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RESEARCH ARTICLE

OPTIMIZATION THE HYDROLYSIS PROCESS OF TANNIC ACID FOR GALLIC ACID PRODUCTION BY TANNASE OF *ASPERGILLUS AWAMORI* USING RESPONSE SURFACE METHODOLOGY

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Abstract

The effect of different assay conditions such as; incubation time, temperature, pH, and tannic acid concentrations on the hydrolytic efficiency of tannase from *Aspergillus awamori* were studied. Response surface methodology (RSM) was used in this optimization by implementing the central composite design (CCD). Statistical analysis of the results showed that linear and quadric terms of these variables had significant effects on the yield of gallic acid formation. However, interactions between the variables were found to contribute to the response at a significant level. The predicted values of studied factors are 30.4 min incubation, 51°C, pH 5.6 and 25.3mg/mL tannic acid concentration, which resulted in 25.281µg/mL gallic acid. The resulted value of gallic acid by this way is very high as compared to the amount obtained by 'one- at- a- time' approach (5.20µg/mL).

Keywords: *Tannase, tannin degradation, gallic acid, Aspergillus awamori, RSM*

Introduction

Tannase (E.C.3.1.1.20) is one of the major groups of industrial enzymes due to its potential applications in a range of industrial processes such as; clarification of fruit juices, manufacture of coffee-flavored soft drinks, manufacture of instant tea and as an analytical probe for determination the structure gallic acid esters (Seth and Chand, 2000; Srivastava and Kar, 2010). Tannase is used for the cleavage of polyphenolics cross-links present in the plant cell walls and for the degradation of tannins present in the effluents of tanneries which represent serious environmental problems (Garca-Conesa *et al.,* 2001; Van de Lagemaat and Pyle, 2006).

The major application of tannase is catalyze the hydrolysis of ester and depside bonds present in hydrolysable tannins to form glucose and gallic acid (3, 4, 5-trihydroxybenzoic acid). Gallic acid is used in the pharmaceutical industry for production of antibacterial drug trimethoprim (Aguilar and Sanches, 2001; Bajpai and Patil, 2008) and in the manufacturing of gallic acid esters such as; propyl gallate and pyrogallol which widely used as food antioxidants (Mondal *et al.,* 2001). In addition, gallic acid has wide ranges of applications as antiviral, analgesic and anti-apoptotic agent to protect human cells against oxidative damage (Treviño-Cueto *et al.,* 2007). Because of the interesting properties and applications, gallic acid is a compound of great interest to pharmaceutical and chemical industries.

Response Surface Methodology (RSM) a collection of mathematical and statistical technique is used for building models which gaining importance for optimizing conditions (Jatinder *et al.,* 2006). This technique has been successfully utilized to optimize media manipulation and overproduction of microbial tannase (Naidu *et al.,* 2008; Beena *et al.,* 2010 and 2011). In this regard, the statistical design is applied to enhance the production of many biological agents, reduce time and costs, and give more precise results. Application of statistical design not only allows quick screen of a large experimental domain, but

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also helps in understanding the interaction between different independent variables to predict and calculate the optimal values of each factor and response (Box and Draper, 1987).

Hydrolysis of tannic acid by tannase is greatly influenced by many assay parameters such as time incubation, pH, temperature and concentrations of tannin (Abdel-Naby *et al.,* 1999; EL-Tanash *et al.,* 2011). In order to obtain the optimum conditions for maximal liberation of gallic acid, development of assay conditions is obligatory. In this communication, the present work is to optimize the conditions for higher yield of gallic acid by the hydrolytic effects of active tannase using RSM.

Material and methods

The fungal strain used in the present study was isolated from a tannery soil and identified as *Aspergillus awamori* by the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt. The strain was sub-cultured on modified Czapek's agar media containing 0.5% tannic acid as sole carbon source at 30°C for 7.0 days and maintained at 4°C. Induced slants of *A. awamori* were used to prepare the spore suspension.

