Enhanced Production of Tannase from Redgram Husk Using Response Surface Methodology

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ABSTRACT

PURPOSE:

To Study the Statistical Optimization of fermentation conditions for maximum production of tannase enzyme by *Aspergillus flavus* (MTCC 3783), using redgram husk as a substrate was studied using the Response Surface Methodology.

METHOD:

The present study was done via two steps. At first to study the maximum production of tannase enzyme by *Aspergillus flavus*, using redgram husk as a substrate followed by Statistical Optimization of Process parameters using Response Surface Methodology.

RESULTS:

The optimum conditions were tannic acid concentration, 2.8 % (w/v); fermentation period, 95 h; temperature, 34ºC; and pH 5.65. Higher value of the regression coefficient (R^2 = 0.9806) indicates excellent evaluation of experimental data by second-order polynomial regression model. The RSM revealed that a maximum tannase production of 143.5 U/ml was obtained at the optimum conditions.

CONCLUSIONS:

The Response Surface Methodology using Central Composite Design was effectively applied for the production of tannase from *Aspergillus flavus*, using redgram husk as a substrate in submerged fermentation.

Key words

Tannase, Response Surface Methodology, *Aspergillus flavus*, Submerged fermentation

INTRODUCTION

Tannin acyl hydrolase also known as tannase (E.C.3.1.1.20) is a hydrolytic enzyme that catalyses the hydrolysis of (hydrolysable) tannins releasing glucose and gallic acid. Tannins are natural compounds which have a number of phenolic hydroxyl groups and can precipitate protein. The ability of these compounds to precipitate protein generates some problems. Tannins are capable of interacting with protein and crude-fibers and also with digestive enzymes so that they could interfere with the digestion process that can inhibit the growth of livestock [1].

Tannase is industrially important inducible enzyme produced by large number of fungi and a few bacteria [2]. The fungal tannase is used in many industrial processing including clarification of fruit juice; detannification of food, high grade leather tannin and coffee flavored soft drinks, but the major commercial application of the tannase is in the manufacture of instant tea and in the production of gallic acid [5]. Garcia-Conesa et al [6] reported its application in plant cell wall digestibility by breakage of polyphenolics such as dehydrodimer cross links present in the cell wall. In Industrial level tannase is mainly produced by Aspergillus species under submerged fermentation (SmF). The SmF is widely used for enzyme production because it offers many advantages like uniform process conditions namely concentration, Temperature, pH, aeration and agitation in the bioreactors [9, 12].

Response Surface Methodology (RSM) is a collection of mathematical and statistical technique, which 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. [7, 15].

In the present work, optimization of tannase production by *Aspergillus flavus* sp. in a batch process using redgram husk as the substrate, was carried out using a sequential strategy of the experimental design.

MATERIALS AND METHODS

CHEMICALS

Chemicals used in the experiments were purchased from Hi-Media, Mumbai and were of the highest purity.

MATERIALS

Redgram husk samples were obtained from the local market and used as a substrate for tannase production. The sample was made into 100 mesh (0.15 mm) fine powder by the use of laboratory blender at 3000 rpm and was preserved in a sealed plastic bag at 4°C to prevent any possible degradation or spoilage.

MICROORGANISM AND MEDIA

The fungal culture, *Aspergillus flavus* (MTCC 3783), was obtained from IMTECH, Chandigarh and used for tannase production. The strain was maintained on Czapek Dox minimal media agar slants supplemented with 1 % (w/v) tannic acid as the sole carbon source. The fungal strain was sub-cultured periodically, grown at 30ºC for 7 days. The well grown culture was stored at 4°C in a refrigerator and used for further subculturing.

The composition of the Czapek Dox minimal medium used for tannase enzyme production was tannic acid - 10 g/L, sodium nitrate - 6 g/L, potassium dihydrogen orthophosphate - 1.52 g/L, magnesium sulphate - 0.52 g/L, potassium chloride - 0.52 g/L, ferrous sulphate - 0.01 g/L and zinc sulphate- 0.01 g/L. Cells were harvested from slants and used to inoculate liquid medium.

PRODUCTION OF TANNASE

The spore suspension was inoculated in 250 ml Erlenmeyer flask containing 100 ml of Czapek Dox minimal medium. 3 gm of substrate (redgram husk) was added separately to the above mentioned medium for studying their effect on the enzyme production. The cultures were grown at 30°C, 140 rpm for 6 days in an incubator shaker. The samples were withdrawn at regular intervals of 24 h. The biomass was separated by filtering through Whatman No.1 filter paper. The cell free culture broth was assayed for the tannase activity.

