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Response Surface Methodology (RSM) Approach for Improved Extracellular RNase Production by a *Bacillus* sp.

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Abstract: The extracellular ribonuclease activity of *Bacillus* sp. was successfully improved by optimization of nutritional and physical parameters in a set of non-statistical and statistical experiments. Plackett-Burman and Central Composite Design in Response Surface Methodology were used to build statistical models to screen out the significant variables and then study the effect of such variables on ribonuclease production. Four significant variables namely malt extract, yeast extract, ammonium molybdate and fermentation broth pH were selected *via* 2^4-factorial Central Composite Design (CCD) for ribonuclease production *Bacillus* sp. The optimized values obtained by the statistical analysis showed that malt extract 1. 40 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v) and pH 8.81 affected maximum ribonuclease production by *Bacillus* sp. The ribonuclease production after optimization increased up to 1.83-fold with 83.52 % yield in comparison to the conventional strategy. Analysis of variance (ANOVA) revealed high coefficient of determination (R^2) of 0.9268 for the respective responses at significant level ($p < 0.05$).

Keywords: *Bacillus* sp.; Optimization; RNase; Response Surface Methodology; Central Composite Design; Plackett–Burman Design.

Introduction

Ribonuclease (RNase) is a type of nuclease that catalyzes the degradation of RNA into smaller components that involves the exo- and endo-ribonucleolytic cleavage of RNA molecules [1]. Exo-ribonuclease degrades starting at their termini in a sequence independent manner whereas endo-ribonucleases cleave internally single or double-stranded RNA (dsRNA) molecule. Many endo-RNases exhibit substrate specificity, but their target site is usually limited to one or a few specific nucleotides in a single-stranded RNA and often in a context of a particular three-dimensional structure of the substrate [2]. In addition, RNases play important roles in the metabolism of all types of cellular RNAs, such as mRNA and rRNA or tRNA maturation. Prokaryotic and eukaryotic cells possess a large number of RNases that participate in many cellular functions such as cell growth and differentiation, control of gene expression, cell protection from pathogens and apoptosis [3]. RNases uniquely influence several functions in the tumor cell simultaneously and demonstrated the ability to overcome multi-drug resistance and to enhance the cytotoxicity of a variety of anti-cancer agents [4]. The microbial RNases have received attention due to difficulties surrounding safety and standards on the use of bovine or other animal-derived RNases. The commercial applications of RNase in various industrial processes to produce nucleotides for clinical use or for the food industry and in molecular biology to study the structure of nucleic acids and proteins require maximum RNase production.

The maximum production of RNase with cost effective way in less time period may be achieved

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by employing alternate strategies of culture medium optimization. Conventional one-variable-at-a-time optimization strategy has traditionally been used to optimize physical and nutritional parameters to enhance the production of RNase enzyme. This approach is time consuming, requires a large number of experiments and do not investigate the interactive effects between variables. Therefore, Response Surface Methodology (RSM) which is a collection of mathematical and statistical techniques for empirical model building was employed by considering several independent (input) variables [5]. An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. RSM also explains the combined effects of all the independent variables in a fermentation process and explores an approximate interaction between a response variable and a set of design independent variables [6]. The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods (*e.g.* finite element method or CFD analysis) and their associated numerical noise [7]. In RSM, Plackett-Burman (PB) design is usually used as the first step to screen main factors from a number of process variables and the design is useful in selecting variables that can be fixed or eliminated in further optimization processes. It is generally followed by the steepest ascent (descent) method and Central Composite Design (CCD) to estimate the relationship between the variables and response.

In the present study, we aimed to produce extracellular RNase from the *Bacillus* sp. with enhanced RNase activity using one-variable-at-a-time strategy and then screened the significant variables using PB design and to further optimize the levels of the screened variables were evaluated by CCD approach.

Materials and methods

Microorganism and screening of RNase activity

The strain of *Bacillus* sp. procured from Department of Biotechnology, Himachal Pradesh University, Shimla (Himachal Pradesh, India) was originally isolated from a soil sample. Bacterial strain was screened on the basis of zone of hydrolysis on the Nutrient agar plates containing 0.1 % (w/v) RNA [8]. The bacterial culture was prepared as suspension and screened for extracellular RNase production by inoculating onto the Nutrient agar plate. The plate was then incubated at 37°C until growth was clearly visible. After incubation the plates were flooded with 3 ml of the precipitant (perchloric acid) and left to stand for 5 min. The plates were then visualized for transparent halos formed around the grown colonies, against an opalescent RNA background. The cfu/culture producing clear zone was preserved and used for the fermentation.

Production of extracellular RNase by *Bacillus* sp.

