity changes as a function of (A) gelatin, (B) peptone, (C) the incubation time and (D) inoculum volume.

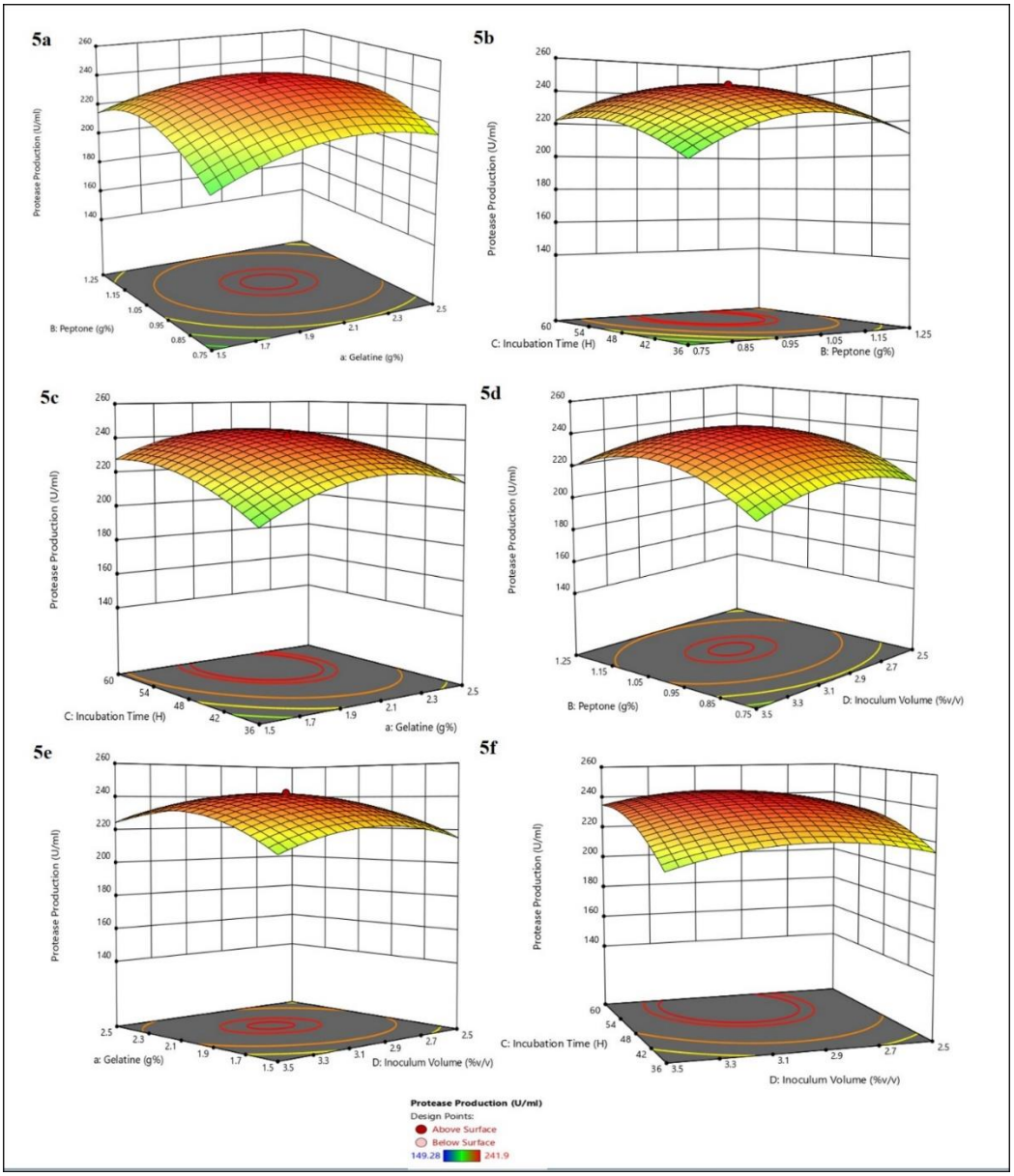

Figure 5: Response Surface 3-D plots showing interactive effects of different independent variables on protease activity. (A) Gelatin, (B) Peptone, (C) Incubation Time and (D) Inoculum Volume.