ASSAY OF TANNASE

0.1 ml of enzyme solution was incubated with 0.3 ml of 1.0% (w/v) tannic acid and 0.2 M acetate buffer (pH 5.0) at 40°C for 10 min and then the enzyme production was stopped by cooling to 0°C by the addition of 2 ml Bovine Serum Albumin (BSA) (1mg/ml), which precipitates the remaining tannic acid. Simultaneously a control without the enzyme was incubated and the samples were analyzed. The tubes were then centrifuged (5,000 x g, 10 min) and the precipitate was dissolved in 2 ml of Sodium Dodecyl Sulphate (SDS) – triethanolamine (1% w/v SDS in 5% v/v triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01N HCl) [11].

One Unit of the tannase enzyme was defined as the amount of enzyme required to hydrolyze 1µ mole of ester linkage of tannic acid in 1 min at specific condition.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

CENTRAL COMPOSITE DESIGN

The Central Composite Design (CCD) using statistical software package “Design Expert 7.1.5” was used to obtain a quadratic model, consisting of factorial trials and star points to estimate quadratic effects and central points to estimate the pure process variability with tannase production as response. RSM was employed to optimize the process parameters namely tannic acid concentration, fermentation period, temperature and P^H which enhances the tannase enzyme production. The four independent variables were studied at five different levels (Table 1), and sets of thirty one experiments were carried out (Table 2).

The statistical software package “Design Expert 7.1.5” was used to analyze the experimental data. All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table 1. Upon the completion of experiments, the average maximum tannase activity was taken as the response (Y). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables.

A second-order polynomial equation is

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad --- \quad (1)$$

Where Y is the measured response, β_0 is the intercept term, β_i are linear coefficients, β_{ii} are quadratic coefficient, β_{ij} are interaction coefficient and X_i and X_j are coded independent variables. When developing the regression equation, the test factors were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad ----- \quad (2)$$

Where x_i is the coded value of the i^{th} independent variable, X_i the actual value of the i^{th} independent variable, X_0 the real value of the i^{th} independent variable at the center point, and ΔX_i is the value of step change.

Statistical analysis of the data was performed by design package Design Expert 7.1.5 to evaluate the analysis of variance (ANOVA) to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case. The fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. In order to verify the accuracy of the predicted model an experiment was conducted with optimized media.The optimal concentrations of the critical variables were obtained by analyzing 3D plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The effect of various process parameters namely tannic acid concentration, fermentation period, temperature and P^H on tannase enzyme activity in submerged fermentation using *Aspergillus flavus* was studied by conducting experiments with substrate redgram husk and optimized with Central Composite Design of RSM. Thirty one experiments were performed at different combinations of the factors shown in Table 1. The predicted and observed responses along with design matrix were presented in Table 2, and the results were analyzed by ANOVA. The second-order regression equation provides the tannase activity as the function of tannic acid concentration, fermentation period, temperature and P^H which can be presented in terms of coded factors as in the following equation:

$$Y = 139.54 - 87.47X_1 + 0.71X_2 - 2.71X_3 + 1.57X_4 + 3.8854\ X_4 - 97.40\ X_1^2 - 29.04\ X_2^2 - 28.59\ X_3^2 - 25.18\ X_4^2 + 0.808\ X_1\ X_2 - 0.780\ X_1\ X_3 + 1.632\ X_1\ X_4 + 3.042\ X_2X_3 - 3.695\ X_2X_4 - 2.408\ X_3\ X_4 \quad ---------- (3)$$

Where Y is the tannase activity (U/ml) produced as a function of the coded levels of Tannic acid concentration (X_1), Fermentation period (X_2), Temperature (X_3) and pH (X_4).

Based on the experimental response, the quantity of tannase enzyme produced by *A.flavus* ranged from 93.20 U/ml to 149.8 U/ml. The Analysis of Variance (ANOVA) for tannase production was given in the Table 3. Values of "Prob>F" less than 0.05 indicate the model terms were significant. Values greater than 0.1 indicate the model terms were not significant. In the present work, the linear effect of tannic acid concentration and square effect of tannic acid concentration were found to be significant for maximum tannase enzyme production. The coefficient of determination (R^2) for the tannase activity was calculated as 0.9806, which is close to 1 and can account for up to 98.06% of the variability of the response.