The *Bacillus* sp. was inoculated into broth containing (g/L) glucose 1.0, beef extract 1.5, yeast extract 1.5, NaCl 5.0 and pH was adjusted to 7.4±0.2. The broth amended with different carbon sources (glucose, malt extract, galactose, sucrose, sorbitol, lactose, fructose, starch soluble and xylose) with concentration ranging from 0.5 % (w/v) to 3.0 % (w/v) and nitrogen source (yeast extract, beef extract, tryptone, casein hydrolysate, peptone as well as inorganic nitrogen sources such as ammonium nitrate, ammonium sulphate, sodium nitrate, ammonium chloride, potassium nitrate and ammonium molybdate) in different concentrations from 0.5 % (w/v) to 2.0 % (w/v) was subjected to physical conditions such as wide range of pH, ranging from 4.0 to 10.5, various temperatures ranging from 25 to 55°C and agitation rates ranging from 100 to 220 RPM. Out of a total of 18 compounds tested, the best carbon and nitrogen sources were selected on the basis of response of the organism in terms of ribonuclease production. The components which showed relatively good response for ribonuclease production by *Bacillus* sp. were identified and the levels of these components were studied. The best response was selected as center point to be applied in response surface methodology.

RNase activity

RNase activity of the *Bacillus* sp. was determined by the method of [9]. The reaction mixture contained 200 µL sodium acetate buffer (0.1 M, pH 5.0) and 200 µL of culture filtrate/purified en-

zyme. After equilibrating the enzyme assay mixture at 37°C for 5 min, 200 µL of freshly prepared yeast RNA (0.1 %, w/v in sodium acetate buffer) was added and incubated for 15 min. After incubation, 200 µLof chilled absolute alcohol was added to stop the reaction and the entire solution was cooled at 20°C for 10 min. The undigested RNA was precipitated by centrifugation at 10,000 *g* for 10 min and the soluble ribonucleotides were estimated spectrophotometrically at 260 nm. One unit of enzyme activity is defined as the activity to release acid soluble oligonucleotides per minute to increase one unit of A_{260} value under assay conditions.

Experimental design

After optimizing the nutrients by primary screening using a one-variable-at-a-time approach for the production of extracellular RNase, Plackett-Burman experimental design was used to screen the significant variables that influenced ribonuclease production by *Bacillus* sp. There were 7 variables screened in 12 experiments with four dummy variables. The experimental design with the name, symbol code, and the actual level of thevariables is shown in Table 1. Each variable is represented in two levels: a high level denoted by (+) and a low leveldesignated by (-). All experiments were performed in duplicate and mean values were presented.

In order to determine the optimum levels of significant variables for ribonuclease production, the RSM, using a CCD, was adopted for the augmentation of ribonuclease production. A 2^4 factorial central composite experimental design with eight start points ($\alpha = 2$) and six replicates at the central point, which results in 30 experiments, was used to optimize the screened variables grouped as malt extract (X_1), yeast extract (X_2), ammonium molybdate (X_3) and pH (X_4). Each of the four significant variables is assessed at five coded levels (-2, -1, 0, 1 and 2) as shown in Table 2,

Table 1. Plackett-Burman experimental design matrix for screening of culture conditions for extracellular ribonuclease production by *Bacillus* sp.

Standard order	Variables/levels											RNase (U/mL)
	X_1	X_2	X3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	
1	+	+	-	+	+	+	-	A1	B1	C2	D1	5.94
2	+	+	+	-	-	-	+	A1	B2	C2	D1	4.68
3	-	-	-	-	-	-	-	A1	B1	C1	D1	1.86
4	+	-	-	-	+	-	+	A2	B1	C2	D2	3.13
5	-	+	-	+	+	-	+	A2	B2	C1	D1	0.58
6	+	-	+	+	+	-	-	A1	B2	C1	D2	2.01
7	-	-	+	-	+	+	-	A2	B2	C2	D1	1.65
8	+	-	+	+	-	+	+	A2	B1	C1	D1	4.44
9	-	+	+	-	+	+	+	A1	B1	C1	D2	2.89
10	-	+	+	+	-	-	-	A2	B1	C2	D2	2.64
11	+	+	-	-	-	+	-	A2	B2	C1	D2	3.93
12	-	-	-	+	-	+	+	A1	B2	C2	D2	2.39

X_1, Malt extract at a high level of 1.0 % (w/v) and a low level of 0.5 % (w/v)
X_2, Yeast extract at a high level of 2.0 % (w/v) and a low level of 1.0 % (w/v)
X_3, Ammonium molybdate at a high level of 1.0 % (w/v) and a low level of 0.5 % (w/v)
X_4, Culture temperature at a high level of 40°C and a low level of 30°C
X_5, Inoculum size at a high level of 9 % (v/v) and a low level of 5 % (v/v)
X_6, Initial pH at a high level of 8.5 and a low level of 6.5
X_7, Agitation rate at a high level of 160 RPM and a low level of 120 RPM
X_8, X_9, X_{10} and X_{11}, dummy variables

