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APPLICATION OF RESPONSE SURFACE METHODOLOGY FOR THE MAXIMUM PRODUCTION OF TANNASE USING *ASPERGILLUS FOETIDUS* (MTCC 3557)

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ABSTRACT

Production of tannase by *Aspergillus foetidus* (MTCC 3557) using redgram husk as substrate was studied in submerged fermentation. The optimization of process parameters was carried out using Response Surface Methodology (RSM). RSM is applied for the Design Of Experiments (DOE) to evaluate the main effects, squared effects and interactive effects of the process parameters affecting the production of tannase in a submerged fermentation using *Aspergillus foetidus* (MTCC 3557) through a full factorial design. The optimum conditions were found to be a tannic acid concentration of 3.1 %, fermentation period of 97 h, temperature of 35.5°C and pH of 5.5 with a higher R^2 value of 0.9251.The RSM revealed that a maximum tannase production of 157.6 U/ml was obtained at the optimum conditions.

Keywords: *Aspergillus foetidus*, Response surface methodology, Submerged fermentation, Tannase.

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]. Tannase has several applications in the food, juice, beer, cosmetic, pharmaceutical and chemical industries [2, 3], but its main uses are in gallic acid production, tea production and the stabilization of wine color and coffee flavor [4]. Fungi cultures are mainly used for tannase production [5, 6] and some yeast [7] and bacteria [8] also could be used.

Agro industrial residues are continuously being generated in vast quantity especially in developing countries and their disposal is associated with several environmental problems. Utilization of these agro residues and byproducts of agro industries as nutrient sources for microbial tannase production may reduce the final enzyme production cost, which is one of the major challenges affecting the large scale production of enzymes [9]. Realizing the importance of the tannase enzyme, efforts are made to find suitable process parameters, which may produce higher amounts of tannase.

Commercial production of tannase is mainly by microbial fermentation using *Aspergillus* species under Submerged Fermentation (SmF) in industries. The SmF is widely used for enzyme production because it offers many advantages like uniform process conditions throughout the medium, concentration, temperature, pH, aeration and agitation in the bioreactors [10].

MATERIALS AND METHODS

Chemicals

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

Microorganism and Growth Conditions

The fungal culture, *Aspergillus foetidus* (MTCC 3557) was obtained from IMTECH, Chandigarh, India 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.

Submerged Fermentation (SmF)

100 ml of Czapek Dox minimal medium in 250 ml Erlenmeyer flask was inoculated with the *Aspergillus foetidus* spore suspension. The composition of the Czapek Dox minimal medium used for tannase enzyme production

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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. Commercial quality redgram husk was procured from the local market and used as a substrate for tannase production. 3 gm of redgram husk (substrate) was added separately to the Czapek Dox minimal medium for studying their effect on the enzyme production. The cultures were grown at 30°C, 140 rpm for six days in an incubator shaker. The samples were withdrawn at regular intervals of 24 h. The biomass was separated by the filtration through Whatman No.1 filter paper. The cell free culture broth was assayed for the tannase activity.

Assay of Tannase

0.05 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) (1 mg/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 530 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.

Optimization of Tannase Production

Response Surface Methodology

Response Surface Methodology (RSM) is an empirical statistical technique, based on the fundamental principles of statistics, randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner [12, 13]. It is an efficient statistical technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments. Central Composite Design (CCD) is one of the response surface methodologies usually utilized to obtain data that fits a full second order polynomial model [14].

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 foetidus* was studied by conducting experiments with substrate redgram husk and optimized with Central Composite Design.

Analysis and Interpretation of Results

Response Surface Methodology used in the present study is a Central Composite Design (CCD) involving four different factors. Experiments were conducted in a randomized fashion. The CCD contains a total of 31 experimental trials involving the replications of the central points as given in the Table 2. The dependent variable selected for this study was tannase activity (U/ml). The independent variables chosen were Tannic acid concentration, X_1(%); Fermentation period, X_2(h); Temperature, X_3(°C) and pH, (X_4). Once the experiments were performed, a second order regression equation (1) shown below was used to describe the effect of variables in terms of linear, quadratic and cross product terms

$$Y = \beta_0 + \sum_{i=1}^{k} b_i Xi + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j + e \quad (1)$$

Where, *i, j* are linear, quadratic coefficients, respectively, while '*b*' is regression coefficient, Y is the tannase activity (U/ml), *k* the number of factors studied and optimized in the experiment, 'e' is random error and β_0 is the intercept, When developing the regression equation, the test factors were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i \qquad i = 1,2,3,\ldots\ldots,K \quad (2)$$

Where x_i is the coded value, X_i is the actual value of an independent variable; X_0 is the real value of X_i at center point, ΔXi is the step change of the variable.

