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RESEARCH ARTICLE

Optimization and modeling studies on the production of a new fibrinolytic protease using *Streptomyces radiopugnans*_VITSD8

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BACKGROUND: The current study demonstrated the possibility of statistical design tools combination with computational tools for optimization of fermentation conditions for enhanced fibrinolytic protease production.
METHODS: The effects of using different carbon and nitrogen sources for protease production by *Streptomyces radiopugnans*_VITSD8 were examined by a full factorial design method. The incubation time, temperature, pH of the medium, and RPM were assessed by the predictable one factor at a time (OFAT) method. Optimization was carried out using starch and oat meal as carbon source, nitrogen source as peptic and malt extract using Fractional Factorial Design (FFD). The analysis was further continued for medium volume, temperature, initial medium pH, inoculum concentration, high determination co-efficient as (R'-0.965), and lower determination co-efficient of variation (CV-8.19%), which defines a reliable and accurate experimental value.
RESULTS: Analysis of variance by the fixed slope effect by temperature and starch; temperature and L-aspargine, temperature and oat meal, temperature and peptic extracts, temperature and pH, temperature and duration of incubation were more vital for protease production at an interactive level. Response surface plots revealed that temperature, starch, and peptic extracts affix critical concerning in temperature. Programming estimated a 28% increase in protease production. Incubation temperature and medium volume portrayed extreme impact among all factor. Starch, peptic and temperature play an important regulatory role in protease production. Optimium temperature for protease production was 33°C. The ratio of carbon and nitrogen sources and pH were the major regulatory factors in protease production by *Streptomyces radiopugnans*_VITSD8. It demonstrated a 4% noteworthy change in condition.
CONCLUSION: Among all the selected parameters, temperature was the most intuitive factor, demonstrating a notable connection with the type of media and pH, while inoculum fixation had a direct impact on protein production.

Keywords Protease, *Streptomyces radiopugnans*_VITSD8, Full Factorial design (FFD), fermentation, optimization, RSM

Introduction

Proteases, especially alkaline proteases, constitute 60%–65% of the global industrial enzyme market. In fact, it is reported that the global proteolytic enzyme demand will increase dramatically to 1.0–1.2 billon dollars, because of their application potential in several industrial sectors, especially in the food industry for meat tenderization, peptide synthesis, infant formula preparations, baking, and brewing, for use as pharmaceuticals and for medical diagnosis, in the textile industry, and in the detergent industry, where they are used as additives. Marine actinomycetes gained importance due to their potential for producing these extracellular enzymes under submerged fermentation conditions (Mu et al., 2009). It is well documented that each microbial strain differs in its enzyme production efficiency, which mainly depends on its fermentation ability and on its nutritional, physiologic and genetic characteristics (Liu and Wang, 2007). The composition of the fermentation media plays an important role in the production of primary and secondary metabolites. Moreover, the production characteristics would offer a competitive advantage over existing products. Discovering new species and producing proteases with novel characteristics will be of great value to the enzyme industry; these new species may be used in different applications (Li et al., 2008; Lotfy, 2007). Hence, designing an appropriate fermentation medium is of critical importance as the medium composition, product

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concentration, yield, and volumetric productivity influence the rate of enzyme production (Abdeltif, 2000; Krishnan et al., 1998). Achieving the basic industrial objective of producing enzymes using a new strain will be possible when the isolated strain is characterized for its growth and enzyme production potential (Knight et al., 2003; Jonsbu et al., 2002). This can be achieved by optimizing the different parameters. The current study demonstrated the possibility of combining statistical design tools with computational tools for the optimization of fermentation conditions for enhanced fibrinolytic protease production.

Materials and methods

Media optimization by statistical analysis

Selection of process parameters by fractional factorial design

The primary screening of the media parameters was done to analyze the utility of modified fibrinolytic protease production medium. Several parameters that influence the fibrinolytic protease production, such as nutritional components (starch, L-asparagine, peptic extracts, oat-meal, and malt Extract) and physiologic factors (temperature, pH, and duration) were selected (Table 1). Optimization of the experimental design and analysis of data was achieved using Minitab 17.0 (Minitab Inc., PA, and USA). In fractional factorial design, all the factors were evaluated at low (−1) and high levels (+ 1), with a center point to determine the linear and curvature effects of the variables (Table 2). The fibrinolytic protease was produced performed by inoculating 25 mL of the optimized production medium with 1% (v/v) of *Streptomyces radiopugnans*_VITSD8, and incubating at 33°C for 72 h at 150 rpm.

