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OPTIMIZATION OF EXTRACELLULAR TANNASE PRODUCTION BY *ASPERGILLUS NIGER* VAN TIEGHEM USING RESPONSE SURFACE METHODOLOGY

Hamada Abou-Bakr, Malak El-Sahn, Amr El-Banna*

Address(es): Hamada Abou-Bakr
[1]Department of Food Science and Technology, Faculty of Agriculture, Alexandria University, Aflaton St., El-Shatby, P.O.Box 21545, Alexandria, Egypt.

*Corresponding author: hamada.metwali@alex-agr.edu.eg

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ABSTRACT

Response surface methodology (RSM) was used to optimize the production of tannase by a newly isolate of *Aspergillus niger* Van Tieghem using rotatable central composite design (RCCD). This statistical optimization process was carried out involving four of quantitative growth parameters (variables), namely tannic acid concentration, nitrogen source concentration, initial pH of the medium and inoculum size. A mathematical model expressing the production process of tannase by submerged fermentation (SmF) technique was generated statistically in the form of a second order polynomial equation. The model indicated the presence of significant linear, quadratic and interaction effects of the studied variables on tannase production by the fungal isolate. The results showed maximum tannase production (580 U/50 ml medium) at 2% tannic acid, 4 g/l sodium nitrate, pH 4 and inoculum size of 5×10^7 spores/50 ml medium, which was also verified by experimental data.

Keywords: Tannase optimization, response surface methodology, rotatable central composite design, *Aspergillus niger* Van Tieghem, fungal tannin acyl hydrolase

INTRODUCTION

Tannase (tannin acyl hydrolase, EC 3.1.1.20) hydrolyses the ester bond between sugar and gallic acid in hydrolysable tannin molecules (**Bradoo *et al.*, 1997**). The enzyme has been used extensively in the food, beverage, pharmaceutical and chemical industries (**Kumar et al. 2010**).Tannase has been isolated from bacteria, moulds and yeasts (**Albertse, 2002; Banerjee and Pati, 2007; Rodrigues *et al.*, 2008**). Few works exist on process optimization of tannase production (**Bradoo *et al.*, 1997**).

Realizing the importance of the enzyme, efforts are underway to develop processes for the economically production of tannase. Two kinds of variables are affecting tannase production, qualitative and quantitative variables. Generally, one variable at a time (OVAT) procedure is suitable to study the qualitative variables, where quantitative variables need statistically based experimental designs.

Lekha *et al.* (1994) pointed out that inspit of the importance of studying the interactions between factors influencing tannase production, there are few researches about this point. They studied the effects of five independent factors [initial pH (3.5-7.5), fermentation temperature (20-35 ° C), initial moisture content (30-70%), inoculum ratio (5-20%) and fermentation time (1-7 days)] on tannase production by *Aspergillus niger* PKL 104 under solid state fermentation (SSF) using response surface methodology. They found a positive interaction between the initial pH and initial moisture level but no interaction between initial pH and other factors. Also, they confirmed that initial moisture level and inoculum ratio showed negative interaction in contrast to positive interaction between inoculum ratio and fermentation period. Likewise, **Sharma et al. (2007)** studied the interaction between four independent factors (% tannic acid; % sodium nitrate; agitation rate, and incubation time) affecting production of tannase by *Aspergillus niger* under submerged fermentation (SmF). The statistical study was carried out using the rotatable central composite design (RCCD) method. Results confirmed the presence of only an interaction between agitation rate and % tannic acid at constant sodium nitrate concentration (0.8 %).

In a previous work, *Aspergillus niger* Van Tieghem was selected as a promise producer of tannase after screening of 105 fungal isolates from environmental samples. Furthermore, two stages of a three stages-optimization process for tannase production were carried out. The effect of qualitative variables (medium constituents, type of substrate and fermentation techniques) was studied in the first stage using one variable at a time (OVAT) procedure. In the second stage of optimization, the effects of fermentation time and temperature, under the optimum conditions obtained from the first stage, were studied using split plot design (**Abou-Bakr, 2010**).

In present study the third stage of optimization process were carried out statistically using RCCD which is one of RSM designs. The effect of four quantitative factors affecting the production of extracellular tannase by *Aspergillus niger* Van Tieghem has been studied in addition to their interaction effects.