The quality of fit of the second order regression equation was expressed by the coefficient of determination R^2, and its statistical significance is determined by *F*-test. The significance of each coefficient was determined using Student's *t*-test. The coefficients of the equation were determined by employing MINITAB software version 15. Analysis of variance (ANOVA) for the final predictive equation and optimization values were also obtained using the same software package which indicate whether the variable is more significant or less significant. The response surface equation was optimized for maximum activity in the range of process variables using MATLAB software version 7.0.1. The response surface plots were obtained based on the effect of the levels of two parameters and their interactions on the activity of tannase enzyme by keeping the other two parameters at their optimal concentrations. From these response surface plots, the interaction of one parameter with another parameter was studied and also the RSM model was developed to explain the quadratic interaction effects by conducting the pair wise regression analysis of experimental data.

RESULTS AND DISCUSSIONS

Optimization of Process Parameters by RSM

The level of the factors namely tannic acid concentration, fermentation period, temperature and pH and the effect of their interactions on tannase production were determined by Central Composite Design of RSM. Table 1 shows the four independent variables namely tannic acid concentration, fermentation period, temperature and pH and their concentrations at different coded values and actual values of the variables employed in the design matrix. The predicted and observed responses along with the design

matrix are presented in the Table 2. The results were analyzed by ANOVA software. The second-order regression equation (3) provided the levels of tannase activity as the function of tannic acid concentration, fermentation period, temperature and pH which can be presented in terms of coded factors as in the following equation:

$$Y = 149.27 + 0.887\ X_1 + 1.483\ X_2 + 2.083\ X_3 + 0.962\ X_4 - 8.429\ X_1^2 - 13.241\ X_2^2 - 13.341\ X_3^2 - 13.129\ X_4^2 - 0.243 X_1 X_2 - 1.658\ X_1 X_3 + 0.288\ X_1 X_4 - 0.574\ X_2 X_3 - 3.221\ X_2 X_4 - 0.406\ X_3 X_4 \quad (3)$$

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.foetidus* ranged from 83.6 U/ml to 149.8 U/ml. The ANOVA for tannase production was given in the Table 3 and Table 4. 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 squared effect of tannic acid concentration, fermentation period, temperature and pH were found to be significant for maximum tannase enzyme production as the "Prob>F" is < 0.001. The coefficient of determination (R^2) for the tannase activity was calculated as 0.9251, which is close to 1 and can account for up to 92.51% of the variability of the response. The predicted R^2 value of 0.7689 is in reasonable agreement with the adjusted R^2 value of 0. 8595.

Response Surface Plots

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.

Fig.1 shows the dependency of tannase on tannic acid concentration and fermentation period. The tannase activity increased with an increase in tannic acid concentration to about 3.22 % (w/v) and thereafter tannase activity decreased with a further increase in tannic acid concentration. From Fig. 4, it is also inferred that an increase in fermentation period resulted in an increase in tannase activity up to 97 h after that tannase activity is found to decrease. A decline in enzyme activity after 97 h of fermentation may be due to decrease in nutrient availability in the medium. These are in agreements with the results obtained by elsewhere [15, 16]. Fig.2 and Fig.6 show the response surface plots obtained at various values of temperature and tannic acid concentration at the fixed pH of 5.5 and fermentation period of 96h. As temperature is increased from 25^{o}C to 35.5^{o}C the tannase production is found to increase with further increase in temperature the tannase activity was found to decrease. Kar [17] and Lekha [18] studied similar work using response surface methodology for tannase production, obtained similar values for the temperature range between 32^{o}C and 28^{o}C using *Rhizopus oryzae* and *Aspergillus niger* PKL 104. Fig.3 and Fig.5 show the response surface plots obtained with various values of pH and tannic acid concentration at the fixed temperature of 35^{o}C and fermentation period at 96h. As can be seen from Fig. 3 and Fig 5 an increase in pH from 4.5 to 5.5 the tannase production is found to increase with further increase in pH the tannase activity is found to decrease. Tannase activity was maximum in the acidic pH range, and its activity decreases in the alkaline range [19].

The optimum conditions for the maximum production of tannase were found to be the tannic acid concentration of 3.1 % (w/v), fermentation period of 97 h, temperature of 35.5 °C and pH of 5.5. The predicted results of RSM are shown in Table II. The coefficient of regression (R^2) value of 0.9251 indicates that the predicted values closely agree with the experimental values.