The production of tannase by *A. awamori* was performed through batch SSF using China green tea as the source of tannin (Sherief *et al.,* 2011). One gram of China green tea was transferred to a 250 mL Erlenmeyer flask and mixed with 3.0 mL of basal medium (0.3% peptone; 0.1% $CaCl_2.2H_2O$, 0.1% KH_2PO_4 and 0.05% $MgSO_4$-$7H_2O$); then the mixture was autoclaved at 121°C at 15 psi (100 kPa) for 20 min. After cooling, the medium was inoculated under aseptic conditions by 1.0 mL of spore suspension (2.0 x 10^7spores) and incubated at 30°C under static conditions. The final moisture content was 80%. After 4.0 days incubation, the fermented substrates were eluted and recovered using 30 mL of 0.1 M acetate buffer (pH 5.5). The flasks were then kept on a rotating shaker for 1.0 h at 10°C and centrifuged at 5000 rpm for 10 min to remove all fungal cells and substrate residues. The clarified extract was collected and stored in a freezer (-20°C) until use as crude tannase.

For optimization the assay conditions, 0.5 mL of enzyme solution were incubated in 1.0 mL freshly prepared tannic acid (Sigma). Conditions such as: incubation period (X_1), incubating temperature (X_2), pH (X_3) and concentration of tannic acid (X_4) were adjusted according to the experimental design. The liberated gallic acid in each run (30 runs) was estimated through two steps against boiled enzyme. The first step was the precipitation of un-hydrolyzed tannic acid by Quinine HCl solution (Nishira and Mugibayashi, 1958). The second step was to detect the released gallic acid (Deschamps *et al.,* 1983). Enzyme mixture (1.5 mL) was terminated by adding 2.5 mL of 1.0 % Quinine HCl solution prepared in 1.0% NaCl solution. After strong shaking, all the tubes were kept for 20 min at room temperature to precipitate the residual tannins and subsequently centrifuged at 5000 rpm for 20 min. The librated gallic acid was estimated by diluting 200 µl of the supernatant in 5.0 ml distilled water, then the mixture was mixed with 1.0 mL $FeSO_4$ (colour reagent). The absorbance of developed colour (blue-violet) was read at 555 nm in a Spectro UV-VIS RS spectrophotometer. The liberated gallic acid (µg mL^{-1}) was estimated using standard curve of µg gallic acid.

Table 1 *Levels of the independent variables used in the central composite experiment design*

Independent Variables		Range of coded levels				
		-α (-2)	**-1**	**0**	**1**	**α (2)**
X1	Incubation time (min)	5.0	15	25	35	45
X2	Incubating temperature (°C)	40	45	50	55	60
X3	pH value (degree)	3.5	4.5	5.5	6.5	7.5
X4	Concentration of tannic acid (mg ml^{-1})	5.0	15	25	35	45

To determine the response pattern and synergy of variables the full 2^k composite design was performed giving 2^k+2k+n_0 combinations where k is the number of independent variables and n_0 is

the number of replications of the experiments at centre point. This provided 30 experimental runs performed with four factors at five coded levels (– 2 –1, 0, +1 and +2) in duplicate, with central points in triplicate to determine the experimental error (Box and Draper, 1987). The coded and actual values of the variables are presented in Table 1. The responses of the input variables were evaluated as a function of gallic acid, measured as the amount of gallic acid liberated and coded by Y_p (µg/mL). The relationship of variables was determined by fitting a second order polynomial equation to data obtained from the 30 runs. Design-based experimental data were matched according to the following second-order polynomial equation Eq. (1):

$$Y_p = \beta_0 + \sum_{i=1}^{4} \beta i x i + \sum_{i=1}^{4} \beta i i x i^2 + \sum_{i,j=1}^{4} \beta i j x i x j \quad (1)$$

Where Y_p is the predicted response (liberated gallic acid in µg mL^{-1} from the hydrolysis of tannic acid by tannase of *A. awamori*); β_0 is constant; βi, linear terms coefficients; βii, quadratic terms coefficients and βij interaction coefficients. The relation between the coded forms of the input variable and the actual values of chosen variables is described as Eq. (2).

$$xi = Xi - X0 / \delta Xi \quad \ldots.. (2)$$

Where *xi* is the coded value, *Xi* is the actual value of an independent variable; *X0* is the value of *Xi* at center point, δ*Xi* is the step change of the variable and *i* = 1–4. Regression analysis of obtained data and the calculations were performed using Design Expert 7.0 (Stat- Ease, Minneapolis, USA).