MATERIAL AND METHODS

Microorganism

Aspergillus niger Van Tieghem was isolated from tannery soil sample (**Abou-Bakr, 2010**). It was identified by Assiut Univirsity Mycological Center, Egypt. The maintenance code number is "AUMC 5614".

Chemicals

All chemicals used were of analytical grade and obtained from Sigma, Aldrich, Merck and VEB Laborchemie Apolda (Germany); BDH, JUDEX chemicals, LAB M™ and Oxoid (England); Biolife (Italy); Chemapol (Czechoslovakia) and El-Nasr Pharma-ceutical Chemicals Co. "ADWIC" (Egypt).

Cultural media

The modified Czapek-Dox's minimal (MCDM) medium was used as fermentation medium and prepared from the following ingredients (g/l): tannic acid (10), $NaNO_3$ (6), KCl (0.52), $MgSO_4{\cdot}7H_2O$ (0.52), KH_2PO_4 (1.52), $FeSO_4{\cdot}7H_2O$ (0.01), $ZnSO_4{\cdot}7H_2O$ (0.01), $Cu(NO_3)_2{\cdot}3H_2O$ (0.01). The medium was adjusted at pH 4.5± 0.2 and sterilized at 121 ºC for 15 min. The solution of tannic acid was sterilized separately by passing through MILLEX®-OR membrane filter (33 mm diameter, 0.22 µm pore size, Millipore, France) and adjusted separately at pH 4.5 ± 0.2, then added to the medium (**Bradoo *et al.*, 1996**).

Potato dextrose agar (PDA) supplemented with 0.01% tannic acid was used as maintenance medium. It was prepared as mentioned by **Bajpai and Patil (1997)**.

Inoculum preparation

Inoculum was prepared by the method described by **Ramirez-Coronel *et al.* (2003)**. The fungal isolate was cultivated on PDA slant then incubated for 4 days at 30 ºC until a good sporulation was obtained. Spores were then scraped into a sterile 0.02 % Tween 80 solution and counted in a Neubauer chamber (Hemocytometer slide) by the method described by **Harisha (2007)**. The suitable volume which contains the needed number of spores was calculated.

Harvesting the enzyme and enzyme assay

The fermentation medium was filtered through Whatman No.1 filter paper. The obtained filtrate was used for extracellular tannase determination (**Gupta *et al.*, 1997**). Tannase activity was assayed using the spectrophotometric method of **Sharma *et al.* (2000)**. Methyl gallate was used as substrate. The method is based on the chromogen formation between gallic acid and rhodanine. The method was modified by using 0.2 ml of 1 N potassium hydroxide solution instead of 0.5 N for enhancing the colour formation. One unit of the enzyme (U) was defined as micromoles of gallic acid formed per minute by one ml of enzyme extract.

Standard protocol

Sterilized MCDM medium (pH 4.5±0.2) containing 1% filter sterilized tannic acid was distributed in 50 ml portions into 250 ml Erlenmeyer flasks. Each flask was inoculated with 5×10^7 spores from 4-days-old culture and incubated for 96 h at 35 ºC (**Saxena and Saxena 2004**). The incubation was carried out with intermittent shaking three times a day at 200 rpm for 2 min each time using an orbital incubator (Model INR-200, Gallenkamp, UK). Deviations from this standard protocol have been indicated in respective experiments. After harvesting the culture filtrate, the enzyme was assayed.

Statistical analysis and modeling

Rotatable Central Composite Design (RCCD), which falls under response surface methodology (RSM), was used to study the main effects (linear and quadratic) and interactions of four factors (tannic acid concentration, nitrogen source concentration, initial pH and inoculum size). The factors are usually referred to as independent variables. Each variable in this design was studied at five coded levels **(**–α, –1, 0, +1, +α). The actual levels of variables versus their coded levels are shown in Table 1. The relation between the coded values and actual values were as described in the equation (1).

$$X_i = \frac{U_i - U_0}{\Delta U} \qquad (1)$$

Where X_i is the coded value of independent variable, U_i the actual value of the independent variable, U_0 is the actual value of the independent variable at the central level (zero level) and ΔU is the step change.

