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# Statistical Optimization of Culture Conditions for Tannase Production by *Aspergillus awamori* MTCC 9299 under Submerged Fermentation

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**Abstract:** Optimization of culture conditions for tannase production by *Aspergillus awamori* MTCC 9299 was studied using the response surface methodology. Maximum response for tannase production was obtained at 5.0 pH, 35°C incubation temperature, 125 rpm agitation speed and incubation period of 48 h. Under the proposed optimized conditions, the tannase experimental yield (1.45 U $mL^{-1}$) closely matched the yield predicted by the statistical model (1.43 U $mL^{-1}$) with $R^2 = 0.99$ and highly significant F-value of 173.79.

**Key words:** Tannase, response surface methodology, tannic acid, *Aspergillus awamori* MTCC 9299

## INTRODUCTION

Tannase or tannin acyl hydrolyase (EC 3.1.1.20) is an extracellular inducible enzyme that catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins, releasing glucose and gallic acid. Gallic acid is extensively used as an ingredient of developer in photography and printing inks. It also serves as a precursor for the commercial production of an anti-microbial drug-trimethoprim, a food preservative-propylgallate and some dyestuffs. Besides this, gallic acid possesses wide range of biological activities, such as antioxidant, antibacterial, antiviral, analgesic etc. As antioxidant gallic acid acts as an antiapoptotic agent and helps to protect human cells against oxidative damage. Gallic acid is also found to show cytotoxic activity against cancer cells, without harming normal cells (Bajpai and Patil, 2008). Besides gallic acid production, the enzyme is extensively used in the preparation of instant tea, wine, beer and coffee-flavored soft drinks and also as additive for detannification of food (Lekha and Lonsane, 1997; Seth and Chand, 2000).

In biotechnology, optimization of culture condition through Fractional Factorial Design (FFD) and Response Surface Methodology (RSM) is a common practice. The FFD and RSM are statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions, have successfully been employed for screening significant factors and optimization the medium composition in many bioprocess (Elibol, 2004; Lin *et al*., 2007; Zhao *et al*., 2008). These have several advantages that included less experiment numbers, suitability for multiple factor experiments, search for relativity between factors and finding of the most suitable condition and forecast response (Chang *et al*., 2006). In the present study, we aimed to optimize culture conditions for an efficient tannase production using response surface methodology.

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## MATERIALS AND METHODS

### Microorganism and Maintenance of Culture

A tannase producing fungus was isolated from the local garden soil of Guru Jambheshwar University of Science and Technology Campus, Hisar, India in the month of February 2009 and identified as *Aspergillus awamori* MTCC9299. The strain was sub-cultured at an interval of 4-5 weeks and routinely maintained on Potato Dextrose Agar (PDA) slants.

### Preparation of Spore Inoculum

Fungal spore inoculum was prepared by adding 2.5 mL of sterile distilled water containing 0.1% Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions and the number of spores in the suspension was determined using the Neubauer chamber. The volume of 1 mL of the prepared spore suspension was used as the inoculum, with concentration of $5\times10^9$ spores.

### Fermentation Medium

For the fermentation process, a 250 mL Erlenmeyer flask with 50 mL of Czapek Dox minimal medium containing (g $L^{-1}$): $NaNO_3$, 6; $KH_2PO_4$, 1.52; KCl, 0.52; $MgSO_4$. $7H_2O$, 0.52; $FeSO_4$. $7H_2O$, 0.01; $ZnSO_4$. $7H_2O$, 0.01 was employed (Bradoo *et al*., 1996). The medium was adjusted to pH 5.0 and then sterilized at 121°C for 15 min. Tannic acid solution was prepared separately and the solution was adjusted to pH 4.5 with 0.1 M NaOH. The solution was sterilized by filtering through a sterile membrane (pore size 0.2 μm) and added to the medium to have a final tannic acid concentration of 1%.

### Tannase Assay

Tannase activity was determined calorimetrically using the method of Mondal *et al*. (2001). The reaction mixture containing 0.3 mL of tannic acid (0.5% in 0.2 M sodium acetate buffer, pH 5.5) and 0.1 mL of enzyme was incubated at 30°C for 20 min. The enzymatic reaction was stopped by addition of 3 mL BSA solution, which precipitates the remaining tannic acid. The tubes were centrifuged (5000x g 10 min) and the resultant precipitate was dissolved in 3 mL SDS-triethanolamine solution. One milliliter of $FeCl_3$ reagent was added to each tube and was kept for 15 min at room temperature for stabilization of the color. The absorbance was read at 530 nm against the blank. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 mMol of tannic acid in 1 min under assay condition and expressed as U $mL^{-1}$.