Table 2. Experimental range and levels of the variables for RSM experiments

Variables	Symbol code	Range and levels -2	-1	0	1	2
Malt extract	X_1	0	0.63	1.25	1.88	2.5
Yeast extract	X_2	1.0	1.5	2.0	2.5	3.0
Ammonium molybdate	X_3	0	0.25	0.5	0.75	1.0
pH	X_4	6.0	7.25	8.5	9.75	11.0

while the detailed experimental design was presented separately (**Table 3**). The variable levels of Xi were coded as xi according to the following equation:

$$i = \frac{X_i - X_0}{\Delta X_i} i = 1, 2, 3, 4, \ldots\ldots, j, \quad (1)$$

Where xi is the dimensionless value of the variable X_i, X_i is the real value of the variable X_i, X_0 is the real value of the variable X_i at the center point and X_i is the step change [10].

From the experimental results, an approximate polynomial relationship for dependent variables of ribonuclease activity was obtained. The result of this design was used to fit a second-order polynomial equation:

$$y = \beta_0 + \sum_i \beta_i x_i + \sum_{ii} \beta_{ii} x_i^2 + \sum_{ij} \beta_{ij} x_i x_j \quad (2)$$

Where y is the predicted response, β_0 is the offset term, β_i is the *i*th linear coefficient, β_{ii} is the *i*th quadratic coefficient and β_{ij} is the *ij*th interaction coefficient [11].

Statistical analysis

The Design-Expert Software (Version 7.0, Stat-Ease Inc., Minneapolis, USA) was used for the regression analysis o fthe data obtained and to plot the response surface graphs. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Results and discussion

Effect of process variables on ribonuclease production

Among the selected variables, inoculum size of *Bacillus* sp. enhanced the ribonuclease production (2.27 U/mL) on addition of 7 % (v/v) inoculum. Different carbon sources were tested, out of which malt extract induced a higher production of RNase at 0.75 % (v/v) concentration (2.97 U/mL), while the ribonuclease production was reduced continuously on increasing the concentration up to 3.0 % (2.42 U/mL). Since the highest RNase production was at 0.75 % (v/v) inoculum concentration, it was selected as the center point for the RSM-based process optimization. Yeast extract was selected as the organic nitrogen/carbon source since it induced the second highest nuclease production, next to malt extract, in Nutrient broth among the four different concentrations tested. The highest ribonuclease production was recorded at 1.5 % (3.7 U/mL) while at 1 and 2 % (v/v) yeast extract, the RNase production was 3.35 and 3.41 (U/mL), respectively. Hence, 1.5 % yeast extract was chosen as the center point for RNase production by *Bacillus* sp. for RSM based optimization. Inorganic nitrogen source (Ammonium molybdate) along with yeast extract also enhanced the RNase production (3.87 U/mL) at 0.75 % (w/v) concentration by *Bacillus* sp. The temperature and pH play major roles in the production of ribonuclease and were also included in the optimization process using a one-parameter-at-a-time approach. Nutrient broth, containing the selected carbon/nitrogen sources, was incubated at various temperatures to select the best temperature for the highest production of ribonuclease. Maximum production of RNase was recorded at 35°C (4.09 U/mL) but dropped to 3.56 U/mL at 30°C. RNase production was drastically reduced at higher temperatures; therefore, temperature 35°C was selected for the RNase by *Bacillus* sp. The *Bacillus* sp. showed highest RNase production (4.17 U/mL)

Table 3. Experimental design used in RSM studies by using four variables with six center points showing observed and predicted ribonuclease activity

Standard order	X_1	X_2	X_3	X_4	RNase (U/mL) Observed	Predicted
1	-1	-1	-1	-1	1.58	2.64
2	1	-1	-1	-1	1.24	1.62
3	-1	1	-1	-1	1.83	2.48
4	1	1	-1	-1	0.93	0.82
5	-1	-1	1	-1	1.96	2.25
6	1	-1	1	-1	1.87	2.71
7	-1	1	1	-1	4.72	4.60
8	1	1	1	-1	4.00	4.42
9	-1	-1	-1	1	0.38	1.03
10	1	-1	-1	1	1.31	1.90
11	-1	1	-1	1	1.24	0.85
12	1	1	-1	1	0.32	1.07
13	-1	-1	1	1	1.87	2.44
14	1	0	1	1	4.37	6.43
15	-1	1	1	1	4.00	4.73
16	0	1	1	1	7.03	6.43
17	-2	0	0	0	0.34	0.00
18	2	0	0	0	0.68	0.07
19	0	0	-2	0	4.17	2.53
20	0	2	0	0	3.96	4.05
21	0	0	-2	0	0.48	0.00
22	0	0	2	0	5.00	4.47
23	0	0	0	-2	6.93	5.97
24	0	0	0	2	7.00	6.41
25	0	0	0	0	7.60	6.57
26	0	0	0	0	6.48	6.57
27	0	0	0	0	6.12	6.57
28	0	0	0	0	6.34	6.57
29	0	0	0	0	6.41	6.57
30	0	0	0	0	6.48	6.57