Table 1 Coded levels and actual values of the variables in RCCD

Variables	Units	Coded value	Range of levels (coded and actual)				
			- α (-2)	-1	0	+1	+α (+2)
Tannic acid concentration	%	X_1	0.5	1	1.5	2	2.5
Nitrogen source concentration	g/L	X_2	2	4	6	8	10
pH value	–	X_3	3	4	5	6	7
Inoculum size	spores / 50 ml medium	X_4	10^7	$5x10^7$	$9x10^7$	$1.3x10^8$	$1.7x10^8$

A five levels-four variables RCCD, was adopted in this study, consisted of 31 treatments, which included sixteen factorial points, eight star (axial) points, and seven central points (Table 2).

The mathematical relationship of the independent variables and the responses (tannase U/50 ml medium) were calculated by the second-order polynomial equation (equation 2).

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \qquad (2)$$

Where Y is the response, β_0 intercept, β_i the linear regression coefficient, β_{ii} the quadratic regression coefficient, and β_{ij} the interaction regression coefficient. X_i and X_j are the independent variables.

The quality-of-fit of the second-order polynomial model was expressed by the determination coefficient (R^2), and its statistical significance was examined by F value. The significance of the regression coefficients was determined by *t* value. The analysis was made using actual units. The data of tannase production obtained was subjected to analysis of variance (ANOVA), appropriate to the design of experiments, using the statistical software package "STATESTICA 10", StatSoft, Inc., USA. Surface response three dimensional plots describe the interaction effects of each two variables on the response, when the other variables of the design were set to a constant value (generally the coded level zero).

RESULTS AND DISCUSSION

Regression analysis and mathematical model

Thirty one treatments were carried out to study the effects of four factors on extracellular tannase activity (U/50 ml medium). The experimental and predicted responses of all 31 treatments (variable combinations) are presented in Table 2. Regression analysis of the data presented in Table 3 resulted in the second-order polynomial equation (3), which describes the tannase activity (*Y*) as a function of the coded levels of tannic acid concentration (X_1), nitrogen source concentration (X_2), initial pH of the medium (X_3) and inoculum size (X_4).

$$\boldsymbol{Y} = -35.67 + 863.59\boldsymbol{X_1} + 21.23\boldsymbol{X_2} - 66.57\boldsymbol{X_3} - 5.4\times10^{-6}\boldsymbol{X_4} - 69.95\boldsymbol{X_1^2} - 1.84\boldsymbol{X_2^2} + 5.43\boldsymbol{X_3^2} - 1.07\times10^{-14}\boldsymbol{X_4^2} - 33.16\boldsymbol{X_1X_2} - 56.89\boldsymbol{X_1X_3} + 4.3\times10^{-7}\boldsymbol{X_1X_4} + 2.48\boldsymbol{X_2X_3} + 3.9\times10^{-7}\boldsymbol{X_2X_4} + 7.3\times10^{-7}\boldsymbol{X_3X_4} \qquad (3)$$

Regression coefficients of the model were evaluated by testing their significance using both regression analysis (Table 3) and analysis of variance (ANOVA) (Table 4). It was found that eight effects **{**two linear effects ($X_1$and X_4), two quadratic effects (X_1^2 and X_4^2), and four interaction effects (X_1X_2, X_1X_3, X_2X_4 and X_3X_4)**}** were significant.

Table 2 The full design, experimental and predicted responses of RCCD

Treatment No.	Coded levels of variables				Actual Levels of variables				Response (Y) Tannase activity (U/50 ml medium)		
	X_1	X_2	X_3	X_4	X_1 Tannic acid conc. (%)	X_2 Nitrogen source conc. (g/l)	X_3 pH	X_4 Inoculum size (spores/50ml medium)	Experimental	Predicted	Residual
1	- 1	- 1	- 1	- 1	1	4	4	5×10^7	255.9	264.8	-8.9
2	+1	- 1	- 1	- 1	2	4	4	5×10^7	598.0	580.0	18.0
3	- 1	+1	- 1	- 1	1	8	4	5×10^7	214.0	246.8	-32.8
4	+1	+1	- 1	- 1	2	8	4	5×10^7	441.9	429.4	12.5
5	- 1	- 1	+1	- 1	1	4	6	5×10^7	161.9	219.5	-57.6
6	+1	- 1	+1	- 1	2	4	6	5×10^7	432.6	421.0	11.6
7	- 1	+1	+1	- 1	1	8	6	5×10^7	260.5	221.4	39.1
8	+1	+1	+1	- 1	2	8	6	5×10^7	240.0	290.2	-50.2
9	- 1	- 1	- 1	+1	1	4	4	1.3×10^8	79.1	75.6	3.5
10	+1	- 1	- 1	+1	2	4	4	1.3×10^8	418.6	425.5	-6.9