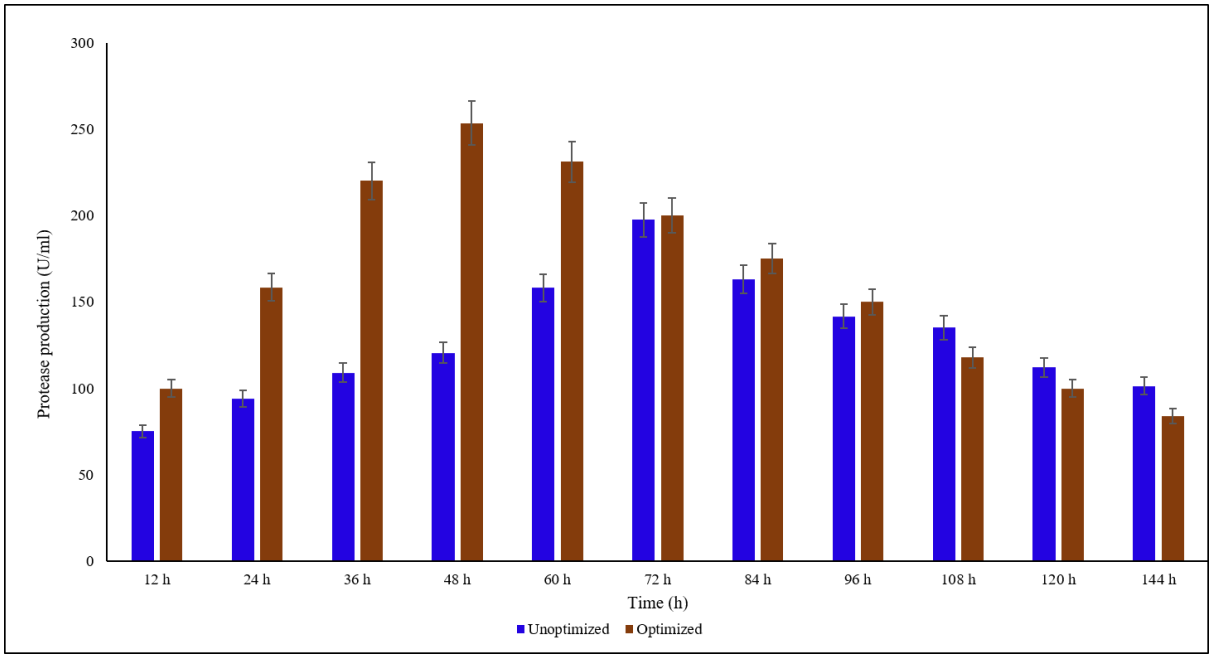

Figure 6: Comparative assessment of protease production by K-15-9-6 under optimized and unoptimized media conditions

Figure 5 depicts the variance in enzyme production when the value of each component varies independently of the other parameters (kept constant). The significant impact of gelatin on protease activity is shown in figure 5A. As the concentration of gelatin increased, the activity of proteases also increased. When the incubation time and peptone concentrations increased, a parabolic increase in protease production was observed (Figure 5B). Figures 5C, 5D and 5E show comparable profiles for incubation time and gelatin, gelatin and inoculum volume, as well as peptone and inoculum volume respectively. Protease production, on the other hand, increased with incubation time and inoculum volume whereas lower values of both factors reduced biosynthesis (Figure 5F).

Triplicate independent trials were used to verify the appropriateness of the projected model. The experimentally measured production values were nearly equal to those anticipated statistically, supporting the model's validity. The multiple correlation coefficients (R^2) and the coefficient of variation (CV) are 0.98 and 1.88 respectively. These indicate that the experimental and projected values have a good correlation and that the experiments performed are very dependable. Furthermore, the average error (difference between actual and anticipated value) is nearly zero, demonstrating that the model's predictions are not biased.

Figure 6 depicts the time of protease production of K-15-9-6 in optimized and unoptimized conditions. Results indicate that during the exponential growth phase, protease activity increased up to 253.45 U/ml after 48 h. Protease production increased modestly at the beginning of the stationary phase. However, after 48 h under unoptimized conditions, the protease activity was only 120 U/ml. *Nesterenkonia* sp. K-15-9-6 produced 2.11 times higher alkaline proteases when the medium composition and culture conditions were optimized.