Validation of the Experimental Model

The validation of the RSM experimental model was tested by carrying out a batch experiment under optimal operating conditions with optimal media using MATLAB software version 7.0.1. Three repeated experiments were performed and the results were compared. The tannase activity obtained from the experiments was very close to the response predicted by the regression model, which proves the validity of the model. At these optimized conditions the maximum tannase activity was found to be 157.6 U/ml.

Table 1. Coded values and Actual values of the Independent variables

Independent Variables	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 (^{o}C) X_3	25	30	35	40	45
pH X_4	4.5	5	5.5	6	6.5

Table 2. Central Composite Design and the experimental responses of the tannase activity (U/ml) by A.*foetidus* using Redgram husk as substrate

Run No.	X_1	X_2	X_3	X_4	Tannase Activity, U/ml	
					Experimental	Predicted
1	0	0	0	0	149.8	149.27

2	0	0	0	0	149.8	149.27
3	1	1	1	1	99.54	100.73
4	1	-1	1	-1	86.54	97.71
5	1	-1	1	1	100.6	105.84
6	-1	1	-1	1	100.34	96.66
7	-1	1	-1	-1	97.1	100.94
8	-1	-1	-1	-1	83.6	89.90
9	0	2	0	0	105.2	99.27
10	1	-1	-1	-1	90.5	94.90
11	0	0	0	0	149.8	149.27
12	0	0	0	0	149.7	149.27
13	1	-1	-1	1	100.65	104.65
14	-1	-1	1	1	103.2	106.32
15	0	-2	0	0	104	93.34
16	0	0	0	0	149.8	149.27
17	0	0	0	0	149.8	149.27
18	1	1	1	-1	100.2	105.48
19	0	0	0	2	104.2	98.68
20	-1	-1	1	-1	99.5	99.34
21	0	0	0	-2	105.9	94.83
22	0	0	2	0	108.8	100.07
23	-2	0	0	0	116.2	113.78
24	0	0	-2	0	99.6	91.74
25	-1	1	1	1	97.5	102.18
26	-1	-1	-1	1	94.7	98.50
27	1	1	-1	1	92.6	101.84
28	1	1	-1	-1	100.6	104.97
29	-1	1	1	-1	104.6	108.09
30	0	0	0	0	149.8	149.27
31	2	0	0	0	131.5	117.33

Table 3. Regression analysis and corresponding *t* and P- value of second order polynomial model for the optimization of tannase production

Term Constant	Regression coefficient	Std deviation	*t*- value	p- value
Intercept	149.27	3.129	47.71	0.000
X_1	0.887	1.690	0.525	0.607
X_2	1.483	1.690	0.878	0.393
X_3	2.083	1.690	1.233	0.237
X_4	0.962	1.690	0.569	0.577
X_1X_1	-8.429	1.548	-5.445	0.000
X_2X_2	-13.241	1.548	-8.554	0.000
X_3X_3	-13.341	1.548	-8.618	0.000
X_4X_4	-13.129	1.548	-8.481	0.000
X_1X_2	-0.243	2.069	-0.117	0.908
X_1X_3	-1.658	2.069	-0.801	0.435
X_1X_4	0.288	2.069	0.139	0.891
X_2X_3	-0.574	2.069	-0.278	0.785
X_2X_4	-3.221	2.069	-1.556	0.139
X_3X_4	-0.406	2.069	-0.196	0.847
R^2 = 0.9251				

Table 4. Analysis of Variance (ANOVA) for the quadratic polynomial model for tannase production by *A.foetidus* using redgram husk as substrate

Source	Seq SS	Degrees of freedom (DF)	Mean square (MS)	*F*-value	p –value
Regression	13532.5	14	966.61	14.11	0.000

Linear	198.0	4	49.50	0.72	0.589
Square	13114.4	4	3278.60	47.85	0.000
Interaction	220.1	6	36.69	0.54	0.774
Residual Error	1096.4	16	68.52	-	-
Lack-of-Fit	1094.3	10	109.43	322.77	0.000
Pure Error	2.0	6	0.34	-	-
Total	14628.9	30		-	-

Fig 1. Response Surface plot showing the effect of fermentation period and tannic acid concentration on tannase production by *A.foetidus* using Redgram husk as substrate.

