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ORIGINAL ARTICLE

Statistical optimization for tannase production from *Aspergillus niger* under submerged fermentation

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Abstract Statistically based experimental design was employed for the optimization of fermentation conditions for maximum production of enzyme tannase from *Aspergillus niger*. Central composite rotatable design (CCRD) falling under response surface methodology (RSM) was used. Based on the results of 'one-at-a-time' approach in submerged fermentation, the most influencing factors for tannase production from *A. niger* were concentrations of tannic acid and sodium nitrate, agitation rate and incubation period. Hence, to achieve the maximum yield of tannase, interaction of these factors was studied at optimum production pH of 5.0 by RSM. The optimum values of parameters obtained through RSM were 5% tannic acid, 0.8% sodium nitrate, 5.0 pH, 5 x $I0^7$ spores/50mL inoculum density, 150 rpm agitation and incubation period of 48 h which resulted in production of 19.7 UmL^{-1} of the enzyme. This activity was almost double as compared to the amount obtained by 'one- at- a- time' approach (9.8 UmL^{-1}).

Keywords Tannase · Response surface methodology · *Aspergillus niger* · Fermentation · Statistical analysis

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Introduction

Tannase (tannin acyl hydrolase, E.C.3.1.1.20) is an inducible enzyme that catalyses the breakdown of ester linkages in hydrolysable tannins resulting in the production of gallic acid and glucose[1]. The major applications of this enzyme are in the production of gallic acid, which is used in the manufacture of antimalarial drug, trimethoprim[2] and in the synthesis of propyl gallate used as antioxidants in the food industry[3]. The enzyme also has applications as a clarifier in the production of beer and fruit juices, in the manufacture of instant tea and in the treatment of wastewater contaminated with polyphenolic compounds[2,4]. Conesa *et al.*[5] reported the hydrolysis of diethyldiferulates by tannase from *Aspergillus oryzae* for animal feed improvement.

Realizing the importance of the enzyme tannase, efforts were made to find a suitable micro-organism/s, which may produce higher amounts of tannase. Also, processes are to be developed for their economically viable production. Though, generally, 'one-at-a-time' procedure on low cost substrate and media components results in a process optimization to a certain extent, however, the limitations of this process lies when a large number of factors have to be investigated, as the statistical interactions between factors could not be examined by this approach[6,7]. Optimization through response surface methodology (RSM) is now widely used to evaluate and understand the interactions between different physiological and nutritional parameters[8–11]. This technique is an empirical modeling technique devoted to the evaluation of relations existing within a group of controlled experimental factors and observed results of one or more selected criteria[12]. This includes factorial design and regression analysis which helps in evaluating the effective

factors and building blocks to study interactions and select optimum conditions of variables for a desired response [13,14]. In the present investigation, an attempt was made to optimize the culture conditions for maximizing the production of tannase from *A. niger* using RSM.

Materials and Methods

Micro-organism and its maintenance: A natural isolate of *A. niger* isolated from soil was found to be a potent tannase producer after the extensive screening carried out in our laboratory. This fungus was grown on potato dextrose agar (PDA) slants supplemented with 0.01% tannic acid at 37 ± 1°C for 72 h. The purity of the sporulated culture was checked microscopically and sub-cultured every week.

Enzyme Production: Conidia were harvested from 72 h old culture in 10 mL of sterilized normal saline containing 0.01% sterile Tween-80. Modified Czapek's Dox medium with the composition (g/l): tannic acid (x g); $NaNO_3$ (y g); KCI (0.52 g); $MgS0_4.7H_20$ (0.52 g); KH_2PO_4 (1.52 g); glucose (2.0 g) was used. $Cu(NO_3)_2.3H_2O$; $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$ (1.0 mg each) were added as traces. The pH of the medium was adjusted to 5.0 by 1 N HCl. Stock solution of filter-sterilized tannic acid (25% w/v) was prepared and used at the desired concentration. Approximately, 5×10^7 conidia (in 0.5 mL of sterilized normal saline) were inoculated in each 250 mL flask containing 50 mL of the Czapek's Dox medium. These flasks were incubated at 37 ± 1°C in a New Brunswick Incubator shaker (Model G-26 R) at different agitation rates for different time intervals. After the desired incubation period, biomass was harvested using Whatman filter paper no. 1 and the culture filtrates were analyzed for tannase activity.