at the pH value of 7.4, whereas use of other pH values (6 and 7) of the fermentation medium resulted in lower ribonuclease production (3.35 and 3.68 U/mL, respectively). Hence the pH 7.4 was selected for the further optimization process. Besides temperature and pH, agitation rate also increased the RNase production (4.31 U/mL) at 140 RPM. This study of classical optimization showed better extracellular RNase production (4.31U/mL) by *Bacillus* sp. than earlier work in which RNase activity of 3.8 U/mL was reported for *Streptomyces* sp. and 0.18 U/mL for *Streptomyces aurofaciens* [12].

In the initial screening, *Bacillus* sp. produced the maximum RNase using malt extract, yeast extract, ammonium molybdate, pH 7.4, temperature 35°C under shaking at 140 RPM. Based on the above results, use of malt extract as a carbon source, yeast extract as an organic nitrogen source, ammonium molybdate as an inorganic nitrogen source, inoculum size 7 % (v/v), pH 7.4, temperature 35°C and agitation rate 140 RPM

were predicted for the maximum RNase production by *Bacillus* sp. using RSM.

Screening of significant variables for RNase production using PB design

Based on the above results, the relatively important seven variables were selected for the RNase production by the *Bacillus* sp., *i.e*. incubation temperature, initial pH, inoculum size, agitation rate, carbon source, organic nitrogen source and inorganic nitrogen source were screened by Plackett-Burman experimental design. The experimental design and corresponding ribonuclease yields were presented (Table 1). Among selected variables, the most effective factors with high significance level were concentration of malt extract, yeast extract, ammonium molybdate and pH (Table 4), which were hence considered for further optimization for the RNase production by the *Bacillus* sp.

Statistical analysis of the Plackett-Burman design demonstrated that model F value of 4.56 was significant. The value of $p<0.05$ indicated that the model terms were significant (Table 4). The R^2 value (multiple correlation coefficient) closer to 1 denoted a better correlation between the experimental and predicted responses. In this case, the value of R^2 (0.79) indicated good correlation between experimental and predicted values. The coefficient of variation indicates (CV) degrees of precision with which the experiment compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case, a low CV (30.88 %) denoted that the experiment performed are highly reliable. The *p* value denoted the significance of coefficient and also important in understanding the pattern of mutual interaction between the variables. Consequently, based on the results from this experiment, statistically significant variables *i.e.*, malt extract, yeast extract, ammonium molybdate and pH with positive effect were further investigated with central composite design to find the optimal range of these variables.

Optimization of significant variables for RNase production by using CCD

In order to search for the optimum culture conditions to enhance the ribonuclease production, experiments were performed according to CCD experimental plan together with experimental results obtained from PB design. Based on the Plackett-Burman design malt extract, yeast extract, ammonium molybdate and pH were further selected for optimization by CCD for 4 significant variables and the corresponding experimental data were presented (Table 2). The response generated from the results of CCD showed that Quadratic Model was best one and hence selected (Tables 3, 4, and 5) while other regression models were aliased. The results obtained were subjected to analysis of variance on Stat-Ease package with the regression model given as:

$$Y= 6.57+0.17\ X_1+0.38\ X_2+1.25\ X_3+0.11\ X_4-1.71\ X_1^2-0.82X_2^2-1.15X_3^2-0.094\ X_4^2-0.16\ X_1X_2+0.37\ X_1X_3+0.47\ X_1X_4+0.62\ X_2X_3-0.011\ X_2X_4+0.44\ X_3X_4$$

Results from the second order RSM fitting in the form of analysis of variance (ANOVA) were recorded (Tables 5, 6 and 7). The regression model, which consisted of one offset, eight quadratic and

Table 4. Results of the screening experiments for RNase production by *Bacillus* sp.