The interaction effects of variables on the tannase production were studied by plotting response surface curves against two independent variables, while keeping the other variable at its central (0) level. Fig.1 – Fig. 6 represent the response surface plots for the tannase enzyme production during batch submerged fermentation.

The effect of fermentation period and tannic acid concentration by fixing other two variables at central points namely temperature and pH on tannase activity is shown in Fig.1. As the tannic acid concentration was increased upto 2.8% (w/v), the tannase activity was found to increase and thereafter tannase activity was decreased with a further increase in tannic acid concentration. The optimum concentration of 2.8% tannic acid obtained in the present investigation was in agreement with previous report of Bradoo et al [3]. Fig.2 shows the dependency of tannase activity on temperature and tannic acid concentration. The tannase activity was increased with increase in temperature to about 34°C and thereafter tannase activity was decreased with further increase in temperature. Kar et al [8] and Lekha and Lonsane [10] obtained similar values for the incubation temperature using response surface methodology for tannase production from *Rhizopus oryzae* and *Aspergillus niger* PKL 104, respectively. Fig.3 and Fig.5 show three dimensional response surface plots obtained with various values of pH and tannic acid concentration at the fixed temperature of 35°C and fermentation period at 96h. As can be seen from Fig 3 and Fig 5 an increase in pH from 4.5 to 5.65 the tannase production was found to increase with further increase in pH the tannase activity was found to decrease. Tannase activity was maximum in the acidic pH range, and its activity decreases in the alkaline range [13, 14]. Fig.4 shows the dependency of tannase activity on temperature and fermentation period. The tannase activity was increased with increase in fermentation period to about 95h and

thereafter tannase activity was decreased with further increase in fermentation period. The same trend was observed in Fig.6. A similar optimal pH 5.5 and optimal incubation period 120hr have been reported for tannase from *Paecilomyces variotii* [4]. The optimum conditions for the maximum production of tannase were determined by response surface analysis and also estimated by regression equation.

The optimum conditions for the maximum production of tannase were found to be the tannic acid concentration of 2.8 % (w/v), fermentation period of 95 h, temperature of 34 °C and pH of 5.65. The coefficient of regression (R^2) value of 0.9806 indicates that the predicted values closely agree with the experimental values.

CONCLUSIONS

The response surface methodology was effectively applied for the production of tannase from *Aspergillus flavus* (MTCC 3783), using redgram husk as a substrate in submerged fermentation. Central Composite Design was employed to evaluate the effects of tannic acid concentration, fermentation period, temperature and pH on production of tannase by *Aspergillus flavus.* Using the above optimized process parameters and fermentation conditions, Maximum tannase activity of 143.5 U/ml was obtained at the tannic acid concentration of 2.8% (w/v), temperature of 34ºC, pH of 5.65, and fermentation period of 95h. The statistical Design Of Experiment (DOE) offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for maximum tannase production by *Aspergillus flavus.*

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Figure Legends

Fig 1: Three dimensional Plot showing the effect of Fermentation period and Tannic acid concentration on tannase activity.

Fig 2: Three dimensional Plot showing the effect of Temperature and Tannic acid concentration on tannase activity.

Fig 3: Three dimensional Plot showing the effect of pH and Tannic acid concentration on tannase activity.

Fig 4: Three dimensional Plot showing the effect of Temperature and Fermentation period on tannase activity.

Fig 5: Three dimensional Plot showing the effect of pH and Fermentation period on tannase activity.

Fig 6: Three dimensional Plot showing the effect of pH and Temperature on tannase activity.

Table 1 Coded and actual levels of the independent variables for the design of experiment for tannase production using *Aspergillus flavus* on Redgram Husk

Independent Variables	Symbols	Coded levels				
		– 2	**–1**	**0**	**1**	**2**
Tannic Acid conc. (%)	X_1	1	2	3	4	5
Fermentation period (h)	X_2	48	72	96	120	144
Temperature (^oC)	X_3	25	30	35	40	45
pH	X_4	4.5	5	5.5	6	6.5

Table 2:Five level Central Composite Rotatable Design (CCRD) and the experimental responses of dependent variable of tannase activity (U/ml) by *Aspergillus flavus* using Redgram Husk