Table 1 Analysis of variation

Source of variation	Df
Replicates	r-1
Whole- plot analysis	2^{p1}-1
WP effect WP error	(r-1)(2^{p}-1)
Subplot analysis	2^{p}-2^{p1}
WP*SP interaction effects SP error	(r-1)(2^{p}-2^{p1})
Total	ra^{p}-1

The effect of each variable on the production of SK was calculated by regression equation Eq. (1): $Y = X\beta + \varepsilon^{wp} + \varepsilon^{sp}$.

Regression parameters for the complete factorial effects were represented by Xβ, and the whole-plot and sub-plot error vectors are ε^{wp} and ε^{sp}, respectively. Independent and normally distributed random variables shared mutual error terms. Additionally, the investigation of a split-plot design fluctuates from the completely randomized design. The complete analysis of variance for a 2p1 + p2 factorial split-plot design with r replicates was performed.

Analogous of block designs, the set of investigational units, are distributed into subsets (sub-plots). There was an inaccessible source data, which depicts the significance of the factors used for segregating the investigational units. Factorial effects have to be calculated for the entire source. If the FFD replicates $r > 1$ times, then the normal ANOVA-based hypothesis tests can be executed. If the strategy was replicated, two distinct half-normal plots are mandatory to evaluate the implication of the 2^{pl_1} factorial effect (Table 1).

Selection of independent variables by response fractional factorial design

Fractional factorial design is an empirical combination of statistical and mathematical techniques. It is a powerful tool for optimizing the process parameters, interactive modeling, and improving the yield (Shabbiri et al., 2012). The effect of three independent variables viz. carbon, nitrogen, and potassium dihydrogen on fibrinolytic protease production was studied at 5 different levels (-α,-1, 0, + 1 and + α), where $\alpha = 2^{n/4}$; here, n denotes the number of variables used for the study (Table 2). A fractional factorial design was performed to build a total of 16 experimental runs with 8 cube points, 8 star points, and 5 center points. The optimization of the experimental design and statistical analysis of the runs were performed by MINITAB version 17.0, USA.

The test factors on fibrinolytic protease production were evaluated using regression equation Eq. (3): $Y = X\beta + \varepsilon^{r} + \varepsilon^{c} + \varepsilon$.

Factorial effects of regression parameter consist of Xβ as the mean term, the error vectors related with the rows and

Table 2 Fractional factorial design- range and coded values of the variables for fibrinolytic protease production

Factors	Independent variable	Coded values	
		(+ 1)	(−1)
A	Temperature (°C)	30	40
B	pH	7	7.5
C	Duration (h)	72	96
D	Starch (g/100mL)	1.0	1.2
E	L-asparagine (g/100mL)	0.1	0.2
F	Peptic (g/100mL)	0.8	1.2
G	Oat meal (g/100mL)	1.2	1.5
H	Malt extract (g/100mL)	0.2	0.4

columns are ε^r and ε^c, respectively, and the replication error conjuncts with the experimental units are ε.

The second order polynomial general equation was used to calculate the correlation between independent factors (Table 3) and response fibrinolytic protease activity. The linear, square, and linear interaction terms were developed as quadratic regression models and illustrated as Eq. (4):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ii} x_i x_j \qquad (4)$$

where, Y represents the predicted response value, β_0 represents the intercepts of β_i, β_{ii} and β_{ij}, which are straight, quadratic, and interaction co-efficients, respectively, and x_i and x_j are the coded values of independent variables.

Validation of the experimental model

The results obtained on using the model were validated using the statistical software MINITAB ver. 17.1.0, (USA). The optimum process parameter for the production of fibrinolytic protease production was predicted and analyzed (Minitab 17.1.0 Statistical software 2013). All the experiments were executed thrice within the respective level ranges. The actual responses and predicted and coefficient values for each solution were calculated by the correlation and regression co-efficients.

Results

Totally, eight factors were analyzed by fractional factorial design for their effect on fibrinolytic protease production. The design matrix with significant variables and the corresponding fibrinolytic protease activity was represented in Table 1. The fibrinolytic protease activity varied from 178.496±0.989 to 620.196±0.989 U/mL. The significant factors from the fibrinolytic protease production medium such as temperature, pH, incubation time, starch, L-asparagine, peptic, oat-meal, and malt extract, were selected for the optimization studies. The design matrix was developed by the fractional factorial design and the results are shown in Table 4.