11	- 1	+1	- 1	+1	1	8	4	1.3×10^{8}	203.7	183.1	20.6
12	+1	+1	- 1	+1	2	8	4	1.3×10^{8}	411.2	400.3	10.9
13	- 1	- 1	+1	+1	1	4	6	1.3×10^{8}	167.4	147.7	19.7
14	+1	- 1	+1	+1	2	4	6	1.3×10^{8}	369.8	383.7	-13.9
15	- 1	+1	+1	+1	1	8	6	1.3×10^{8}	210.2	274.9	-64.7
16	+1	+1	+1	+1	2	8	6	1.3×10^{8}	419.5	378.4	41.1
17	- α	0	0	0	0.5	6	5	9×10^{7}	99.5	66.2	33.3
18	+ α	0	0	0	2.5	6	5	9×10^{7}	466.0	484.8	-18.8
19	0	- α	0	0	1.5	2	5	9×10^{7}	337.7	327.7	10.0
20	0	+ α	0	0	1.5	10	5	9×10^{7}	308.8	304.3	4.5
21	0	0	- α	0	1.5	6	3	9×10^{7}	385.1	400.8	-15.7
22	0	0	+ α	0	1.5	6	7	9×10^{7}	363.7	333.5	30.2
23	0	0	0	- α	1.5	6	5	10^{7}	354.4	327.5	26.9
24	0	0	0	+ α	1.5	6	5	1.7×10^{8}	214.0	226.4	-12.4
25	0	0	0	0	1.5	6	5	9×10^{7}	352.6	345.4	7.2
26	0	0	0	0	1.5	6	5	9×10^{7}	368.4	345.4	23.0
27	0	0	0	0	1.5	6	5	9×10^{7}	362.8	345.4	17.4
28	0	0	0	0	1.5	6	5	9×10^{7}	380.5	345.4	35.1
29	0	0	0	0	1.5	6	5	9×10^{7}	300.5	345.4	-44.9
30	0	0	0	0	1.5	6	5	9×10^{7}	343.3	345.4	-2.1
31	0	0	0	0	1.5	6	5	9×10^{7}	310.0	345.4	-35.4

Table 3 Regression coefficients of the second order polynomial model

	Factor's coefficients	Estimated regression coefficient	Standard error	$t_{(16)}$	p-value
Linear effects	β_0 Intercept	-35.6724	353.99	-0.1008	0.92098 ^{ns}
	β_1 Tannic acid concentration	863.5879	153.61	5.6220	0.000038 **
	β_2 Nitrogen source concentration	21.2345	38.40	0.5530	0.58794 ^{ns}
	β_3 pH	-66.5663	88.60	-0.7513	0.46339 ^{ns}
	β_4 Inoculum size	-5.4×10^{-6}	1.8×10^{-6}	-2.918	0.01005 *
Quadratic effects	β_{11}	-69.9470	29.70	-2.3552	0.03161 *
	β_{22}	-1.8404	1.86	-0.9915	0.33619 ^{ns}
	β_{33}	5.4257	7.42	0.7308	0.47549 ^{ns}
	β_{44}	-1.07×10^{-14}	4.6×10^{-15}	-2.3064	0.03481 *
Interaction effects	β_{12}	-33.1562	9.923	-3.3404	0.00415 **
	β_{13}	-56.8875	19.86	-2.8656	0.01121 *
	β_{14}	4.3×10^{-7}	4.96×10^{-7}	0.8721	0.39606 ^{ns}
	β_{23}	2.4781	4.963	0.4993	0.62435 ^{ns}
	β_{24}	3.9×10^{-7}	1.2×10^{-7}	3.1590	0.00608 **
	β_{34}	7.3×10^{-7}	2.5×10^{-7}	2.9538	0.00934 **