Run No.	X_1	X_2	X_3	X_4	Tannase Activity, U/ml	
					Experimental	**Predicted**
1	1	-1	-1	-1	97.98	104.45
2	0	0	2	0	109.70	103.93
3	0	0	0	0	139.50	139.51
4	-1	-1	1	-1	105.40	109.14
5	-1	1	-1	-1	109.40	115.40
6	1	1	1	1	101.40	106.91
7	0	0	-2	0	117.40	114.79
8	2	0	0	0	111.50	101.55
9	0	-2	0	0	110.20	106.14
10	-1	-1	-1	1	123.60	126.36
11	1	-1	1	-1	93.20	96.19
12	0	0	0	0	139.60	139.51
13	-1	1	1	1	108.60	110.11
14	1	1	1	-1	107.50	112.71
15	0	0	0	0	139.60	139.51
16	1	1	-1	1	108.40	112.63
17	-2	0	0	0	113.00	114.57
18	-1	-1	-1	-1	119.40	114.28
19	1	-1	1	1	103.20	105.18
20	-1	1	-1	1	115.30	112.70
21	-1	-1	1	1	110.50	111.60
22	1	1	-1	-1	109.50	108.80
23	0	0	0	0	139.70	139.51
24	0	0	0	2	130.50	126.15
25	0	0	0	0	139.60	139.51
26	1	-1	-1	1	120.50	123.06
27	-1	1	1	-1	124.60	122.43
28	0	0	0	-2	123.90	119.86
29	0	0	0	0	139.40	139.51
30	0	0	0	0	139.20	139.51
31	0	2	0	0	113.30	108.98

Table 3: Regression analysis and corresponding *t* and p- value of second order polynomial model for the optimization of Tannase production by *A.flavus* using Redgram Husk

Term constant	Coefficient factor	Std Deviation	*t*- value	p –value
Intercept	139.514	2.341	2.271	0.037
X_1	-87.472	1.264	-2.636	0.018
X_2	0.713	1.264	0.021	0.983
X_3	-2.712	1.264	-0.082	0.936
X_4	1.572	1.264	0.047	0.963
X_1X_1	97.408	1.158	3.204	0.006
X_2X_2	-29.042	1.158	-0.955	0.354
X_3X_3	-28.592	1,158	-0.941	0.361
X_4X_4	-25.180	1,158	-0.828	0.420
X_1X_2	0.808	1.548	0.020	0.984
X_1X_3	-0.780	1.548	-0.019	0.985
X_1X_4	1.632	1.548	0.040	0.968
X_2X_3	3.042	1.548	0.075	0.941
X_2X_4	-3.695	1.548	-0.091	0.929
X_3X_4	-2.408	1.548	-0.059	0.953
R^2 = 0.9806				

X_1, X_2, X_3, X_4 - Linear effects
$X_1X_1, X_2X_2, X_3X_3, X_4X_4$ - Quadratic effects
$X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4$ - Interaction effects
X_1 - Highly Significant
X_1X_1 - Highly Significant

Table 4 : Analysis of variance (ANOVA) for the quadratic polynomial model for Tannase production by *Aspergillus flavus* using Redgram Husk

Source	Seq SS	Degrees of freedom (DF)	Mean square (MS)	*F*-value	p –value
Regression	5454.10	14	389.58	13.91	0.000
Linear	502.25	4	125.56	4.48	0.013
Square	4429.75	4	1107.44	39.55	0.000
Interaction	522.10	6	87.02	3.11	0.033
Residual Error	448.06	16	28.00	-	-
Lack-of-Fit	447.89	10	44.79	1594.19	0.000
Pure Error	0.17	6	0.03	-	-
Total	5902.16	30	1782.42	-	-

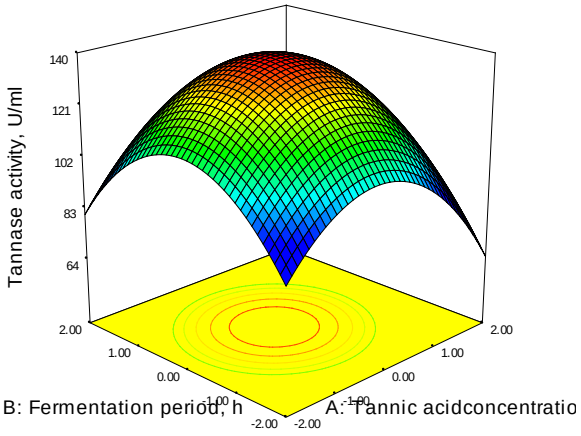

Fig 1: Three dimensional Plot showing the effect of Fermentation period and Tannic acid concentration on tannase activity.