Variables	Sum of squares	Coefficient estimate	Standard error	F value	P value (Prob>F)
Model	19.70	3.01	0.27	4.56	0.0461*
Malt extract	12.24	1.01	0.27	14.15	0.0094
Yeast extract	2.24	0.43	0.27	2.59	0.1590
Ammonium molydate	3.35	0.40	0.27	0.22	0.8864
pH	1.86	0.53	0.27	3.87	0.0966

R^2 - 0.79; CV - 30.88 %

Table 5. Sequential model sum of squares

Source	Sum of squares	df	Mean square	F value	p-value (Prob>F)	
Mean vs Total	379.07	1	379.07			
Linear vs Mean	41.99	4	10.50	1.87	0.1480	
2FI vs Linear	15.40	6	2.57	0.39	0.8767	
Quadratic vs 2FI	111.91	4	27.98	31.36	<0.0001	Suggested
Cubic *vs* Quadratic	4.54	8	0.57	0.45	0.8575	Aliased
Residual	8.84	7	1.26			
Total	561.76	30	18.73			

Table 6. Lack of fit tests

Source	Sum of square	df	Mean square	F value	p-value (Prob>F)	
Linear	139.34	20	6.97	25.65	0.0010	
2FI	123.94	14	8.85	32.59	0.0006	
Quadratic	12.02	10	1.20	4.43	0.0572	Suggested
Cubic	7.48	2	3.74	13.77	0.0093	Aliased
Pure error	1.36	5	0.27			

Table 7. Model Summary Statistics

Source	Standard deviation	Adjusted R-sSquared	Predicted R-squared	R-squared	Press	
Linear	2.37	0.2299	0.166	-0.0525	192.28	
2FI	2.57	0.3142	-0.4417	-0.4417	263.39	
Quadratic	0.94	0.9268	0.8584	0.6102	71.21	Suggested
Cubic	1.12	0.9516	0.7995	-4.9083	1079.40	Aliased

six interaction terms, was generated using the Design Expert Software. The predicted level of ribonuclease production by *Bacillus* sp. at each of the points was determined using a regression equation (Table 3) along with the observed data. The coefficient values of the regression equation(s) are listed in Table 8. The *P*-value serves as a tool for checking the significance of each coefficient and also important in understanding the mutual interactions between the variables. Smaller is the *P*-value, more significant the corresponding coefficient [13]. The coefficients of X_2, X_3, X_1^2, X_3^2 and had remarkable effects on ribonuclease production, followed by X_1, $X_4$and. The interaction terms of X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_3X_4appeared to be insignificant, which showed that there was relatively no mutual interaction between any two variables. The probability value of the interactive effect of yeast extract and ammonium molybdate and yeast extract and pH were 0.9647, respectively indicated that those variables have less significant effects on the model. The ANOVA of quadratic regression model demonstrated that the model was highly significant, which was evident from the Fisher's F-test with a very low probability value [(P_{model}> F) = 0.0001] (Table 8). The model F value of 13.56 implied that the tested model was significant. The fit of the model was also expressed by the coefficient of determination, R^2, which was 0.9268, in-

Table 8. ANOVA for Response Surface Quadratic Model

Source	Coefficient estimate	Standard error	F value	*P*-value Prob>F
Model			13.56	<0.0001
Intercept	6.57	0.38		
X_1	0.38	0.19	0.81	0.3817
X_2	0.17	0.19	3.84	0.0688
X_3	1.25	0.19	42.12	0.5926
X_4	0.11	0.19	0.30	0.5165
$X_1 X_2$	-0.16	0.24	0.44	0.1361
$X_1 X_3$	0.37	0.24	2.48	0.0629
$X_1 X_4$	0.47	0.24	4.04	0.0196
$X_2 X_3$	0.62	0.24	6.83	0.9647
$X_2 X_4$	-0.011	0.24	2.025E-003	0.9647
$X_3 X_4$	0.44	0.24	3.48	0.0817
X_1^2	-1.71	0.18	89.68	<0.0001
X_2^2	-0.82	0.18	20.63	0.0004
X_3^2	-1.15	0.18	40.69	<0.0001
X_4^2	-0.094	0.18	0.27	0.6096

CV- 26.57 %; R^2- 0.9268; Adj. R^2- 0.8584; *: non significant

dicating that 92.68 % of the variability in the response could be explained by the model and only 7.32 % of the total variation were not explained by the model. The R^2 value higher than 0.9 was considered to have a very high correlation [14]. The value of the adjusted determination coefficient (Adj R^2= 0.8584) was also high enough to advocate for a high significance of the model [15]. Adeq precision measures the signal to noise ratio that should be greater than 4 [16]. The observed ratio of 10.749 indicated an adequate and desirable signal. The coefficient of variation (CV) indicated the degree of precision with which the treatments are compared [17,18], and the lower the value of CV the higher the reliability of experiment [19]. Here, a lower value of the CV (26.57 %) indicated a higher reliability of the experiment. The 'lack of fit F value' of 4.43 indicated the lack of fit is not significant relative to the pure error suggesting it is a good fit for this model (Table 6).