Legends: * = Significant at $P< 0.05$, ** = Significant at $P< 0.01$, ns = Non significant at $P< 0.05$

Adequacy of the model

The ANOVA was constructed for the check of the model adequacy (Table 4). It showed that the model was significant. The determination coefficient ($\mathbf{R^2}$) of the obtained second-order polynomial equation was 0.94. It means that the model can explain 94 % of the variation in the response and indicates that the equation is capable of representing the system under the given experimental domain. The adjusted determination coefficient (**adjusted $\mathbf{R^2}$**) was 0.88, indicating a good agreement between the experimental and predicted values of tannase activity. The "**lack of fit**" MS was not significant indicating the adequacy of the present equation.

Interaction effects

The above-mentioned equation was employed to obtain the three-dimensional plots (Fig. 1, 2, 3 and 4) showing the effect of significant variables on tannase production. The effect of tannic acid and nitrogen source concentrations on tannase production by the fungus at pH 5 and inoculum size of 9×10^{7} spores/50 ml medium is shown in Fig. 1. There was a clear positive effect of tannic acid concentration which was indicated also by the highly significant positive regression coefficients of linear (B_1) parameter of tannic acid (Table 3). Production of tannase increased from 66 to 485 U/50 ml medium when concentration of tannic acid increased from 0.5 to 2.5 % (Table 2 and Fig. 1). **Bradoo *et al*. (1997)** and **Banerjee *et al*. (2001)** found that maximum production of tannase was obtained at 2% tannic acid as carbon source in MCDM medium using *Aspergillus japoicus* and *Aspergillus aculeatus* DBF. **Sharma *et al.* (2007)** reported that the statistical optimization of tannase production in MCDM medium by *Aspergillus niger* showed that maximum tannase was yielded at 5 % tannic acid. **Raaman *et al.* (2010)** revealed that *Paecilomyces variotii* tannase production under SmF technique using tannic acid medium increased with increasing substrate concentration up to 1.5 % beyond which it decreased. When adding the levels of nitrogen source and tannic acid concentration into consideration, there was a low increase in tannase activity with the increase of tannic concentration at the higher level of nitrogen source concentration whereas, at lower level of nitrogen source concentration the increase in tannic acid concentration led to higher increase in tannase activity. This confirms the presence of the highly significant negative interaction effect on tannase activity between these two variables (Table 3). This significant interaction effect between tannic acid concentration and nitrogen source (sodium nitrate) concentration on tannase production is in contradictory with the result of a similar study of **Sharma *et al*. (2007)**. They reported that there was no interaction effect between tannic acid concentration and sodium nitrate concentration on tannase production by *Aspergillus niger* under SmF. They also found that sodium nitrate concentration in MCDM was effective for tannase production by *Aspergillus niger*. They observed that increasing sodium nitrate concentration from 0.6 to 0.8 w/v was accompanied by an increase in tannase activity from 9.7 U/ml to 19.4 U/ml.

Table 4 Analysis of variance of RCCD

Source of variation S.O.V	Sum of squares SS	Degree of freedom DF	Mean square MS	F	*P*
X_1 Tannic acid conc. (L)	262901.7	1	262901.7	166.7751	< 0.000001**
X_2 Nitrogen source conc.(L)	817.8	1	817.8	0.5188	0.481744 ns
X_3 pH (L)	6777.1	1	6777.1	4.2992	0.054637 ns
X_4 Inoculum size (L)	15306.6	1	15306.6	9.7099	0.006652 **
X_1^2 Tannic acid conc. (Q)	8744.2	1	8744.2	5.5470	0.031608 *
X_2^2 Nitrogen source conc. (Q)	1549.8	1	1549.8	0.9831	0.336191 ns
X_3^2 pH (Q)	841.8	1	841.8	0.5340	0.475487 ns
X_4^2 Inoculum size (Q)	8385.4	1	8385.4	5.3194	0.034806 *
X_1X_2 (Interaction)	17589.4	1	17589.4	11.1581	0.004151 **
X_1X_3 (Interaction)	12944.8	1	12944.8	8.2117	0.011213 *
X_1X_4 (Interaction)	1198.9	1	1198.9	0.7605	0.396061 ns
X_2X_3 (Interaction)	393.0	1	393.0	0.2493	0.624347 ns
X_2X_4 (Interaction)	15731.4	1	15731.4	9.9794	0.006079 **
X_3X_4 (Interaction)	13753.4	1	13753.4	8.7247	0.009337 **
Residual	**25222.2**	**16**	**1576.4**		
Lack of Fit	19833.0	10	1983.3	2.2081	0.172326 ns
Pure Error	5389.2	6	898.2		
Total SS	391034.5	30			

