

Statistical optimization of extracellular tannase production by *Streptomyces sp.* AT 13 using response surface methodology and Plackett-Burmen design

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ABSTRACT

Tannase has many important applications in animal feed, chemical, food, and pharmaceutical industries. In the present study, optimization of tannase production by *Streptomyces sp.* AT13 was carried out using statistical experimental designs. Initially, a Plackett-Burmen design (PBD) was employed to screen the preferable nutriments (carbon and nitrogen sources of the medium) to produce tannase. The result showed that only tannic acid was found to be significant for the production of tannase by *Streptomyces sp.* AT 13. The significant factor was further optimized by using Box-Behnken design under response surface methodology (RSM). From among 6 fermentative variables that were studied, 5 significant variables were picked up by PBD. Among 5 variables from PBD, 3 were further optimized by Box-Behnken design. The parameters studied through RSM were 1% Tannic Acid, 0.5 % KCl and 0.1 % KH_2PO_4 . Under optimized conditions tannase activity was 18.12 U/ml/min. This activity was almost three times higher as compared to the amount obtained by 'one-at-a-time' approach. (5.19 U/ml/min)

INTRODUCTION

Tannins are polyphenolic compounds with molecular weights ranging from 500 to 3000 daltons which occur widely in a variety of plants including monocots, dicots and ferns (Bate-Smith and Swain 1962; Mcleod 1974; Haslam 1989). They are the fourth most abundant plant constituents after cellulose, hemicellulose and lignin.

Based on their structure and properties, tannins are distributed into two major groups – hydrolysable and condensed tannins. Hydrolysable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar core, which is usually glucose, and are readily hydrolysed by acids and enzymes into monomeric products. Hydrolysable tannins are notably absent in monocots. Commercially, hydrolysable tannins

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are extracted from Chinese gall (*Rhus semialata*), Sumac (*R. coriara*), Turkish gall (*Quercus infectoria*), Tara (*Caesalpinia spinosa*), Myrobalan nuts (*Terminalia chebula*) and chestnuts (*Castania sativa*) (Bhat *et al.*, 1998).

Tannase (tannin acyl hydrolase) catalyses the hydrolysis of ester and depside bonds of hydrolysable tannins to produce glucose and gallic acid (Barthomeuf *et al.*, 1994). Hydrolysis of tannic acid by tannase results in the liberation of glucose, gallic acid and various galloyl esters of glucose (Van de Lagemaat and Pyle, 2006). This enzyme finds widespread applications in food processing, brewing, pharmaceuticals, medicine, textiles, detergents and tea industry (Lekha and Lonsane, 1997). A combination of trimethoprim and sulphonamide is effective against many antibiotic resistant species of bacteria. More than 8000 tonnes per annum of gallic acid is manufactured out of which, almost 70% is used in production of trimethoprim (Beena, 2010).

The use of a sequential experimental design strategy is a useful tool for process optimization. Response surface methodology (RSM) provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield. It reduces the number of experiments without neglecting the interactions among the parameters. This multivariate approach also improves statistical interpretation possibilities and evaluates the relative significance of several contributing factors even in the presence of complex interactions (Dilipkumar *et al.*, 2011). RSM is widely used for multivariable optimization studies in several biotechnological processes such as the optimization of media, process conditions etc. (Mannan *et al.*, 2007; Pan *et al.*, 2008). Statistical optimization allows the interaction amid possible influencing parameters to be evaluated with a limited number of experiments (Rodriguez *et al.*, 2008). Statistical designs such as Plackett-Burman and Response surface methodology are common in practice for testing multiple factors and interactions that can be quantified in an error-free and robust manner (Mohan, 2014).

In order to fully exploit the prospective of this enzyme for various applications, it is imperative to investigate the possibility of enhancing its production by using more efficient production strategies (Rana and Bhat, 2005). Hence in this study the process parameters were optimized for maximum enzyme production adopting Plackett-Burman (PB) and Response Surface Methodology (RSM) with Box Behnken design. The statistical software package DesignExpert® 7.0 (Stat Ease Inc., Minneapolis, and USA) was used.