The 2D contour plots and 3D response surface plots which were generated from the regression equation by keeping the 2 variables at zero and changing the other 2 variables with different combinations (Figs. 1 to 2), thus exhibited the maximum RNase production of 7.6 U/mL at the predicted level of the four variables. The main aim of the RSM was pursued efficiently for the optimum values of the variables, to maximize the response [20]. Six plots showed the response surface curves for the variation in the yields of RNase as a function of concentrations of two variables with the other variables being at their optimum levels [21,22]. By using RSM, the interaction between two variables and their optimum levels could be understood and located easily.

If the response surface is elliptical, the maximum point can be obtained at the point of intersection of major and minor axes of the ellipse. As shown in Fig. 1 the maximum production of ribonuclease from *Bacillus* sp. was obtained when the concentration of malt extract (Fig. 1A, B), yeast extract (Fig. 1A, C) and ammonium molybdate (Fig. 1B, C) was 1.25-1.56 % (w/v), 2.00-2.25 % (w/v) and 0.50-0.63 % (w/v), respectively. According to this model high yeast extract in the

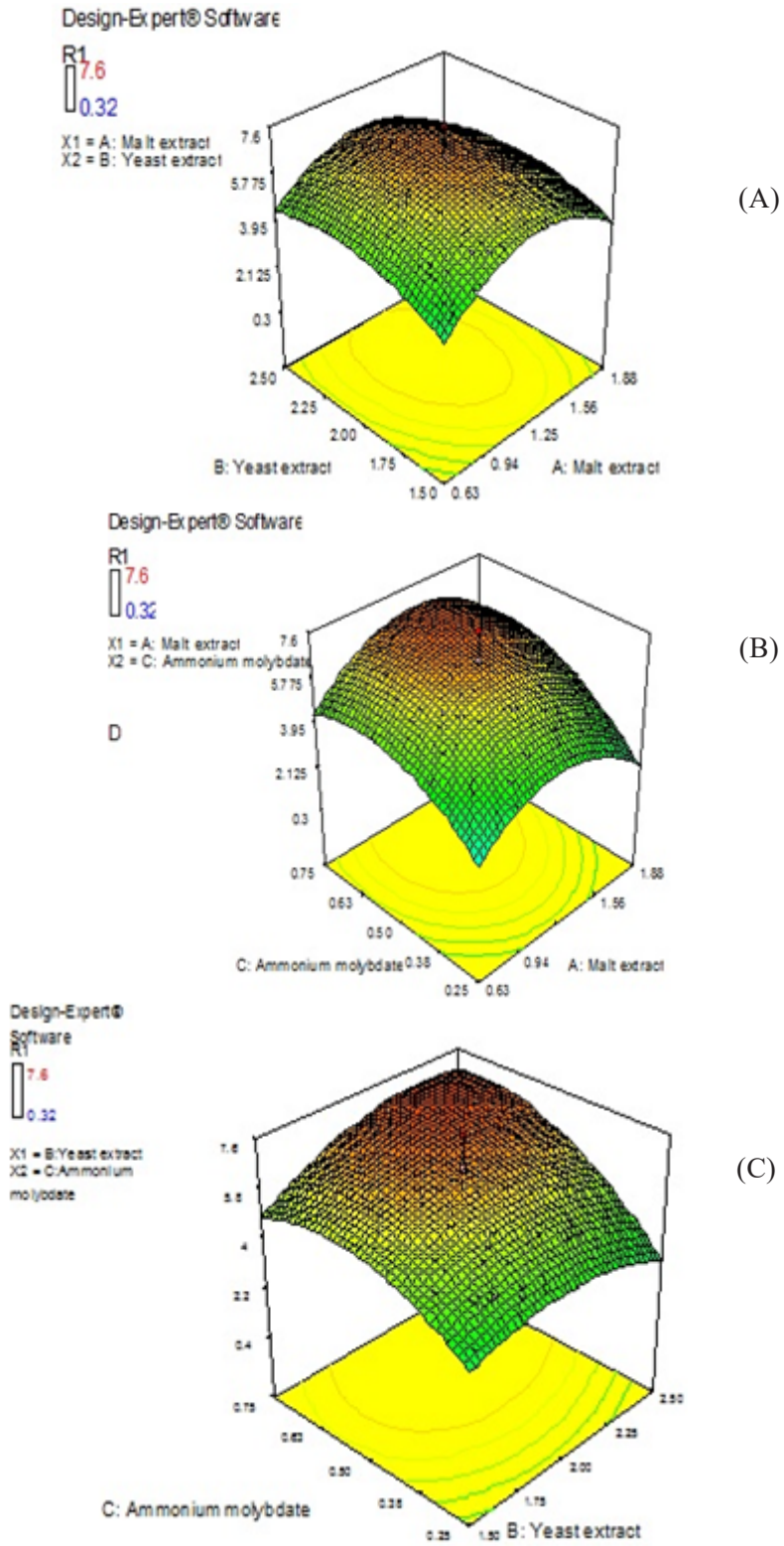


Fig. 1. Response surface of ribonuclease production by *Bacillus* sp.ZH14 as a function of (A) malt extract concentration and yeast extract concentration, (B) malt extract concentration and ammonium molybdate concentration (C) yeast extract concentration and ammonium molybdate concentration.