Legends: * = Significant at *P*< 0.05, ** = Significant at *P*< 0.01, ns = Not significant at *P*< 0.05, (L)= Linear effect, (Q)= Quadratic effect

Determination coefficient of the model (R^2) = 0.94 (means that the model can explain 94% of the variation in the response and indicates that the second-order polynomial equation is capable of representing the system under the given experimental domain). **Adjusted R^2= 0.88** (indicates a good agreement between the experimental and predicted values of tannase activity). The insignificance of '**lack of fit'** indicates the adequacy of the present equation.

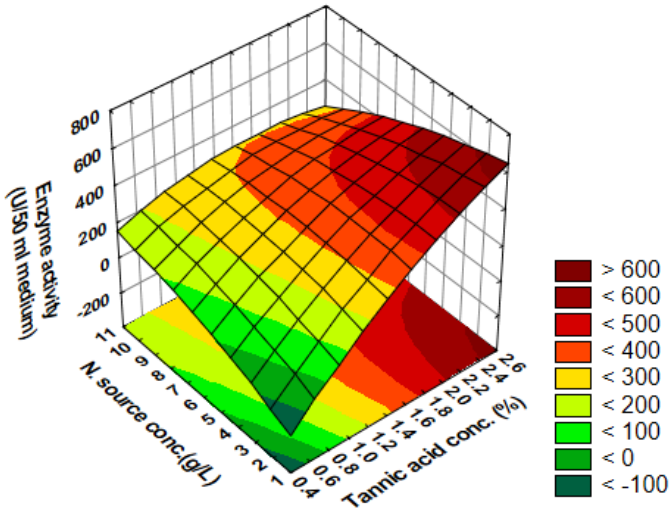

Figure 1 Response surface and contour diagrams for tannase activity considering tannic acid concentration and nitrogen source concentration at the central levels of initial pH (5) and inoculum size (9×10^7 spores/50 ml medium).

Figure 2 shows the effect of tannic acid concentration and medium initial pH on tannase production by the fungus at 6 g nitrogen source/liter of the medium and inoculum size of 9×10^7 spores/50 ml medium. There was a low increase in tannase activity with the increase of tannic concentration at the higher level of pH whereas, at lower level of pH the increase in tannic concentration led to higher increase in tannase activity. This indicates the presence of significant negative interaction effect between these two variables on tannase activity (Table 3).

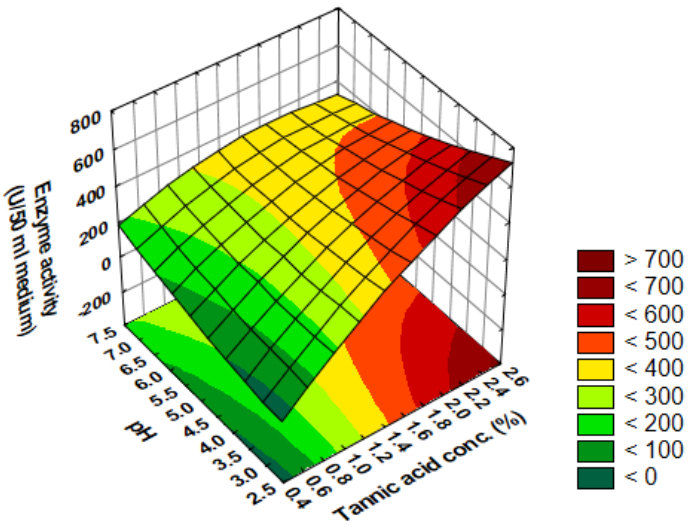

Figure 2 Response surface and contour diagrams for tannase activity considering tannic acid concentration and initial pH of the medium at the central levels of nitrogen source concentration (6 g/L) and inoculum size (9×10^7 spores/50 ml medium).