Enzyme assay: Tannase activity was estimated by the procedure of Deschamps *et al.*[15] The reaction mixture (4 mL) contained 1 mL of 1% tannic acid (in citrate-phosphate buffer pH 5.0), 2 mL of 0.5 M citrate-phosphate buffer (pH 5.0) and 1 mL of the culture filtrate. The mixture was incubated at 50°C for 30 min in a water bath. The enzyme reaction was terminated by adding 4 mL of 2 % bovine serum-albumin (BSA) solution prepared in citrate-phosphate buffer (pH 5.0). For control preparation, BSA was added in the reaction mixture prior to incubation. All the tubes were kept for 20 min at room temperature to precipitate the residual tannins and subsequently centrifuged at 3000 x g for 20 min. Tannase activity was estimated by diluting 20 µl of the supernatant, 500 fold using double distilled water. The absorbance was read at 260 nm in a UV spectrophotometer (Shimadzu, Model no. 1601) against double distilled water, which was used as blank.

Tannase unit: One unit of tannase is defined as the amount of enzyme required to release one µmol of gallic acid per milliliter of culture filtrate per minute under the standard assay conditions.

Experimental design and Data analysis: The most influential factors for tannase production found by 'one-at-a-time' approach were tannic acid and sodium nitrate concentrations, agitation rate and incubation period. Hence, Central Composite Rotatable Design (CCRD), which falls under RSM, was used to study the interaction of these factors at the optimum production pH of 5.0. The statistical software package 'Design Expert 6.0', Stat- Ease, Inc., Minnaepolis, USA was used to analyze the experimental design. Each factor in the design was studied at five different levels ($-\alpha$, –1, 0, +1, $+\alpha$) (Table 1). A set of 30 experiments was performed. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables investigated and the full experimental plan with respect to their values in actual and coded form is listed in Table 2. Upon completion of experiment, the tannase production was taken as dependent variable or response (Y).

Experimentally, as per the combinations presented in Table 2, different concentrations of sodium nitrate were taken in separate set of flasks containing minimal medium (pH 5.0). Glucose concentration of 0.2 % w/v in the medium was kept constant throughout. These flasks were autoclaved at 10 psi. Now, filter-sterilized tannic acid was added in different permutations and combinations. All the experiments were performed in triplicates to estimate the experimental error and to check the linearity of the experimental set up.

Statistical analysis and modelling: The data of tannase production thus obtained was subjected to analysis of variance (ANOVA), appropriate to the design of experiments. The mathematical relationship of the independent variables and the responses (tannase activity) were calculated by the second order polynomial equation i.e.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

where Y = predicted response; β_0 = intercept; $\beta_1, \beta_2, \beta_3, \beta_4$ = linear coefficients; $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$ = squared coefficients; β_{12}, $\beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$ = interaction coefficients.

Validation of the model: The model was validated by considering different permutation and combination of medium components, selected within the model range so as to fit the second order polynomial equation. Eight sets of experiments were generated and carried out.

Table 1 Levels of the four independent variables (factors) used in RSM.

Variables	Units	Coded value	Range of levels				
			–α	–1	0	+1	+α
Tannic acid	%	A	1.0	3.0	5.0	7.0	9.0
Sodium nitrate	%	B	0.4	0.6	0.8	1.0	1.2
Agitation rate	rpm	C	50	100	150	200	250
Incubation Period	h	D	0	24	48	72	96

Table 2 Central Composite Rotatable Design of the variables with Tannase activity as response.