The Model F-value was significant; the fibrinolytic protease production was significantly affected by temperature (< 0.05), starch (< 0.005), L-asparagine (< 0.05), Peptic (< 0.05), Oat meal (< 0.05), and malt extract (< 0.05). The "R-squared" value is 99.99%, and the "predicted R-squared" of 96.87% was similar to the "adjusted R-squared" of 99.82%. The physiologic factors, peptic and incubation

Table 3 Central composite experimental design- independent variables and their levels

Factors	Independent variable	Coded values				
		−α	(−1)	0	(+1)	A
D	Starch (g/100mL)	0.5	1.0	1.5	2.0	2.5
E	L-asparagine (g/100mL)	1.5	0.1	0.15	0.2	2.0
F	Peptic (g/100mL)	0.5	1.0	1.5	2.0	2.5
G	Oat meal (g/100mL)	1.5	0.1	0.15	0.2	2.0
H	Malt extract (g/100mL)	0.5	1.0	1.5	2.0	2.5

Table 4 Design matrix of fractional factorial design

Std order	Run order	Centre pt	Block	Temperature	pH	Duration	Starch	L–aspargine	Peptic	Oat meal	Malt extract	Activity	RESI1	RESI2
15	4	1	1	30	7.5	96	0.3	0.05	0.2	0.3	0.05	269.862	0.87452	1.39338
11	15	1	1	30	7.5	72	0.3	0.025	0.3	0.375	0.05	216.597	−1.14879	1.39337
5	11	1	1	30	7	96	0.25	0.05	0.3	0.375	0.05	178.496	−0.21807	1.39338
13	9	1	1	30	7	96	0.3	0.025	0.2	0.375	0.1	257.789	−0.87452	−1.39338
14	12	1	1	40	7	96	0.3	0.025	0.3	0.3	0.05	538.462	0.02698	−1.39337
10	3	1	1	40	7	72	0.3	0.05	0.2	0.375	0.05	329.286	−1.81647	−1.39337
8	7	1	1	40	7.5	96	0.25	0.025	0.2	0.375	0.05	260.517	−0.53955	−1.39337
2	1	1	1	40	7	72	0.25	0.025	0.3	0.375	0.1	397.218	1.24995	1.39337
6	5	1	1	40	7	96	0.25	0.05	0.2	0.3	0.1	343.716	0.53955	1.39338
3	8	1	1	30	7.5	72	0.25	0.05	0.2	0.375	0.1	186.419	0.0562	−1.39337
12	10	1	1	40	7.5	72	0.3	0.025	0.2	0.3	0.1	620.196	1.81647	1.393937
4	2	1	1	40	7.5	72	0.25	0.05	0.3	0.3	0.05	306.28	−1.24995	−1.39337
9	16	1	1	30	7	72	0.3	0.05	0.3	0.3	0.1	447.791	1.14879	−1.39337
16	14	1	1	40	7.5	96	0.3	0.05	0.3	0.375	0.1	483.056	−0.02698	1.39338
1	13	1	1	30	7	72	0.25	0.025	0.2	0.3	0.05	217.987	−0.0562	1.39338
7	6	1	1	30	7.5	96	0.25	0.025	0.3	0.3	0.1	378.29	0.21807	−1.39338

time, expressed a negative effect on the fibrinolytic protease activity. This indicates that starch and peptone had the most notable effect on the enhanced production of fibrinolytic protease from *Streptomyces radiopugnans*_VITSD8.

Based on the experiment design, 1.2 g/100 mL of starch, 1.2 g/100 mL of peptic, 1.2 g/100 mL of oat meal, 0.4 g/100 mL of malt extract, 0.1 g/100 mL L-asparagine, and 40°C were found to be the optimum parameters. pH and incubation time remained the same. The analysis of variance (ANOVA) showed which factors had the maximum effect on fibrinolytic protease production (Table 5).

The decrease of 'Model F- value' of 0.02% error was due to noise. Values of "Prob > F" less than 0.05 represents that the factors in the model were found to be significant. The adequate precision of this model allowed for the measurement of the signal-to-noise ratio. Thus, this model was used to navigate the design matrix. The model equation for fibrinolytic protease activity was illustrated as a final equation, in terms of coded factor.