fermentation medium was required for extracellular ribonuclease production. A previous study reported that high yeast extract concentration in fermentation medium was required for the biosynthesis of RNases [23]. The positive effect of high concentration of yeast extract on the production of RNase was also reported for *B. cereus* ZH14 [22]. The present study indicated that the RNase production by *Bacillus* sp. was also affected by low concentration of ammonium molybdate as inorganic nitrogen source in the fermentation medium. During the studies on fermentation medium optimization, the carbon source needed for maximal yield of the ribonuclease seemed to be different among bacteria, *i.e*., glucose and lactose for better RNase production of *Bacillus intermedius* [24] and glucose for *E. coli* and *A. niger* [25,26]. In our study, malt extract was found to be better than glucose as carbon sources for ribonuclease production by selected *Bacillus* sp. An increase in ribonuclease production was observed when broth pH was increased. The plots depicted the interaction of pH with the three other variables (Fig. 2). The studies related to fermentation condition reported that the lower pH were suitable for fungi whereas bacteria required higher pH [27,28]. An increase in RNase production was observed when the ammonium molybdate concentration was increased to some extent. Finally, with the detailed point prediction of Design Expert Software, the optimum levels of the variables obtained were malt extract 1.40 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v) and pH 8.81.

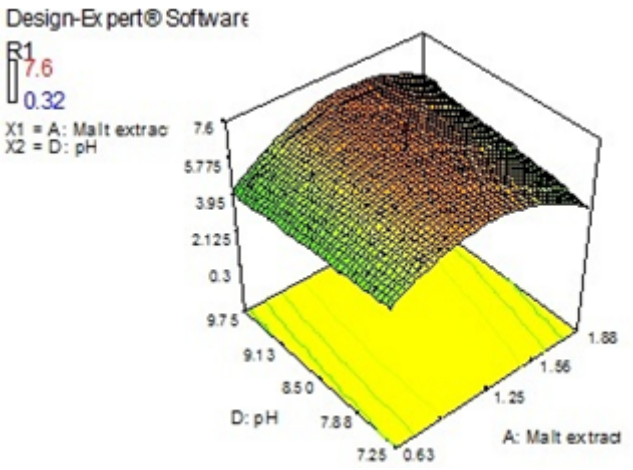
The model predicted that the maximum RNase productionby selected *Bacillus* sp. could be obtained using the above optimumconcentrations of the variables were 8.03 U/ml. A repeated fermentation of RNase under the optimalcondition was carried out, and the maximum enzymeactivity obtained during the experiment was found to be 7.91 U/ml. Evidently, this should be in close agreement with the model prediction. The RNaseactivity underoptimized conditions (malt extract 1.40 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v), pH 8.81, temperature 40°C, inoculum size 9 % (v/v) and agitation rate 120 rpm) was 7.78 U/ml, while the activity under original conditions(malt extract 0.75 % (w/v), yeast extract 1.5 % (w/v), ammonium molybdate 0.75 % (w/v) pH 7.4, temperature 37°C, inoculum size 7 % (v/v) and agitation rate 140 rpm) was 4.31 U/ml. Thus, the optimization enhanced the RNase production by *Bacillus* sp. by 83.5 % in the same fermentation time and the RNase production was greater (60 %) that *Bacillus firmus* VKPACU1 reported in a previous study [19]. Over the last few decades, a few papers reported the optimization of process parameters for ribonuclease [17,19,28,29,30,31] and most of these studies had focus on fungal ribonucleases.In most of the cases, the time required for the optimum RNase production by a fungus was between 2 to 10 days [7,32]. In contrast, in the present study, the RNase production by *Bacillus* sp. occurred within a short duration (post 6-16 h of incubation). Finally, with the detailed prediction of Design Expert Software, the optimum levels of the variables obtained were malt extract 1.4 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v) and pH 8.81 for extracellular RNase production by *Bacillus* sp. The tested model predicted that the maximum ribonuclease production that could be obtained using the above optimum values of the variables was 7.91 U/mL.