Run	Tannic acid (%)	Sodium nitrate (%)	Agitation (rpm)	Incubation (h)	Tannase activity (U/mL)	
					Actual	Predicted
1	0	0	0	0	19.43	19.51
2	0	0	0	0	19.70	19.51
3	+1	+1	+1	+1	13.40	13.59
4	–1	+1	–1	–1	08.54	08.57
5	0	0	0	–α	00.00	00.00
6	0	–α	0	0	15.60	15.77
7	+1	–1	+1	–1	08.20	08.26
8	–1	+1	+1	–1	08.80	08.96
9	+α	0	0	0	07.70	07.73
10	0	0	0	+α	06.50	06.38
11	+1	+1	–1	+1	11.40	11.32
12	+1	–1	–1	+1	11.20	11.22
13	0	0	0	0	19.50	19.51
14	0	0	–α	0	14.20	14.16
15	+1	–1	+1	+1	11.20	11.15
16	–1	+1	+1	+1	13.80	13.72
17	0	0	0	0	19.40	19.51
18	–1	–1	–1	+1	12.20	12.01
19	–1	–1	+1	–1	08.20	08.26
20	–α	0	0	0	08.70	08.51
21	0	0	+α	0	14.61	14.49
22	0	0	0	0	19.60	19.51
23	–1	–1	–1	–1	10.21	10.20
24	+1	+1	–1	–1	08.20	08.21
25	+1	+1	+1	–1	08.80	08.97
26	–1	–1	+1	+1	11.40	11.57
27	0	0	0	0	19.41	19.51
28	0	+α	0	0	16.90	16.57
29	+1	+1	–1	–1	09.50	09.56
30	–1	–1	–1	–1	11.40	11.81

Results and Discussion

On the basis of 'one-at-a-time' approach, four factors (8% tannic acid, 0.6% sodium nitrate, agitation rate of 200 rpm and incubation period of 48 h) showed maximum influence on tannase production resulting in the production of 9.8 UmL^{-1} of tannase. Based on these results, the central point and the range of level for each of the factors were selected in CCRD. The concentrations of non-significant factors were set at their corresponding optima as obtained. The

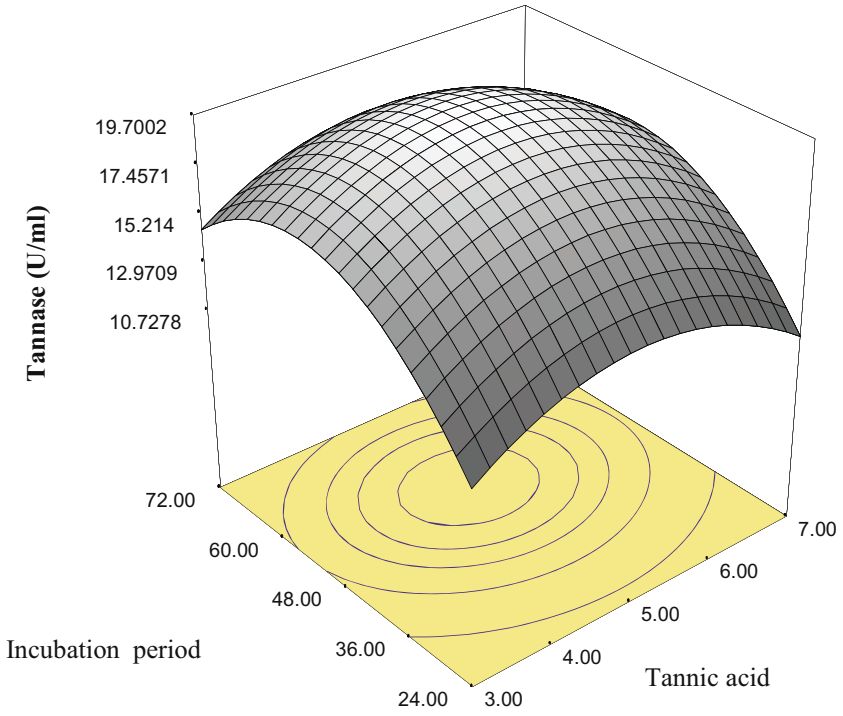

Fig. 1 Three-dimensional plot of tannase activity as a function of tannic acid and incubation period at constant agitation rate (150 rpm) and sodium nitrate concentration (0.8 % w/v).