Regression equation in uncoded units: Activity = 2618- 76.55 temperature – 193.8 pH + 1.503 duration- 5332 starch + 11098 L-aspargine + 1606 peptic – 2943 oat meal + 1992.5 malt extract + 5.61 temperature*pH – 0.0447 temperature*duration + 216.2 temperature* starch + 45.35 temperature*oat meal.

The normal plot implies that starch, peptic, oat meal, malt extract, and temperature are the most significant factors responsible for maximum fibrinolytic activity of 620.196± 1.23 U/mL (Fig. 1). The Pareto chart graphically summarizes and shows the importance of the standardized effects between the media components for fibrinolytic protease production (Fig. 2). The results of fractional factorial design showed the effect of significant factors on the production of fibrinolytic protease (Fig. 3).

The main significant outfits represent all the factors, except pH and incubation period. It recommends that temperature and concentrations of starch and peptic and malt extracts can be increased. Starch, peptic, oat meal, L-asparagine, and Malt extract show optimum range values. Among all these factors, temperature, starch, peptic, and malt extract play major roles. The effect of interaction between significant parameters revealed the enhancement of fibrinolytic protease activity,

Table 5 Design matrix of fractional factorial design

Analysis of variance					
Source	*df*	Adj SS	Adj MS	*F*-value	*p*-value
Model	14	253733	18123.8	583.44	0.032
Linear	8	230749	28843.6	928.53	0.025
Temperature	1	79172	79171.9	2548.68	0.013
pH	1	7	6.9	0.22	0.720
Duration	1	8	8.4	0.27	0.695
Starch	1	49965	49965.2	1608.47	0.016
L-aspargine	1	7317	7316.7	235.54	0.041
Peptic	1	13249	13249.0	426.51	0.031
Oat meal	1	41331	41331.5	1330.53	0.017
Malt extract	1	39699	39699.4	1277.99	0.018
2-way interaction	6	22984	3830.7	123.32	0.069
Temperature*pH	1	788	787.7	25.36	0.125
Temperature*duration	1	115	114.9	3.70	0.305
Temperature*starch	1	11687	11686.8	376.22	0.033
Temperature*l-aspargine	1	8370	8370.4	269.46	0.039
Temperature*peptic	1	868	867.5	27.93	0.119
Temperature*oat meal	1	1157	1156.9	37.24	0.103
Error	1	31	31.1		
Total	15	253764			
Model summary					
		S	R-Sq	R-Sq(Adj)	R-Sq(Pred)
		5.5735	99.99%	99.82%	96.87%
Coded coefficients					
Term	Effect	Coef	SE coef	*T*-value	*F*-value	VIF
Constant	140.69	339.50	1.39	243.65	0.003	
Temperature	140.69	70.34	1.39	50.48	0.013	1.00
pH	1.31	0.65	1.39	0.47	0.720	1.00
Duration	–1.45	–0.72	1.39	–0.52	0.695	1.00
Starch	111.76	55.88	1.39	40.11	0.016	1.00