MATERIALS AND METHODS

Tannic Acid was purchased from Sigma Aldrich, USA. All other chemicals and reagents were used of analytical

grade and purchased from Hi-Media, India. *Sterptomyces* sp. AT 13 used for the present study was screened and isolated from Tannery Effluent at G.I.D.C Naroda, Ahmedabad and the organism was maintained on tannic acid agar (TAA) medium. Tannase production by *Sterptomyces* sp. AT 13 was carried out using tannase production medium containing NaNO₃ 0.6 % w/v, KCl 0.5 % w/v, MgSO₄ 0.05 % w/v, K₂HPO₄ 0.05 % w/v, KH₂PO₄ 0.05 % w/v and 1 % (w/v) filtered sterilized tannic acid (designated as TA broth). The broth was assayed for tannase activity.

TANNASE ASSAY

The tannase activity was estimated by modified spectrophotometric method of Sharma *et al.*, 2000. Tannic Acid was used as substrate. The basic principle of this assay is based on the formation of chromogen between gallic acid (released by the action of tannase on tannic acid) and rhodanine (2-thio-4-ketothiazolidine). A standard curve was prepared using gallic acid. Crude enzyme was used for the assay. All the tests were performed in triplicates. One unit of tannase activity is defined as the amount of enzyme required to liberate 1µM of gallic acid /min under defined conditions. Enzyme activity was expressed as U/ml/min.

Plackett–Burman design

Plackett–Burman design, an efficient technique for medium component selection (Plackett-Burman, 1946) was employed to establish the factors that significantly enhance the tannase production. Five independent variables (Table 1) were analysed in 12 experimental runs (Table 2) for the production of tannase. Triplicates were maintained for each experimental set up. The response of these factors for the production of tannase was measured by Spectrophotometer method as proposed by Sharma *et al.*, (2000). The low level (-1) and the high level (+1) of each factors are demonstrated in Table 2. The statistical software design expert 6.0 was used for analysing the experiment. Total 5 independent variables

Table 1. Screening of variables using a Plackett–Burman design

Variable codes	Variables	Low level (-1)	High level (+1)
A	Tannic Acid	-1	+1
B	KCl	-1	+1
C	NaNO ₃	-1	+1
D	KH ₂ PO ₄	-1	+1
E	K ₂ HPO ₄	-1	+1

Table 2. Plackett Burman design matrix for the screening of variables

Run	F1 A:Tannic Acid	F2 B:KCl	F3 C:NaNO ₃	F4 D:KH ₂ PO ₄	F5 E:K ₂ HPO ₄	F 6 F:F	F 7 G:G	F 8 H:H	F 9 J:J	F 10 K:K	F11 L:L	R1
1	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	1.62
2	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	12.99
3	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	7.17
4	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	-1.00	10.12
5	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	16.53
6	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	5.61
7	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	5.91
8	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	16.2
9	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	2.58
10	-1.00	1.00	1.00	-1.00	1.00	1.00	1.00	-1.00	-1.00	-1.00	1.00	0.3
11	1.00	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	15.75
12	1.00	-1.00	-1.00	-1.00	1.00	-1.00	1.00	1.00	-1.00	1.00	1.00	0.36

+ "sign indicates high level of the variable a nd" - "sign indicates low level"

along with 5 dummy variables generated by the software. All the experiments were done in triplicate and the average of lipase production was taken as response. The obtained results are matched with the polynomial equation 1 in significant order 1:

$$Y = \beta_0 + \sum \beta_i X_i \text{ (i=1, 2, 3.....k)} \quad (1)$$

Where Y is the response (Enzyme activity), β_0 is model intercept, β_i is linear coefficient, X_i is level of the independent variables. The model was statistically analyzed and the overall significance of the model was evaluated by ANOVA (Analysis of variance) involving Fischer's

test (F test). P (probability) values and determination of coefficient obtained determines regression model's goodness of fit.

Box-Behenken Design

After optimizing the various nutritional variables by PBD, the 3 most significant variables (Tannic Acid, KCl and KH₂PO₄) were further chosen for response surface methodology using Box-Behnken design. Design-Expert® statistical software was used to analyze the experimental design. A Box-Behnken design with a set of 12 experiments was generated (Table 4). Each variable was stud-

Table 3. ANOVA for the experiment with PBD for Tannase Production by AT 13

Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	427.76	10	42.78	285.87	0.0460	significant
A-TANNIC ACID	153.08	1	153.08	1023.04	0.0199	
B-KCl	9.12	1	9.12	60.93	0.0811	
C-NaNO ₃	15.01	1	15.01	100.30	0.0634	
D-KH ₂ PO ₄	9.97	1	9.97	66.65	0.0776	
E-K ₂ HPO ₄	11.96	1	11.96	79.93	0.0709	
G-G	77.32	1	77.32	516.71	0.0280	
H-H	69.89	1	69.89	467.08	0.0294	
J-J	43.85	1	43.85	293.07	0.0371	
K-K	19.15	1	19.15	127.99	0.0561	
L-L	18.40	1	18.40	122.98	0.0573	
Residual	0.15	1	0.15			
Cor Total	427.91	11				