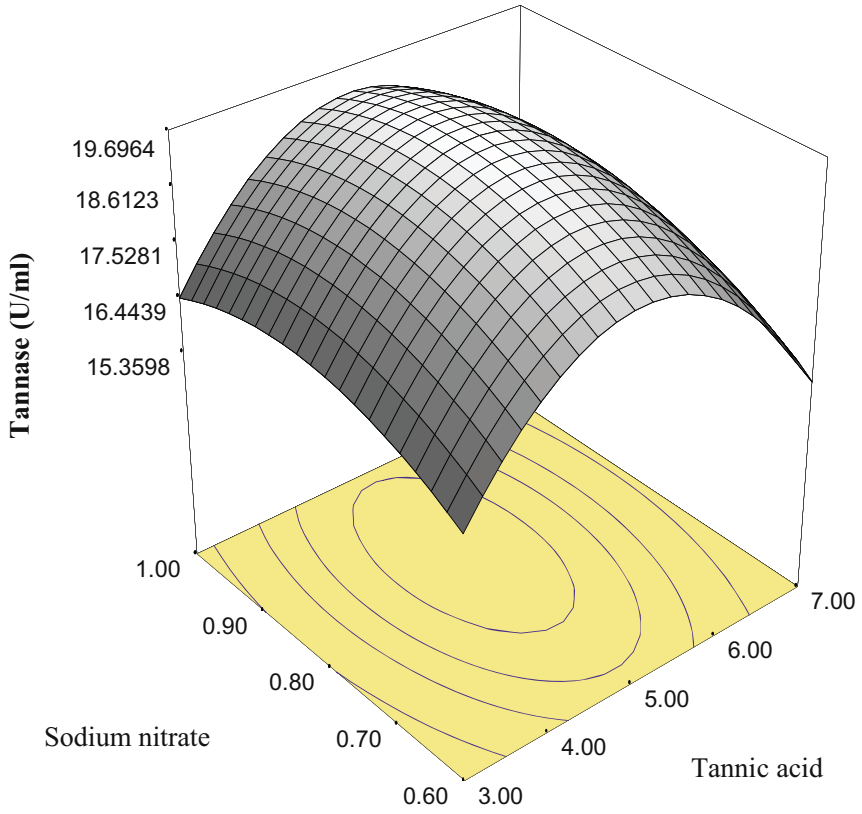

Fig. 2 Three-dimensional plot of tannase activity as a function of tannic acid and sodium nitrate at constant incubation period (48 h) and agitation rate (150 rpm).

Table 3 Analysis of variance for tannase production.

Model terms	**Values**
Std. Dev.	0.21
Mean	12.26
R^2	0.99
Adj R^2	0.99
Pred R^2	0.99
Model F-value	1063.09
Lack of Fit F-Value	4.22
PRESS	3.64
Coefficient of variance	1.74

results of tannase production from these experiments along with the mean predicted and observed response as obtained by CCRD are presented in Table 2. The regression analysis of the experimental data obtained after ANOVA resulted in the following second order polynomial equation-

$$\textit{Tannase activity (Y)} = +19.51 - 0.19 *A + 0.20 *B + 0.082 *C + 1.61 *D - 2.85 *A^2 - 0.83 *B^2 - 1.30 *C^2 - 4.08 *D^2 + 0.073 *A*B + 0.091 *A*C - 0.034 *A*D + 0.59 *B*C + 0.36 *B*D + 0.38 *C*D \quad (2)$$

where Y is the tannase produced as a function of the coded levels of tannic acid (substrate) conc. (A), sodium nitrate concentration (B), agitation rate (C) and incubation period (D).