Discussion

This study focused on the utilization of potent protease-producing bacteria which were previously isolated from the Gulf of Khambhat and identified as *Nesterenkonia* sp. K-15-9-6 (MTCC13247). In the current study, it was highlighted that the statistical tools PB and RSM were used to optimize the process parameters for the synthesis of alkaline protease from the halotolerant alkaliphilic *Nesterenkonia* sp. K-15-9-6. Several studies are looking into ways to boost microbial protease production. For the development of alkaline proteases from various bacteria, a defined or complex medium has been used. Furthermore, each strain has its own set of requirements for maximum enzyme production[22].

Environmental factors that influence the synthesis of extracellular proteolytic enzymes may have a role in the induction or suppression of the enzyme by certain substances. pH, temperature, incubation time, aeration-agitation rate and inoculum volume all had an impact on the protease synthesis[23]. Temperature alters a microbial metabolism and fermentation process which regulates both growth and protease production. The majority of marine bacterial proteases are temperature stable (25-70 °C), but they have lower protease production activity at extremely low and high temperatures. Furthermore, it is reliant on the bacterial isolation source.

Several studies have shown results that are similar to the current study on the optimal pH for protease synthesis[11]. In addition, many studies have found that the highest pH range for protease production in marine bacteria is between 8.0 to 10.0[29]. As a result of the studies, it was determined that

production temperature, pH and isolation source all affect protease synthesis. For isolate K-15-9-6, the optimal temperature and pH were 37 °C and pH 10.0 respectively for protease production. Similar results were also observed for two marine bacteria *Oceanobacillus oncorhynchi* and *Oceanobacillus khimchii* isolated from the seawater of Gujarat for protease production[1].

Consequently, the availability of carbon and nitrogen sources in the medium influences protease production and both of these variables have an impact on enzyme synthesis[4]. Many scientists have attempted combining lactose, glucose, or starch with nitrogen sources like yeast extract, gelatine, peptone, or casamino acids to enhance protease production[20]. Out of all the organic C-N compounds examined, peptone and gelatin produced the highest protease. This conclusion is consistent with prior protease synthesis studies which indicated that gelatin and peptone are better protease substrates than simple carbohydrates like glucose which can promote catabolite repression[28].

Statistical models have lately been employed to enhance production medium ingredients and conditions due to their ease of use and accuracy. The Plackett–Burman design was utilized to find the critical factors that increased protease production in this study. When compared to the Plackett–Burman design trials, a wide range of protease production (39-242.78 U/ml) indicated that further work was needed. In this work, the RSM was employed to optimize protease synthesis and it demonstrated the importance of a variety of factors at different phases. Using the CCD design technique, we were able to study culture conditions that resulted in a 2.11-fold increase in protease production which we also investigated in this work.

The predicted and experimental readings were observed to be very close, indicating the model's precision and utility in determining the best conditions for enzyme synthesis. Wahab and Ahmed[27] discovered for *Aspergillus niger* WA 2017 the alkaline protease synthesis increased up to 3.6-fold[17].

Adetunji and Olaniran[1] discovered that using RSM, *Bacillus aryabhattai* Ab15-ES could increase alkaline protease synthesis by up to 4.4-fold[9]. Similarly, Kumar and his co-workers found that 2.0-fold protease production was enhanced for *Bacillus toyonensis* VKB5[21]. Our experiments also showed an increase in protease production higher than those earlier reported[7,15,18,24].

In conclusion, the marine halo-alkalophilic bacteria *Nesterenkonia* sp. K-15-9-6 might potentially be used in a range of industrial applications. Also, the validation analysis findings confirmed the expected values, indicating that OVAT→PB→RSM may be used to optimize the enzyme manufacturing process, demonstrating the model's usefulness. For industries, the nature, efficacy, application, specificity and stability of enzymes are important. Hence, we are currently purifying and characterizing the protease obtained from this isolate in this context. In the future, we will look into this enzyme for a variety of industrial applications.

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