The effect of nitrogen source concentration and inoculum size on tannase production by the fungus at the central levels of tannic acid concentration (1.5%) and medium initial pH (5) is shown in Fig. 3. There was a negative effect of inoculum size on tannase activity, which was indicated by the negative regression coefficient of linear parameter (β_4) presented in Table 3. Production of tannase decreased from 327 to 226 U/50 ml medium when inoculum size increased from 10^7 to 1.7×10^8 spores/50 ml medium (Table 2 and Fig. 3). When adding the level of nitrogen source and inoculum size into consideration, there was an increase in tannase response with the increase in nitrogen source concentration at the higher level of inoculum size as shown in Fig. 3 whereas, at lower level of inoculum size the increase in nitrogen source concentration led to a decrease in tannase activity response. This confirmed the presence of highly significant positive interaction effect between these two variables on tannase activity as indicated by the positive interaction regression coefficient (β_{24}) presented in Table 3. Also, at lower level of nitrogen source concentration, the increase in inoculum size led to decrease in tannase activity response while the opposite behavior is observed at the higher level of nitrogen source concentration. It is also observed that the higher response attained at lower levels combination of these two variables (Fig. 3).

Figure 4 shows the effect of medium initial pH and inoculum size on tannase production at the central levels of tannic acid concentration (1.5%) and nitrogen source concentration (6 g/l). There was an increase in tannase response with the increase in initial pH of the medium at the higher level of inoculum while, at lower level of inoculum size the increase in pH led to high decrease in tannase activity response. This confirmed the presence of highly significant positive interaction effect between these two variables on tannase activity as indicated by the positive interaction regression coefficient (β_{34}) presented in Table 3. Likewise, at lower level of medium initial pH, the increase in inoculum size led to decrease in tannase activity response while the opposite behavior was observed at the higher level of medium initial pH. It was also observed that the higher

response attained at lower levels combination of these two variables. These results are in disagreement with the study of **Lekha *et al.* (1994)**. They found that there is no interaction effect between initial pH and inoculum size on tannase production by *Aspergillus niger* PKL 104.

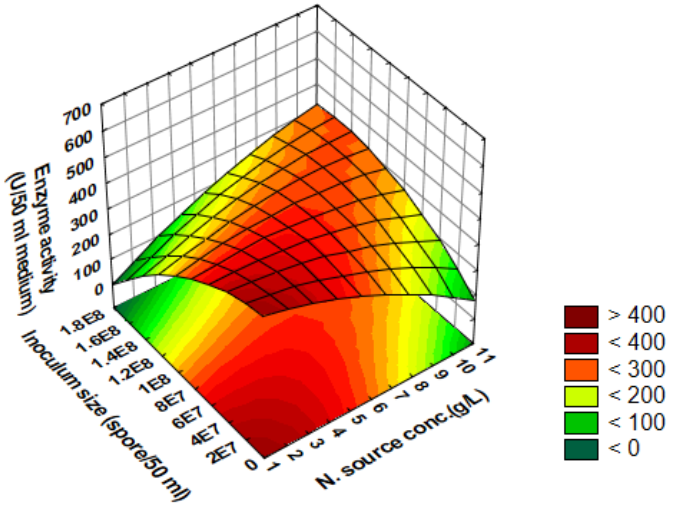

Figure 3 Response surface and contour diagrams for tannase activity considering nitrogen source concentration and inoculum size at the central levels of tannic acid concentration (1.5 %) and medium initial pH (5).