### RSM Experimental Design and Statistical Analysis

A Box-Behnken (Box and Behnken, 1960) factorial design was used in the optimization of culture conditions for tannase production. Four-factors and five-level face-centered cube design requiring a total of 29 experiments were adopted in this study. The independent variables studied were pH (X1), incubation temperature (X2, °C), incubation time (X3, h) and agitation speed (X4, rpm). The response (dependent variable) was tannase activity (U $mL^{-1}$). Each independent variable was studied at three coded levels (-1, 0, +1). The minimum and maximum levels of each independent variable and the experimental design with respect to their coded and uncoded levels are presented in Table 1. The relation between the coded values and actual values were described as in the following Eq. 1:

$$Xi = \frac{xi - x0}{\Delta xi} \quad (1)$$

Table 1: Experimental range and levels of the independent variables

| | Range and levels | | |
|---|---|---|---|
| Independent variables (g $L^{-1}$) | -1 | 0 | +1 |
| pH (X1) | 3 | 5 | 7 |
| Incubation temperature (X2) (°C) | 25 | 35 | 45 |
| Incubation time (X3) (h) | 12 | 42 | 72 |
| Agitation speed (X4) (rpm) | 50 | 125 | 200 |

where, Xi is the independent variable coded value, xi is the independent variable actual value, x0 is the independent variable actual value on the center point and Δxi is the step change value. The second-order model used to fit the response to the independent variables is shown in Eq. 2:

$$Y= \beta_0+ \Sigma\beta i\ xi+ \Sigma\beta ii\ x^2i + \Sigma\beta ij\ xixj \quad (2)$$

where, Y is the response (enzyme activity); $\beta_0$, βi, βii and βij are regression coefficients for intercept, linear, quadratic and interaction terms, respectively and xi and xj are independent variables.

A second-order regression analysis of the data was carried out to get empirical model that define response in terms of the independent variables. Analysis of Variance (ANOVA) was performed in coded level of variables to study the effects of independent variables. The 3D graphs were generated to understand the effect of selected variables individually and in combination to determine their optimum level for maximal production of tannase.

## RESULTS AND DISCUSSION

### Optimization of Culture Conditions using RSM

The statistical technique is widely used as a tool for checking the efficiency of several processes. In the present study it has been used with the purpose of obtaining information about the culture conditions (pH, incubation temperature, incubation time and agitation speed) for the tannase production. To examine the combined effect of four different culture conditions (independent variables) on tannase production, a Box-Behnken factorial design having 5 centre points leading to a total of 29 experiments were performed. Equation 3 represents the mathematical model relating the production of tannase with the independent process variables, Xi and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the design expert. The experimental and predicted values of yields of tannase are given in Table 2. It was observed that the predicted values for tannase production were in good agreement with RSM plots. The coded values of independent variables are also given in Table 2.

### Model Validation

The adequacy of the model and fitness were evaluated by ANOVA (analysis of variance) and regression coefficients for the experimental design used (Table 3, 4). The ANOVA for the quadratic model was highly significant with an F value of 173.79 as shown by Fisher's F-test, along with a very low probability value (Pmodel>F=0.0001), which was significant at 95% confidence interval. At the same time, relatively lower value of coefficient of variation (CV=3.92%) indicated a better precision and reliability of the experiments carried out. The determination coefficient ($R^2$) of the model was 0.9943 indicating that 99.43% of variability in the response could be accounted by the model (Table 4). The highest $R^2$ value

Table 2: Experimental design used in RSM studies by using four independent variables each at three levels showing observed and predicted values of tannase production