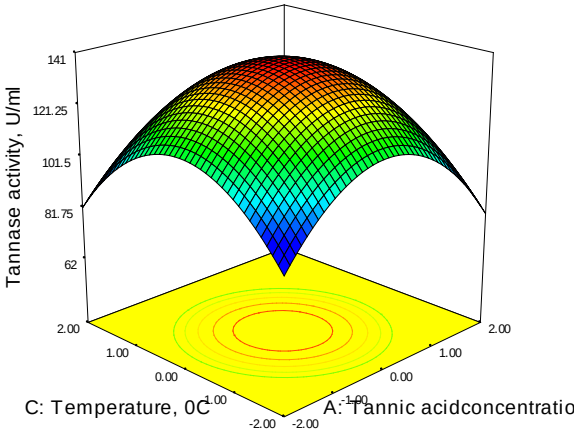

Fig 2: Three dimensional Plot showing the effect of Temperature and Tannic acid concentration on tannase activity.

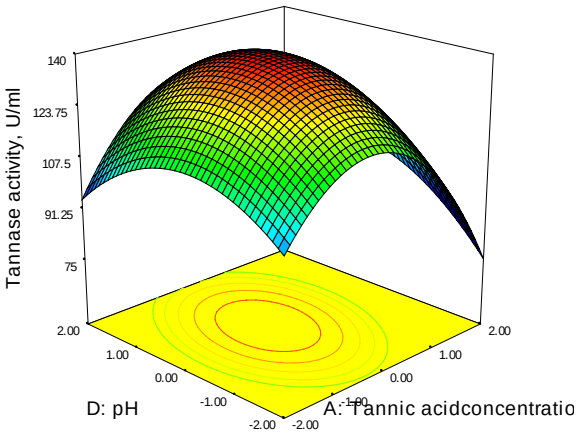

Fig 3: Three dimensional Plot showing the effect of pH and Tannic acid concentration on tannase activity.

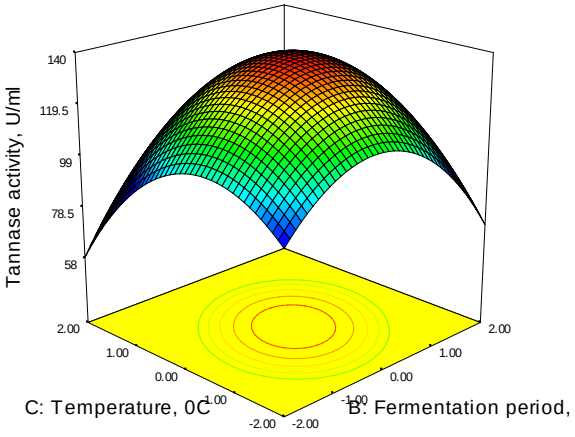

Fig 4: Three dimensional Plot showing the effect of Temperature and Fermentation period on tannase activity.

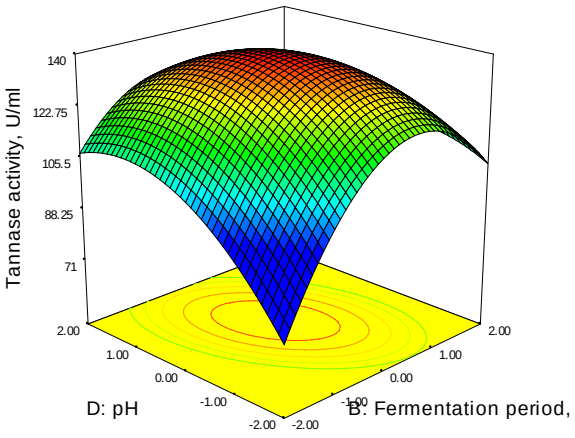

Fig 5: Three dimensional Plot showing the effect of pH and Fermentation period on tannase activity.

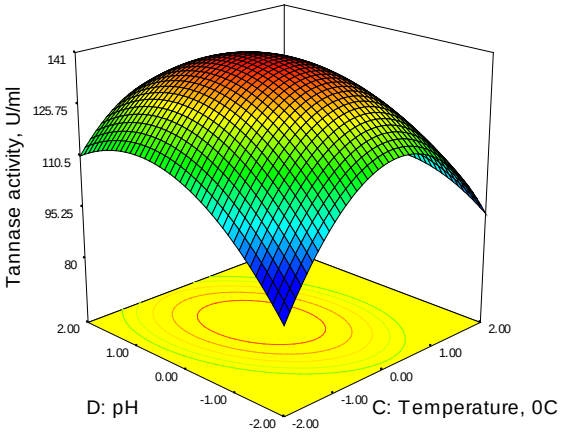

Fig 6: Three dimensional Plot showing the effect of pH and Temperature on tannase activity.