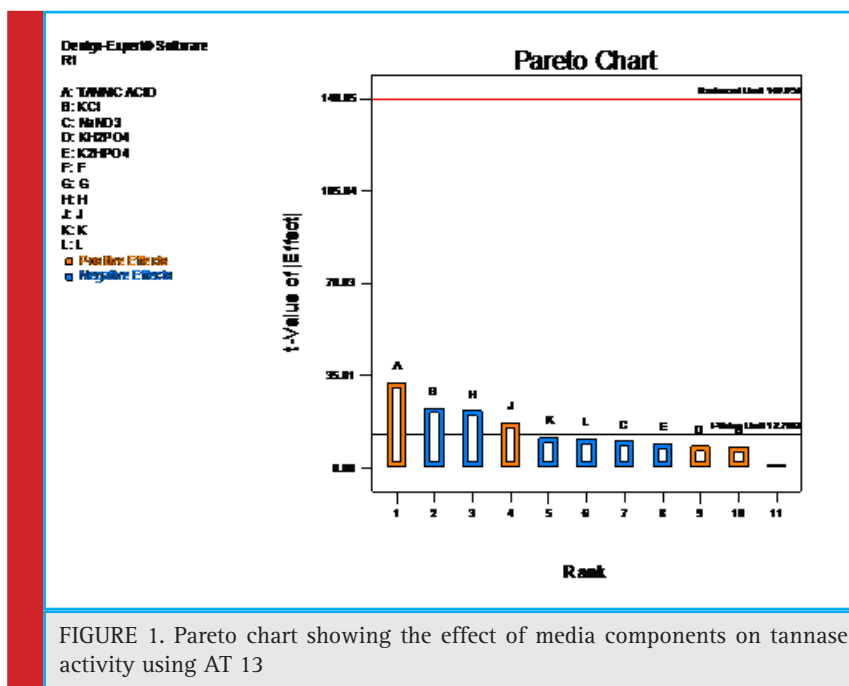


FIGURE 1. Pareto chart showing the effect of media components on tannase activity using AT 13

ied at three levels, +1, 0, -1 where “0” is the central coded value, “+1” high value and “-1” low value. The fermentation was carried out in 250 ml flasks containing 100 ml of the production medium. All experiments were done in triplicate and tannase production obtained was taken as the response.

The obtained results are matched with the polynomial equation 2 in significant order

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β_0 is the intercept term, X_i and X_j are the input variables, β_i the linear

coefficients, β_{ii} the squared coefficients and β_{ij} the interaction coefficients. The model was statistically analyzed and the overall significance of the model was evaluated by ANOVA (Analysis of variance) involving Fischer’s test (F test). P (probability) values and coefficient of determination obtained determines regression model’s goodness of fit. The optimum values of each of the 3 significant variables were determined by solving regression equation.

The interactive effects of the variables on tannase production were studied by analyzing the 3D and counter plots which depicted the interactions graphically.

Validation of the model: The statistical model was again tested by carrying out the fermentation under the optimum conditions that were obtained through the Box-Behnken design.