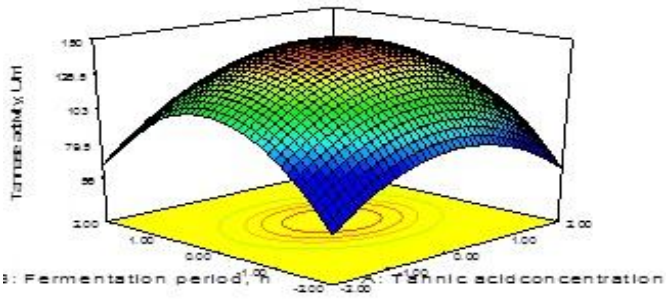


Fig 2. Response Surface plot showing the effect of temperature and tannic acid concentration on tannase production by *A.foetidus* using Redgram husk as substrate.

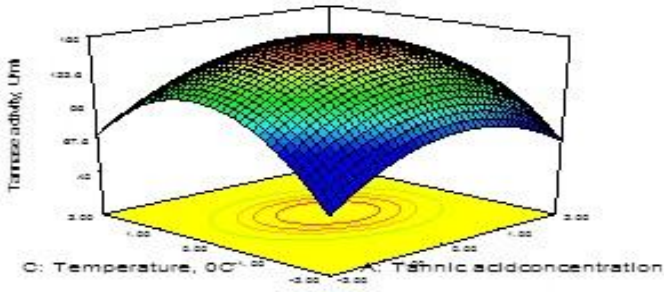


Fig 3. Response Surface plot showing the effect of pH and tannic acid concentration on tannase production by *A.foetidus* using Redgram husk as substrate.

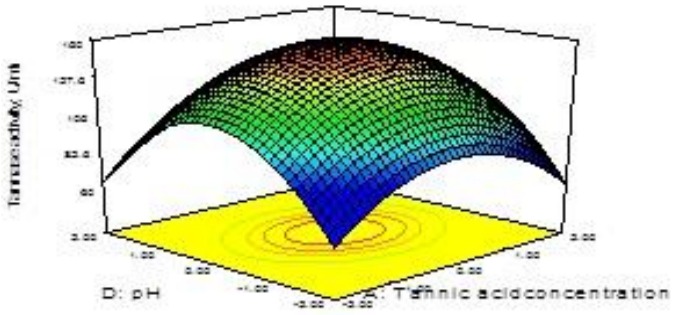


Fig 4. Response Surface plot showing the effect of temperature and fermentation period on tannase production by *A.foetidus* using Redgram husk as substrate.

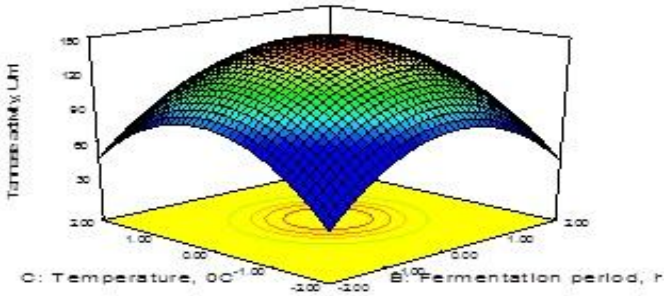


Fig 5. Response Surface Plot showing the effect of pH and fermentation period on tannase production by *A.foetidus* using Redgram husk as substrate.

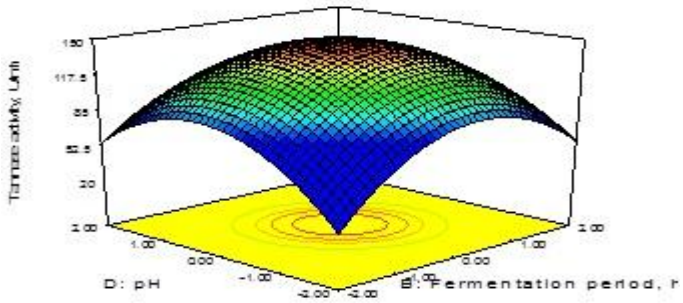
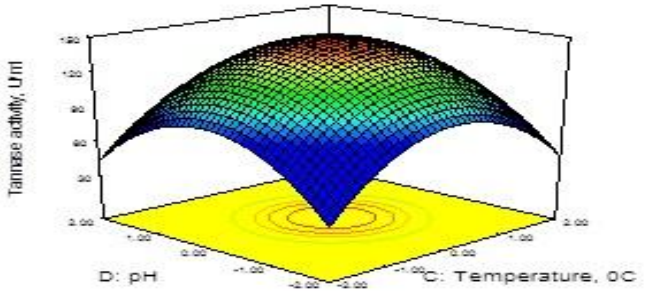


Fig 6. Response Surface plot showing the effect of pH and temperature on tannase production by *A.foetidus* using Redgram husk as substrate.



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