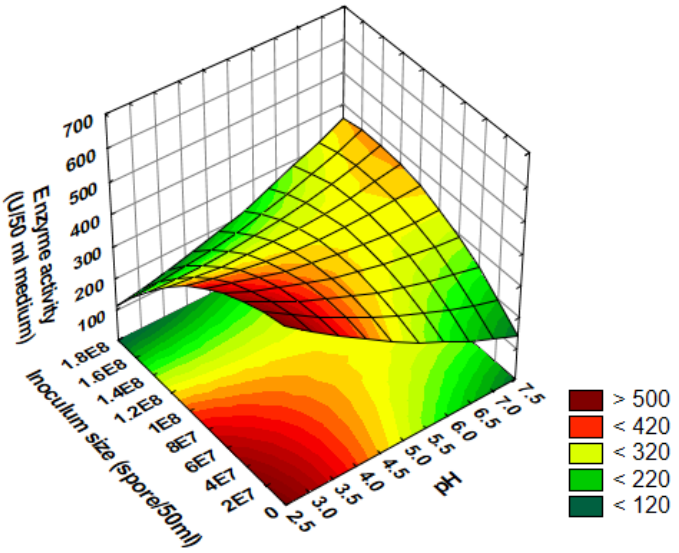

Figure 4 Response surface and contour diagrams for tannase activity considering initial pH of the medium and inoculum size at the central levels of tannic acid concentration (1.5 %) and nitrogen source concentration (6 g/L).

The optimum conditions for maximum tannase production

The optimal set of the four variables levels was found to be 2 % (w/v) tannic acid, 4 g/l nitrogen source, initial pH 4 and inoculum size of 5×10^7 fungal spores/ 50 ml medium. The predicted extracellular tannase production under these conditions was 580 U/50 ml medium while the actual production was 598 U/50 ml medium.

The best-fitting model

The best-fitting mathematical model was determined by stepwise elimination of the non-significant regression coefficients. The best reduced equation is shown below (equation 4). This equation included the linear and quadratic coefficients of both X_1 and X_4 In addition to four linear interactions.
The best-fitting model was determined by stepwise elimination of the non-significant regression coefficients. The best reduced equation is shown below (Equation 4). This equation included the linear and quadratic coefficients of both X_1 and X_4 In addition to four linear interactions.

$$Y = -154.96 + 866.9X_1 - 50.6\times10^{-7}X_4 - 69.2X_1^2 - 1.1\times10^{-14}X_4^2 - 28.46\ X_1X_2 - 55.85X_1X_3 + 4.4\times10^{-7}X_2X_4 + 7.4\times10^{-7}X_3X_4 \quad \textbf{(4)}$$

The analysis of variance of this new model after exclusion of insignificant coefficients showed that the coefficient of determination (R^2) for the new model was slightly reduced (0.923 instead of 0.94 for equation 3) indicating the adequacy of the model and it can explain about 92 % of variation in the response.

Validation of the best-fitting model (Equation 4)

The best-fitting model (Equation 4) was validated, a random set of five treatment combinations was carried out (Table 5) then the experimentally observed responses were compared with the predicted responses calculated by the best-fitting model using Statistica 10 software. Actual levels combinations of X_1, X_2, X_3 and X_4, the predicted, observed responses and residual values are shown in Table 5.
The results clearly showed that experimental values were very close to the predicted values and hence the model was successfully validated. Validation experiment under optimal conditions (treatment No. 2 Table 5) showed that the predicted data of tannase activity (584.1 U/50 medium) was in accordance with the experimental data (595 U/50 medium).

Table 5 Validation of reduced second order polynomial model (Equation 4) using different levels of tannic acid (*X1*), sodium nitrate (X_2), initial pH of the medium (X_3) and inoculum size (X_4)

Treatment No.	Actual level of variables				Response (Y) Tannase activity (U/50 ml medium)		
	X1 Tannic acid conc. (%)	X2 Nitrogen source conc. (g/l)	X3 Initial pH	X4 Inoculum size (spores/50ml medium)	Predicted	Experimental	Residual
1	1	4	4	5×10^7	262.0	301.4	39.4
2	2	4	4	5×10^7	584.1	595	10.9
3	2	8	6	5×10^7	294.6	268.4	-26.2
4	1	8	4	5×10^7	235.4	259.8	24.4
5	1.5	6	5	10^8	336.3	324	-12.3

CONCLUSION

In conclusion, the RCCD led to a predictive equation expressing tannase production as a function of substrate (tannic acid) concentration, nitrogen source concentration, initial pH of the medium and inoculum size. The optimum parameter values obtained were: substrate (tannic acid) concentration, 2%; nitrogen source (sodium nitrate) concentration, 4 g/l; pH 4; and inoculum size, 5×10^7 spores/50 ml medium. This resulted in enzyme production of 580 U/50 ml medium. The data validation under various experimental conditions has also confirmed the correlation between the experimental (observed) and theoretical (predicted) values.

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