Validation of the experiment

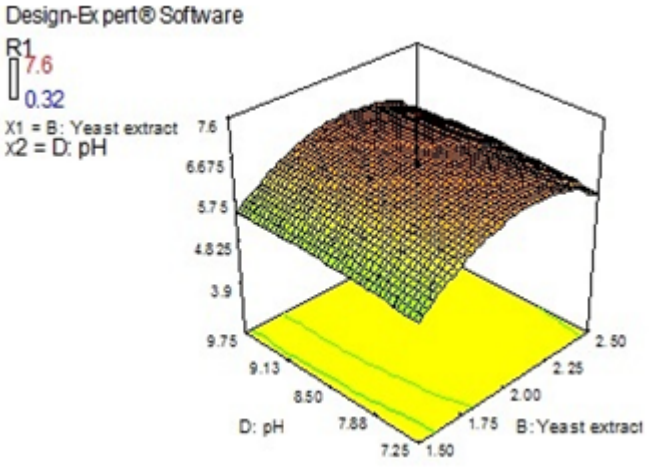
Statistical optimization of culture conditions using PB and CCD appeared to be a valuable tool to obtain high RNase production by *Bacillus* sp. The final optimized condition to produce ribonuclease were: malt extract 1.40 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v), pH 8.81, temperature 40°C inoculum size 9 % (v/v) and agitation rate 120 rpm. Theoretically, this fermentation medium composition produced 8.031 U/ml of RNase and in practice the production titer was about 7.91 U/ml. It proved that the model was adequate to predict the optimization of RNase production by *Bacillus* sp. Compared to the ribonuclease production under original conditions, the ribonuclease production by *Bacillus* sp. after optimization had about 83.5 % increase which was quite remarkable.

Summary

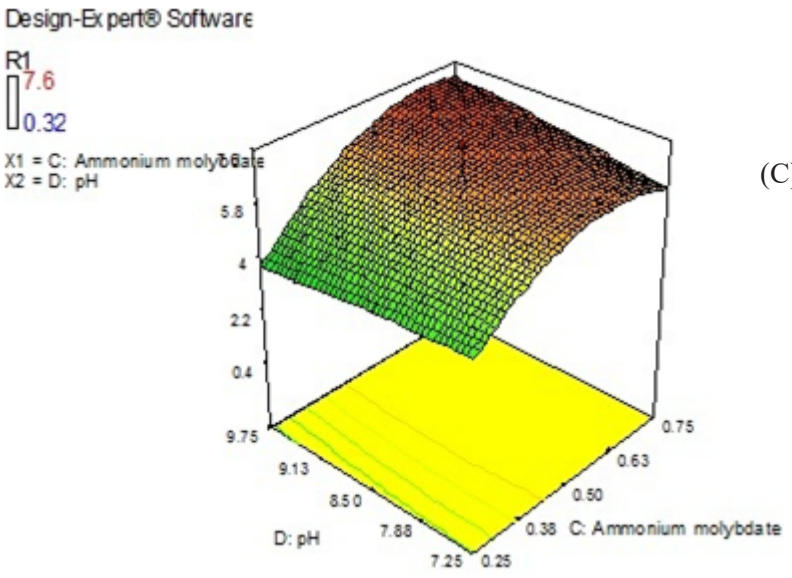
Four significant variables malt extract, yeast extract, ammonium molybdate and pH were



(A)



(BC)



(C)

Fig. 2. Response surface of ribonuclease production by *Bacillus* sp. as a function of (A) malt extract concentration and pH, (B) yeast extract concentration and pH, (C) ammonium molybdate concentration and pH

selected via 2^4 factorial central composite design for ribonuclease production. The optimized values obtained by the statistical analysis showed that malt extract 1.4 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v) and pH 8.81 affected maximum ribonuclease productions. The maximum ribonuclease production after optimization was increased 83.52 % yield over conventional strategy. Analysis of variance (ANOVA) revealed high coefficient of determination (R^2) of 0.9268 for the respective responses at significant level ($p<0.05$). This study will be further helpful for the production of bacterial ribonuclease with cytotoxic activity.

Conclusion

Response Surface Methodology approach using Plackett–Burman design and Central Composite Design appeared to be an influential statistical tool for enhanced production of RNase from *Bacillus* sp. A maximal production 7.78 U/ml of RNase was achieved with the optimized fermentation conditions [malt extract 1.40 % (w/v), yeast extract 2.12 % (w/v), ammonium molybdate 0.56 % (w/v), pH 8.81, temperature 40°C, inoculum size 9 % (v/v) and agitation rate 120 rpm]. Under optimal conditions of independent variables, the experimental responses closely related with predicted responses thus confirming the validity of model. The optimized model through RSM showed 1.83-fold enhancement in RNase production by the selected *Bacillus* sp. Further study is in progress to study the effect of purified RNase on the cancerous mammalian cell lines.

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Conflict of interest

The authors declare no conflict of interests among the authors or with their parent institute.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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