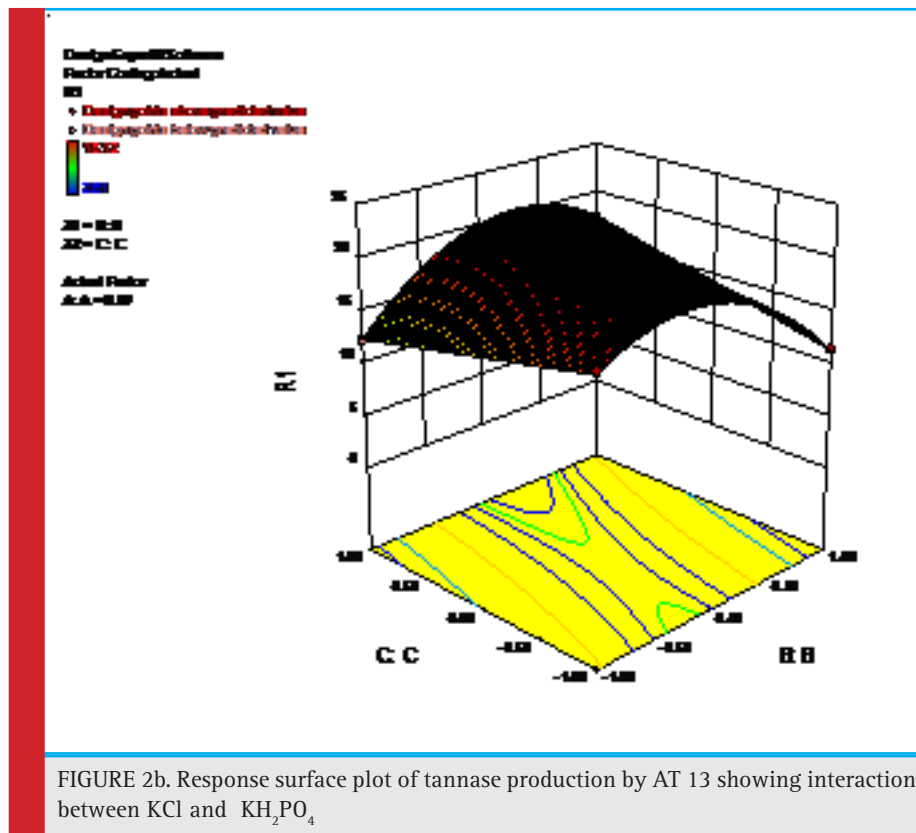
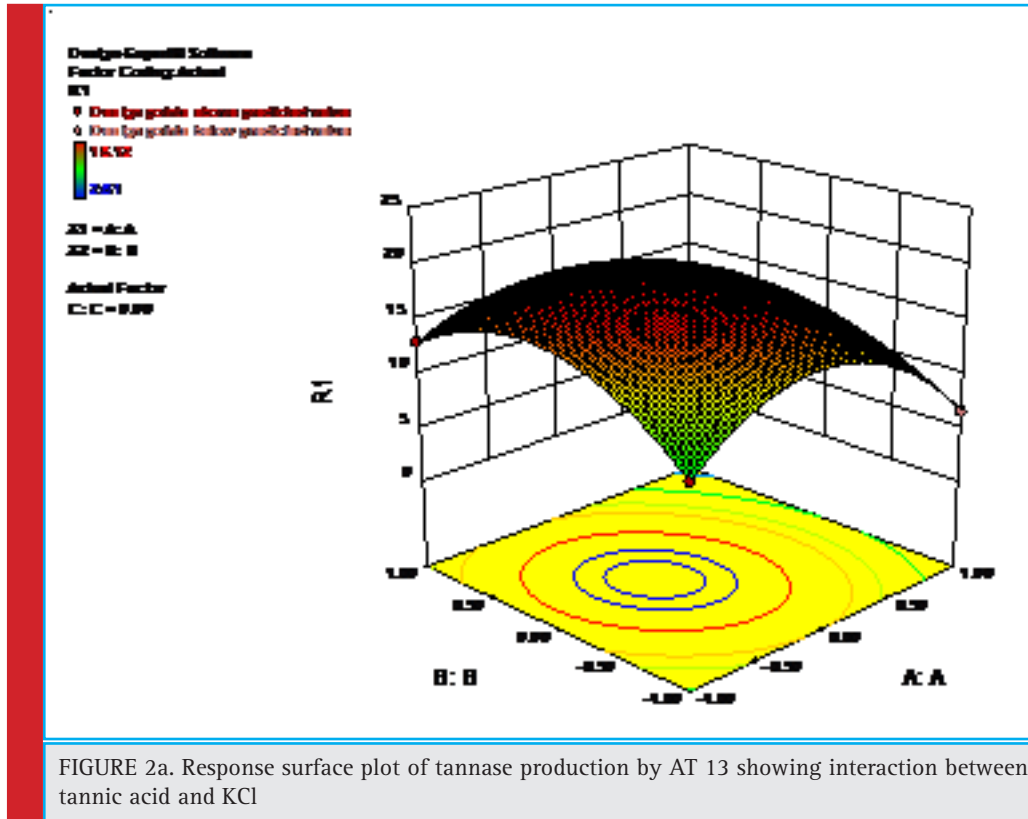
RESULTS AND DISCUSSION

SCREENING OF VARIABLES BY PBD

Important fermentative parameters can be effectively and quickly picked up by applying PBD. The use of statistical tools not only saves time by simultaneously optimizing several process variables with few experimental runs but also reduces the cost of fermentation. A total of 5 important variables Viz., tannic acid, KCl, NaNO_3 , KH_2PO_4 and K_2HPO_4 which were selected for statistical optimization using PBD (Table 1).