| Run | Factor1, A:pH | Factor 2, B: Incubation temperature | Factor 3, C: Incubation time | Factor 4, C: Agitation speed | Observed value | Predicted value |
|---|---|---|---|---|---|---|
| 1 | 5(0) | 25(-1) | 42(0) | 200(1) | 1.12 | 1.13 |
| 2 | 5(0) | 35(0) | 12(-1) | 50(-1) | 0.97 | 0.96 |
| 3 | 7(1) | 45(1) | 42(0) | 125(0) | 0.52 | 0.50 |
| 4 | 7(1) | 35(0) | 72(1) | 125(0) | 0.61 | 0.63 |
| 5 | 5(0) | 25(-1) | 72(1) | 125(0) | 1.06 | 1.08 |
| 6 | 5(0) | 35(0) | 72(1) | 200(1) | 1.14 | 1.10 |
| 7 | 5(0) | 35(0) | 42(0) | 125(0) | 1.45 | 1.43 |
| 8 | 3(-1) | 35(0) | 72(1) | 125(0) | 0.58 | 0.58 |
| 9 | 5(0) | 35(0) | 42(0) | 125(0) | 1.46 | 1.43 |
| 10 | 5(0) | 35(0) | 42(0) | 125(0) | 1.39 | 1.43 |
| 11 | 3(-1) | 35(0) | 12(-1) | 125(0) | 0.49 | 0.50 |
| 12 | 5(0) | 35(0) | 42(0) | 125(0) | 1.43 | 1.43 |
| 13 | 3(-1) | 35(0) | 42(0) | 50(-1) | 0.55 | 0.58 |
| 14 | 5(0) | 45(1) | 72(1) | 125(0) | 0.92 | 0.94 |
| 15 | 3(-1) | 45(1) | 42(0) | 125(0) | 0.57 | 0.52 |
| 16 | 3(-1) | 35(0) | 42(0) | 200(1) | 0.59 | 0.62 |
| 17 | 5(0) | 35(0) | 72(1) | 50(-1) | 1.17 | 1.13 |
| 18 | 5(0) | 25(-1) | 12(-1) | 125(0) | 0.87 | 0.86 |
| 19 | 5(0) | 25(-1) | 42(0) | 50(-1) | 0.92 | 0.94 |
| 20 | 3(-1) | 25(-1) | 42(0) | 125(0) | 0.51 | 0.48 |
| 21 | 5(0) | 45(1) | 42(0) | 50(-1) | 1.03 | 1.06 |
| 22 | 5(0) | 45(1) | 12(-1) | 125(0) | 0.98 | 0.97 |
| 23 | 5(0) | 35(0) | 12(-1) | 200(1) | 1.09 | 1.08 |
| 24 | 5(0) | 45(1) | 42(0) | 200(1) | 0.95 | 0.97 |
| 25 | 7(1) | 25(-1) | 42(0) | 125(0) | 0.57 | 0.57 |
| 26 | 7(1) | 35(0) | 42(0) | 50(-1) | 0.63 | 0.61 |
| 27 | 5(0) | 35(0) | 42(0) | 125(0) | 1.44 | 1.43 |
| 28 | 7(1) | 35(0) | 12(-1) | 125(0) | 0.49 | 0.52 |
| 29 | 7(1) | 35(0) | 42(0) | 200(1) | 0.68 | 0.66 |

Table 3: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of tannase production of *Aspergillus awamori* MTCC 9299

| Source | Sum of squares | Degree of freedom(df) | Mean Square | F-value | p-value Prob>F |
|---|---|---|---|---|---|
| Model | 3.060768 | 14 | 0.218626 | 173.792000 | <0.0001[a] |
| Lack of fit | 0.014692 | 10 | 0.001469 | 2.012557 | 0.2611[b] |
| Pure error | 0.002920 | 4 | 0.000730 | | |
| Cor total | 3.078379 | 28 | | | |

[a]Significant, [b]Non Significant

Table 4: Statistical significance of the tannase production of *Aspergillus awamori* MTCC 9299

| Statistical analysis | Values | Statistical analysis | Values |
|---|---|---|---|
| SD | 0.035468 | R-squared | 0.994279 |
| Mean | 0.902759 | Adj R-squared | 0.988558 |
| CV (%) | 3.928842 | Pred R-squared | 0.971028 |
| Press | 0.089187 | Adeq precision | 37.44848 |

also showed the good agreement between the experimental results and the theoretical values predicted by the model (Weisberg, 1985) and it showed that the model was suitable to represent the real relationship among the selected factors. The insignificant lack of fit test also indicated that the model was suitable to navigate the design space. The final predictive equation was as follows:

$$\text{Tannase activity } (Y) = -7.81835 + 1.70037 * A + 0.20623 * B + 0.030253 * C + 0.011065 * D - 1.37500E\text{-}003 * A * B + 1.25000 * A * C + 1.66667 * A * D - 2.08333E\text{-}004 * B * C - 9.33333 * B * D - 1.66667 * C * D - 0.16508 * A2 - 2.56583 * B2 - 2.36481 * C2 - 2.73926 * D2 \quad (3)$$