The coefficient of determination (R^2) was calculated as 0.99 for tannase activity (Table 3). This explains 99 % R squared of the total variation for tannase activity. This clearly shows that this model is an adequate predictor of the experimental conditions and confirms that the selected process parameters significantly influence tannase activity.

The Model F-value of 1063.09 for tannase production and values of Prob > F (<0.05) demonstrated a high significance for the regression model. For tannase production A, B, D, A^2, B^2, C^2, D^2, BC, BD and CD were significant model terms. The 'Lack of Fit F-value' of 4.22 implied that Lack of Fit was insignificant relative to pure error, which indicated that the model was suitable to represent the experimental data. The predicted sum of squares (PRESS), which is a measure of how particular model fits each point in the design, was 3.64. The model was found to be significant for production within the range of variables employed.

The three-dimensional surface plots for tannase activity were constructed to determine the optimal levels of the parameters (variables) according to equation 1. Here, each response was plotted as the function of substrate (tannic acid) concentration, sodium nitrate concentration, agitation rate and incubation period.

Relative effect of two variables (tannic acid and incubation period) on tannase production when sodium nitrate and agitation rate were kept at their central levels (0.8% sodium nitrate and 150 rpm agitation rate) has been depicted in the response surface plot of Fig. 1. Maximum tannase production was 19.7 UmL^{-1} in 48h when the level of tannic acid was at their central value of 5% (v/v). Increasing the tannic acid concentration beyond 5% (v/v) led to decline in tannase production. Also, increase in incubation period beyond 48 h resulted in decline in the enzyme production.

The optimum concentration of 5% tannic acid obtained in the present investigation was in agreement with previous reports of Akoi *et al*.[16] and Lekha and Lonsane[1]. Aguilar *et al*.[2] also reported that in submerged fermentation, tannase secretion was favored by an initial tannic acid concentration of 50 gL^{-1} from *A. niger* Aa-20. However, Bradoo *et al*.[17] reported 2% tannic acid as optimum for tannase production

Table 4 Validation of CCRD using different levels of tannic acid, sodium nitrate, agitation rate and incubation period for tannase production.

S. No	Tannic acid (%)	Sodium nitrate (%)	Agitation rate (rpm)	Incubation period (h)	Tannase activity (U/mL)	
					Predicted	Observed
1	5.0	0.8	150	48	19.70	19.61
2	3.0	0.8	150	48	17.07	17.20
3	5.0	0.6	150	48	18.48	18.32
4	3.0	0.9	150	48	16.72	16.53
5	5.0	0.8	100	48	18.24	18.42
6	5.0	0.8	150	24	13.99	14.20
7	5.0	0.7	100	48	18.14	18.43
8	5.0	0.9	150	36	17.27	17.42
9	5.0	0.8	150	72	18.20	18.00
10	5.0	0.8	150	96	6.50	6.38