Experiments were carried out based on Plackett–Burman design and the results obtained are given in Table 2.

Table 4. Box-Behenken (BB) design matrix of optimizing factors influence for tannase activity				
	Tannic Acid	KCl	KH_2PO_4	E.A
Run	A:A	B:B	C:C	R1
1	-1.00	1.00	0.00	12.99
2	-1.00	0.00	-1.00	14.61
3	0.00	1.00	1.00	17.34
4	0.00	-1.00	1.00	12.33
5	0.00	1.00	-1.00	11.52
6	1.00	0.00	1.00	9.27
7	1.00	0.00	-1.00	10.71
8	1.00	1.00	0.00	2.61
9	-1.00	-1.00	0.00	9.66
10	1.00	-1.00	0.00	6.57
11	0.00	-1.00	-1.00	17.79
12	-1.00	0.00	1.00	18.12



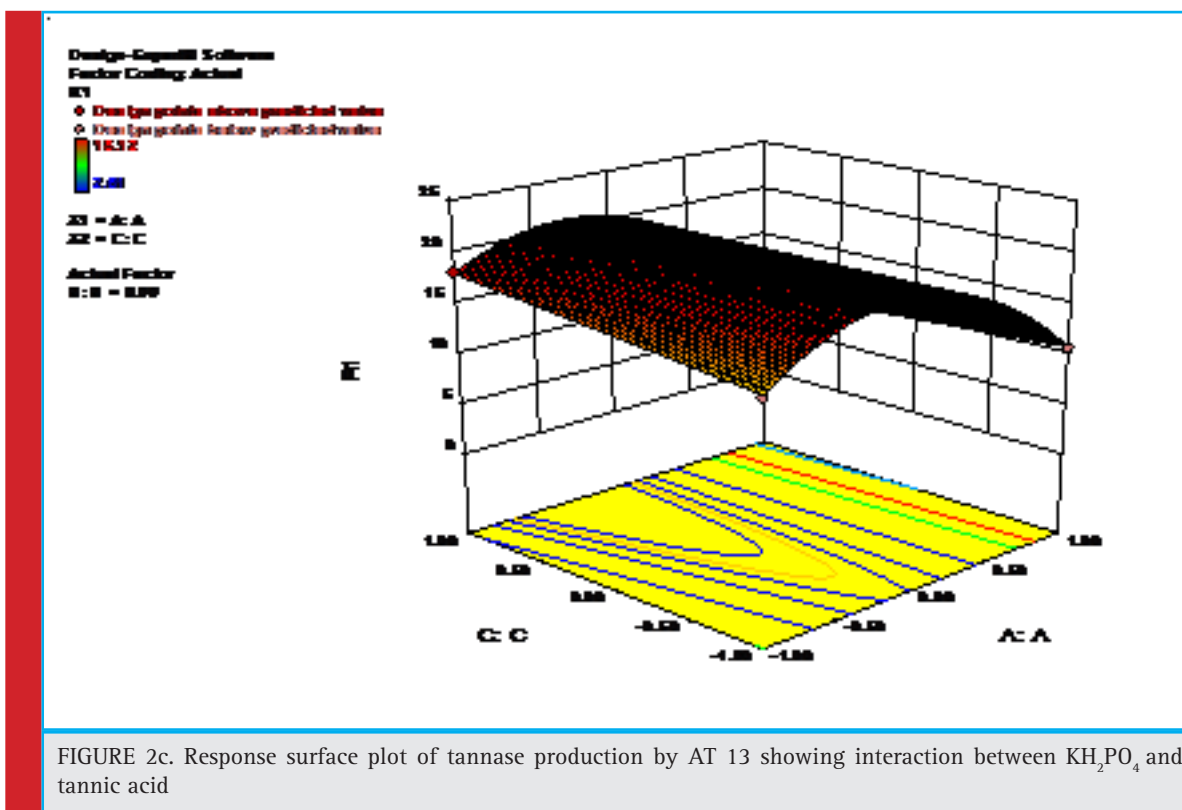


FIGURE 2c. Response surface plot of tannase production by AT 13 showing interaction between KH_2PO_4 and tannic acid

From the table, it was observed that the variation in tannase activity was 0.3–16.53 U/ml/min. From the Pareto chart (Fig. 1), only tannic acid was found to be significant for the production of tannase by AT 13.