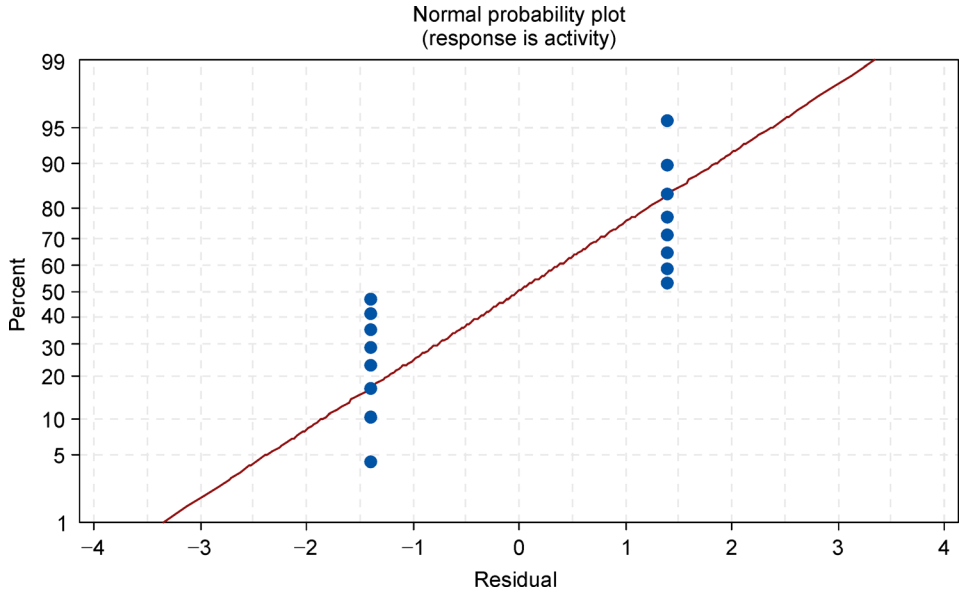

Figure 1 Normal plot of the significant factors on fibrinolytic protease production by (A) Starch (B) Peptic (C) L-Aspargine (D) Oat meal (E) Malt Extract (F) Temperature (G) pH (H) Incubation time.

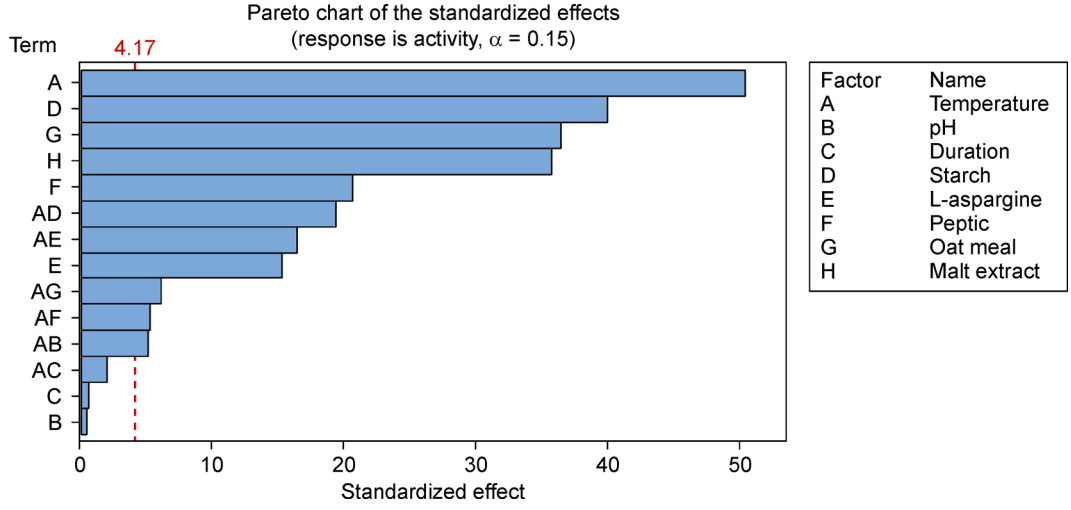

Figure 2 Pareto chart for the standardized effects of different media components on fibrinolytic protease production for various factors (UL1).

associated with an increase in the concentration of starch and peptic. A normal probability plot (Fig. 4) compared the effects of the expected normal values versus residuals, which defines the linearity of all the 16 runs in the design space. The response surface plots generated represented the combined effect of two independent variables on fibrinolytic protease production, while the third variable assumed the center point levels (Fig. 5).

Discussion

Response surface methodology (RSM) has been widely used to evaluate and understand the interactions between different physiologic and nutritional parameters. Prior knowledge and understanding of fermentation parameters were necessary for achieving a more realistic model (Conto et al., 2001). In the present study, an isolated and identified actinomycete strain, which was characterized for fibrinolytic protease production under solid-state fermentation, was used. Based on the results obtained by the classical approach, the parameters significantly affecting protease production were taken into consideration. A 2 [6-2] fractional-factorial central composite design (FFCCD) of RSM was used for the optimization of medium components for the maximal production of protease. Previously, the same method was reported by Naveena et al. (2005). Regression analysis was performed to obtain the optimum medium concentration.