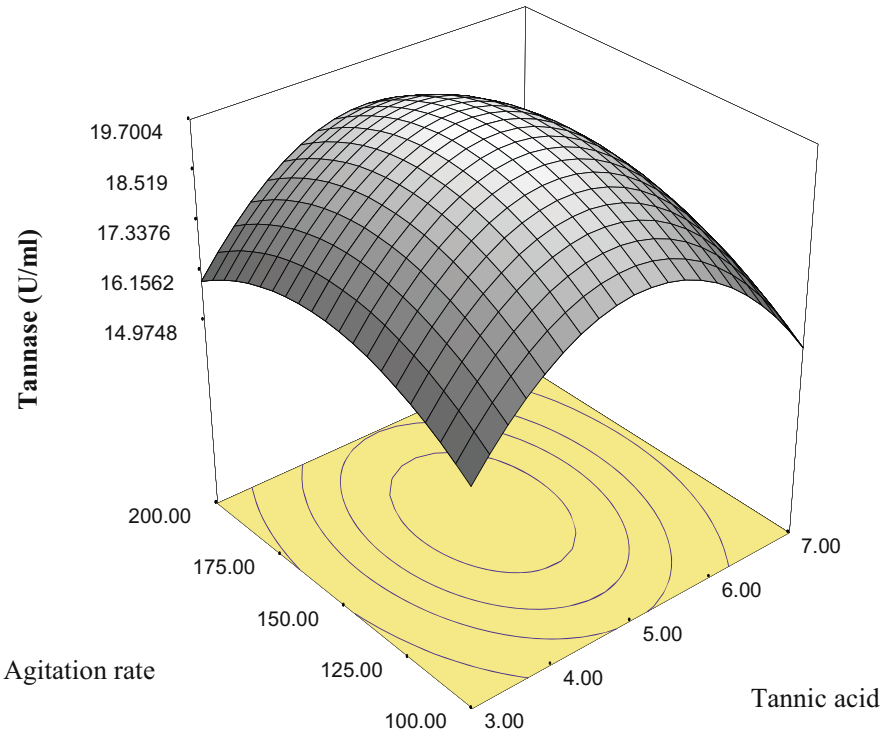

Fig. 3 Three-dimensional plot of tannase activity as a function of tannic acid and agitation rate at constant sodium nitrate concentration (0.8 % w/v) and incubation period (48 h).

from *A. japonicus*. Hadi *et al*.[18] also reported maximum enzyme production of 6.12 UmL^{-1} at 2 % tannic acid from *R. oryzae*.

For maximum tannase production (19.7 UmL^{-1}), an incubation period of 48 h was found to be the optimum in the present investigation, which declines on further incubation. It has also been reported that tannase is produced during the primary phase of growth and thereafter declines[19]. Kar and Banerjee[20] also found 48 h as optimum incubation period for the maximum tannase production (23.86 UmL^{-1}) for *R. oryzae*. However, Hadi *et al*.[18] and Lekha and Lonsane[4] reported maximum tannase production in 120 h and 144 h from *R. oryzae* and *Aspergillus* PKL 104 respectively.

Further, sodium nitrate concentration was also effective for tannase production. An increase in sodium nitrate concentration from 0.6 to 0.8% w/v resulted in an increase in the tannase activity from 9.75 UmL^{-1} to 19.7 UmL^{-1} when incubation period and agitation rate were at their central values of 48h and 150 rpm (Fig. 2). Contrary to our findings, Bradoo *et al*.[17] reported 0.2 % w/v sodium nitrate as optimal for growth and tannase production from *A. japonicus*. However, Hadi *et al*.[18] reported sodium nitrate concentration as low as 0.05% w/v optimal for tannase production from *Rhizopus oryzae*. An interaction between agitation rate and tannic acid at constant sodium nitrate concentration (0.8%) resulted in maximum yield of 19.7 UmL^{-1} of tannase in 48 h at an agitation rate of 150 rpm (Fig. 3). Thus, lowering of agitation rate from 200 to 150 rpm resulted in the rise in enzyme production.

From Figs. 1, 2 and 3 tannic acid (5.0 % w/v), sodium nitrate (0.8 % w/v), agitation rate 150 rpm and incubation period of 48 h were adequate for attaining maximum tannase yield (19.7 UmL^{-1}).

Validation of the model

The results of random set of ten experiments (Table 4) clearly showed that experimental values were found to be very close to the predicted values and hence the model was successfully validated. Validation experiments under optimal conditions showed that the predicted data of tannase activity (19.7 UmL^{-1}) was in accordance with the experimental data (19.61 UmL^{-1}). This confirms the validity of the experimental results carried out for tannase production.

Conclusion

The response surface methodology resulted in a 2.0 fold increase (from 9.8 UmL^{-1} to 19.7 UmL^{-1} in 48 h) in tannase production by *Aspergillus niger.* Therefore, the optimum

composition for tannase production from *A. niger* using RSM was 5% tannic acid, 0.8% sodium nitrate, 5.0 pH, 5 x $I0^7$ spores/50mL inoculum density, 150 rpm agitation and incubation period of 48 h.

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