Figure 1 show the Pareto chart of effects of variables on tannase production which helps in identifying

important factors that are mainly responsible for enzyme production. From the chart it was evident that the most important contributing factor for tannase production was tannic acid only.

The ANOVA results are given in Table 3. The p-value of ANOVA table serves as a tool for checking the sig-

Table 5. ANOVA for Response Surface Quadratic Model for the production of tannase by AT 13

Analysis of variance table [Partial sum of squares - Type III]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	239.38	8	29.92	187.04	0.0006	significant
A-A	85.94	1	85.94	537.18	0.0002	
B-B	0.45	1	0.45	2.79	0.1934	
C-C	0.74	1	0.74	4.61	0.1209	
AB	13.29	1	13.29	83.05	0.0028	
AC	6.13	1	6.13	38.29	0.0085	
BC	31.81	1	31.81	198.84	0.0008	
A2	92.14	1	92.14	575.97	0.0002	
B2	54.50	1	54.50	340.66	0.0003	
C2	0.000	0				
Residual	0.48	3	0.16	187.04		
Cor Total	239.86	11				

nificance of each of the coefficients and is indicative of interaction strength of each independent variable. P-value less than 0.05 indicate that the model terms are significant. In this experiment, the Model F-value of 285.87 implied that the model is significant in enhancing enzyme production. In this case A (Tannic acid), G (Dummy), H (Dummy) and J (Dummy) gave significant terms and B, C, D, K and E gave negative significant terms.

Optimization of significant variables by Response surface methodology (RSM)

Tannic acid was found to be significant by PBD was optimized by RSM using Box-Behnken design for tannase production. The response values in terms of tannase activity and the matrix design were represented in Table 4.

The results obtained by Box-Behnken were analyzed by Analysis of variance (ANOVA) with a model F-value of 187.04 indicating that the model was significant (Table 5). Values of Prob > F less than 0.05 indicate that the model terms are significant. In the present model A, AB, BC, AC, A², B² are significant model terms. The Model F-value of 187.04 implies the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise. The Values of "Prob > F" less than 0.0500 indicate model terms are significant. This fit of the model was checked with the coefficient of determination R², which was calculated to be 0.9680. The determination coefficient (R²) of the model was 0.9980 indicating that 99.80% of variability in the response could be accounted by the model.

The ANOVA gave the following regression equation:

$$R1 = +19.97 - 3.28^* A - 0.24^* B + 0.30^* C - 1.82^* AB - 1.24^* AC + 2.82^* BC - 6.79^* A^2 - 5.22^* B^2 + 0.000^* C^2$$

Where R1 is the tannase activity; A is tannic acid; B is KCl; C is KH₂PO₄ and the interactions AB, BC, AC, A², B² are significant.

3D Surface Plots

The three-dimensional response surface curves were plotted to study the interaction among different physicochemical factors used and to find out the optimum concentration of each factor for maximum tannase production.

For the construction of 3D plots, effect of two variables was considered while the other variable was held at zero. These plots were helpful in understanding both the individual and the interaction effects of the two factors. For the construction of 3D plots, effect of two variables was considered while the other variable was held at zero. From the 3D plots, it was clear that the tannase activity significantly increased with increase in the con-

centration of tannic acid (A) reaching to its maximum at 18.12 U/ml/min, where as the enzyme activity gradually increased with increase KCl (B) concentration and then declined (Figure 2a). Significant interactions were also present between KCl (B) and KH₂PO₄(C) with a low p-value (0.0008) and between KH₂PO₄(C) and tannic acid(A) with a low p-value (0.0085) (Figure 2b and 2c).

Validation of the Model: Validation of the response surface model was confirmed by using optimum conditions obtained by the model. The experimental values were very close to the predicted values and hence it was concluded that the model was successfully validated.

CONCLUSION

The response surface methodology was applicable for the production of tannase from *Streptomyces sp.* AT13. In the present study, optimum conditions for tannase production by *Streptomyces sp.* AT13 was done using Plackett-Burman followed by Response surface methodology using Box-Behnken design. From among 6 fermentative variables that were studied, 5 significant variables were picked up by PBD. Among 5 variables from PBD, 3 were further optimized by Box-Behnken design. Analysis of variance (ANOVA) showed the significance of the model and the validity of the model was confirmed by the verification experiments. P-value less than 0.05 indicate that the model terms are significant. The optimum values of parameters obtained through RSM were 1 % tannic acid, 0.5 % KCl and 0.1% KH₂PO₄ to achieve maximum tannase production.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interests: There are no conflicts of interest.

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