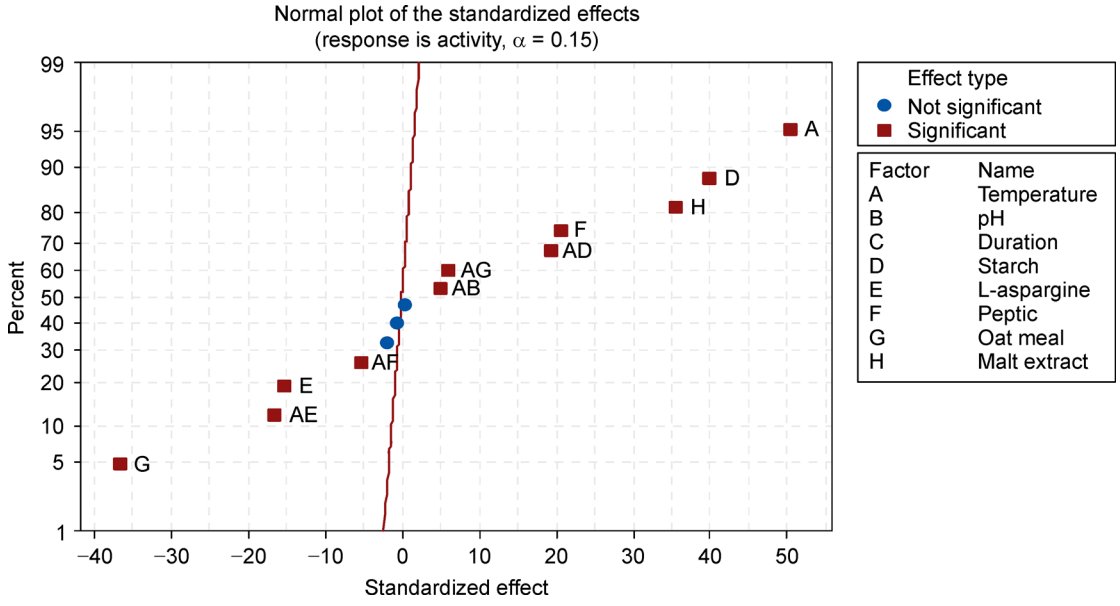

Figure 3 Effect of significant factors on fibrinolytic protease production.

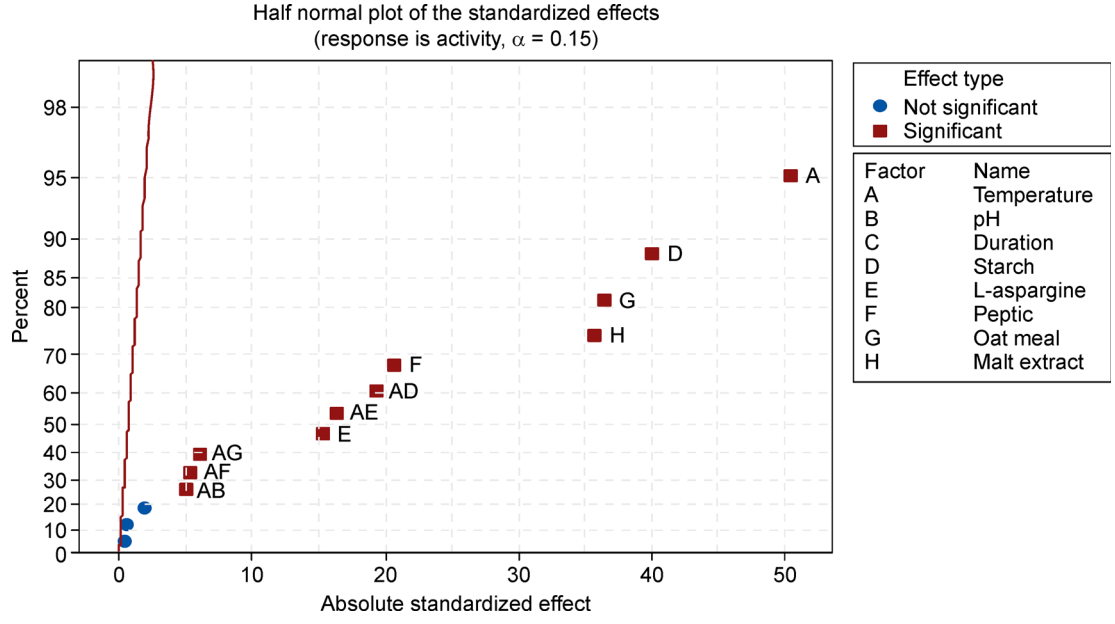

Figure 4 Half-normal plot of the standardized effect.