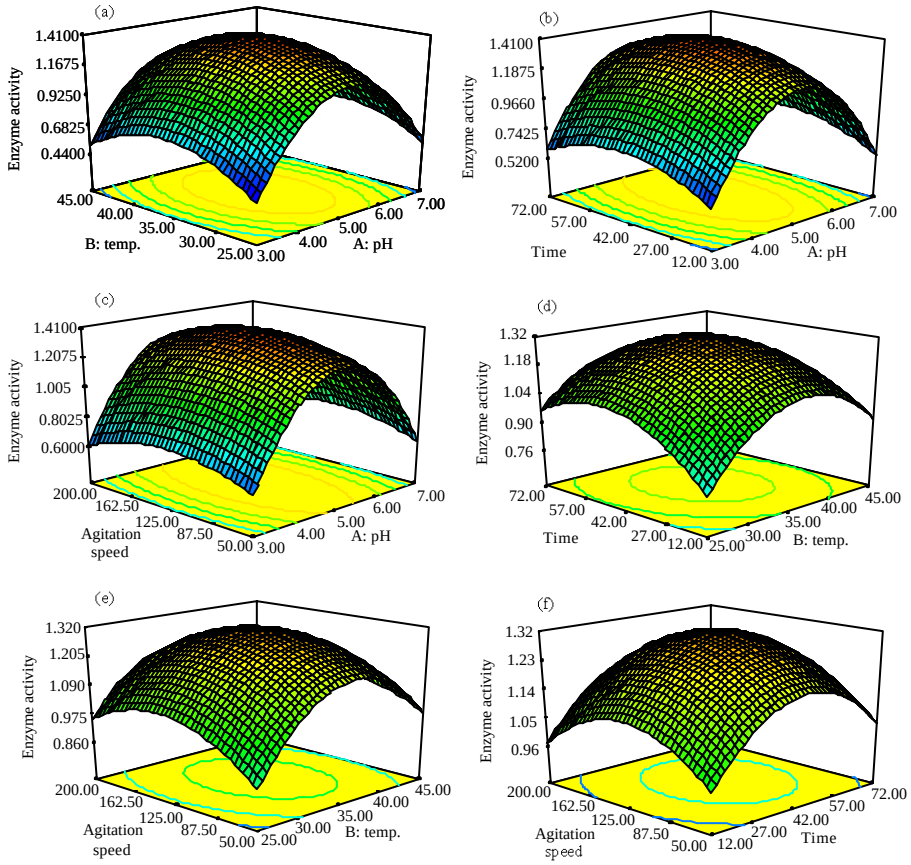


Fig. 1: (a) Effect of pH and temperature on the production of tannase. Other variable was held at zero level (coded), (b) effect of pH and time on the production of tannase. Other variable was held at zero level (coded), (c) effect of pH and agitation speed on the production of tannase. Other variable was held at zero level (coded), (d) effect of time and temperature on the production of tannase. Other variable was held at zero level (coded), (e) effect of agitation speed and temperature on the production of tannase. Other variable was held at zero level (coded) and (f) effect of agitation speed and time on the production of tannase. Other variable was held at zero level (coded)

where, Y is the tannase produced as a function of the coded levels of pH. (A) incubation, (B) temperature, (C) incubation period and agitation (D) rate.

**Response Surface Plots**

The three-dimensional (3-D) response surfaces (Fig. 1a-f) were plotted on the basis of the model equation to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum tannase production by *Aspergillus awamori* MTCC 9299 strain. The response surfaces shown in Fig. 1 were based on the final model, holding two variable constant at its optimum level, while the other two within their experimental range. The three-dimensional plots (Fig. 1) show that the increase in pH and

incubation temperature cause an increase in the tannase production to optimum values of 5 and 35°C, respectively, whereas, further increase leads to the decrease of enzyme production and the maximum tannase production was 1.45 U $mL^{-1}$ in 48 h when the level of agitation speed was at their central value of 125 rpm. Increasing the agitation speed beyond 125 rpm led to decline in tannase production. Also, an increase in incubation period beyond 48 h resulted in decline in the enzyme production.

The 3-D response surface (Fig. 1) corresponding an elliptical contours, suggesting that there were not only well defined optimum operating conditions but also the interaction effect between the two factors was significant (Dutta *et al*., 2004). The optimum pH 5, 48 h incubation period and incubation temperature of 35°C for tannase production obtained in the present investigation was in agreement with the previous reports (Lekha and Lonsane, 1997; Sabu *et al*., 2006). Kar *et al*. (2002) and Lekha and Lonsane (1994) obtained similar values for the incubation temperature using response surface methodology for tannase production from *Rhizopus oryzae* and *Aspergillus niger* PKL 104, respectively. Statistical optimization for tannase production by *Aspergillus niger* showed that 5% tannic acid, 0.8% sodium nitrate, pH 5.0, $5\times10^7$ spores/50 mL inoculum density, 150 rpm agitation speed and 48 h incubation period were optimum for tannase production (Sharma *et al*., 2007).

## CONCLUSION

Response surface methodology was performed to optimize the culture conditions for tannase production by *A. awamori*. Based on the present study, it is evident that the use of statistical optimization tools has helped to locate the optimum levels of the most significant parameters for tannase production, with minimum effort and time.

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