Previously, protease production from *B. amyloliquefaciens* was studied by the Plackett–Burman design (Dworkin et al., 2006). The experimental results indicated that the noteworthy parameters were gelatin concentration, cultivation time, and agitation. The initial pH, glycerol, and $MgSO_4 \cdot 7H_2O$ were the variables affecting protease production from *B. sphaericus* DS11 (Liu et al., 2010). Pillai et al. (2011) demonstrated that soya bean meal and minerals ($BaCl_2$, KH_2PO_4, $CaCl_2$) enhanced the protease production from *B. subtilis* P13.The stretched matrix had specific advantages in large scale production. If protease production of the cells in the matrix displayed higher levels of the free cells, then enzyme activity being engross (Kar and Ray, 2008).

In the present study, there was a twofold increase in enzyme production after optimizing the media components by factorial design. The predicted maximum protease activity assessed as 664.8±1.38 U/mL. The present experimental values (663.5±1.43 U/mL) were similar to the predicted values. Therefore, the experimental model was found to be significantly applicable for the large-scale production of protease. The application of RSM determines maximum enzyme production based on the predicted values. In contrast, it also corelates with the range of variable values and the interactive effects among the process parameters on enzyme

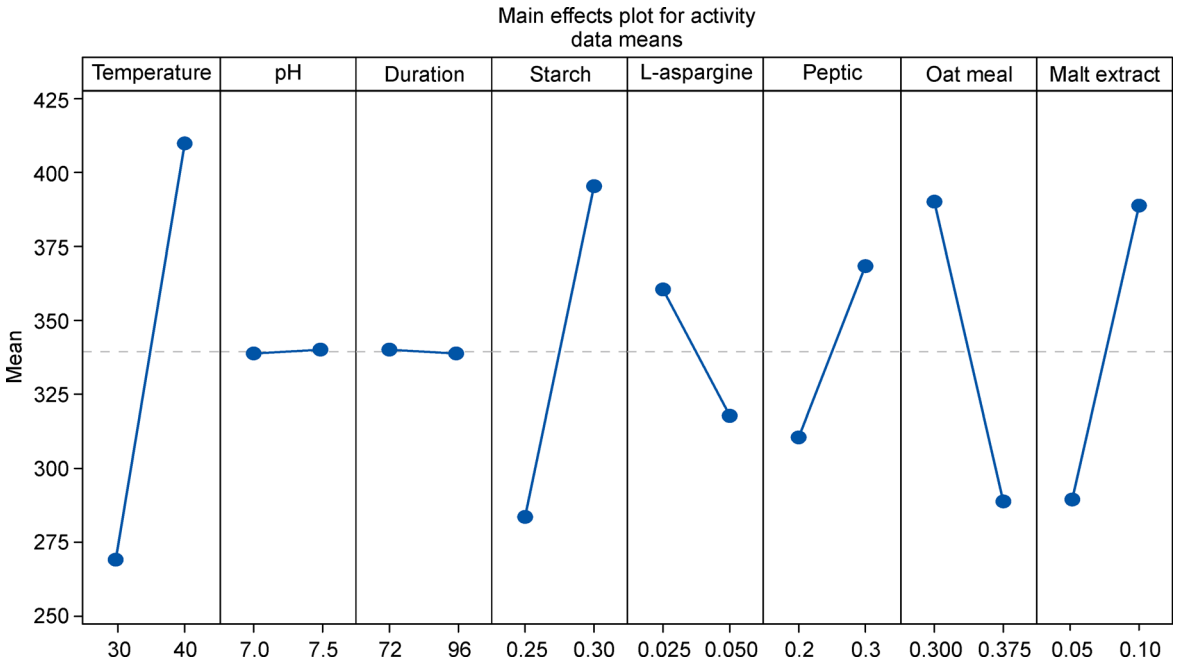

Figure 5 Main effect plot for activity.

production. The statistical optimization strategy was performed to achieve the highest production of protease. The predicted and experimental values were analyzed, and it was found that fructose, tryptose, and sodium di-hydrogen phosphate were the effective significant factors. The optimum conditions for the maximum fibrinolytic protease activity of 663.5±1.43 U/mL were as follows: 8.0 g of fructose, 8.0 g of tryptose, 4.5 g of sodium dihydrogen phosphate, a pH of 7, and an incubation temperature of 40°C. The statistical optimization studies showed a 70% increase in the protease production compared to the initial value. The value of the adjusted coefficient (0.989) proved to be high (Xavier and Lonsane, 1994; Weiyong et al., 2013). The present study depicts the improved fibrinolytic activity of *Streptomyces radiopugnans*_VITSD8.

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Compliance with ethics guidelines

Dhamodharan and subathra declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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