Results and discussion

The CCD experiments and predicted response for each combination of the variable are presented in Table 2. The maximal liberated gallic acid was showed at experimental runs in which the all tested variable were at zero level. In these runs, the amount of liberated gallic acid ranged from 24.12 - 24.70µg/mL.

Table 2 *Central composite design of the variables for gallic acid production by tannase as response*

Run	Independent Variables				Gallic acid (µgml^{-1})		
	X1	X2	X3	X4	Actual	Predicted	Residual
1	-1	-1	-1	-1	10.65	10.71	-0.06
2	-1	1	-1	-1	05.20	05.18	0.02
3	1	-1	-1	-1	17.20	17.06	0.14
4	-1	-1	1	-1	09.77	09.80	-0.03
5	1	1	1	-1	14.80	14.66	0.14
6	-1	1	1	-1	06.73	06.79	-0.06
7	1	1	-1	-1	11.58	11.48	0.10
8	1	-1	1	-1	17.87	17.74	0.13
9	1	-1	1	1	19.01	18.92	0.09
10	-1	1	-1	1	09.60	09.66	-0.06
11	-1	-1	1	1	11.00	11.03	-0.03
12	-1	1	1	1	10.15	10.18	-0.03
13	-1	-1	-1	1	12.99	13.02	-0.03
14	1	1	-1	1	16.05	15.91	0.14
15	1	-1	-1	1	19.46	19.33	0.13
16	1	1	1	1	18.14	18.01	0.13
17	-2	0	0	0	08.87	08.64	0.23
18	2	0	0	0	22.41	22.82	-0.41
19	0	-2	0	0	13.31	13.39	-0.08
20	0	2	0	0	06.85	06.95	-0.10
21	0	0	-2	0	13.76	13.86	-0.10
22	0	0	2	0	14.98	15.06	-0.08
23	0	0	0	-2	09.08	09.18	-0.10

24	0	0	0	2	14.77	14.85	-0.08
25	0	0	0	0	24.60	24.38	0.22
26*	0	0	0	0	24.70	24.38	0.32
27	0	0	0	0	24.12	24.38	-0.26
28	0	0	0	0	24.60	24.38	0.22
29	0	0	0	0	24.12	24.38	-0.26
30	0	0	0	0	24.12	24.38	-0.26

* The most active run

Based on above responses, sequential model sum of squares, Lack of Fit tests and model summary statistics a quadratic model is suggested and represented in Table 3.

Table 3 *ANOVA for selected model*

Source	Sum of squares	df	Mean square	*f*-value	*p*-value*
Model	1077.028	14	76.931	1414.77	0.0001
Residual	0.8157	15	0.0544		
Lack of Fit	0.4137	10	0.0414	0.51467	0.8263
Pure Error	0.4019	5	0.0804		

* Values of *P*-value less than 0.05 indicate significant
R^2 = 0.9992; Adj R^2 =0.9985; Predicted R^2 =0.997, PRESS=2.962; Adequate precision=116.41

The analysis of variance (ANOVA) of the quadratic regression model demonstrated that the computed *f*-value (1414.77) is higher than the tabulated *f*-value indicating that the model is significant at a high confidence level. The significance of the model is also indicated by low probability *P*-value ($P<0.0001$) and the value of the adjusted determination coefficient (Adj R^2=0.9985). Values of *P*-value less than 0.05 indicate model is significant (Box and Wilson, 1951). The 'Lack of Fit *F*-value' of 0.52 is not significant at all observed limits of variables for gallic acid liberation, indicating that the model thus found fit may significantly describe the variation of the responses. The value of the determination coefficient (R^2 = 0.9992) demonstrated that 82.63% of the total variations are explained by the model; while only 17.37% of the total variations are not explained. R^2 value is always between 0 and 1.0, the closer the R^2 to 1.0, the stronger the model and the better it predicts (Haaland, 1989). Furthermore, a lower value of coefficient of variation (CV=1.52%) shows that the conducted experiments are precise and reliable (Lee *et al.*, 2003). The adequate precision measuring the signal to noise ratio is found to be 116.41. The model is thus fit and could be use to navigate the design space. The predicted sum of squares (PRESS), which is a measure of how a particular model fits each point in design, is 2.96.

The significance of each coefficient in the model is established by estimating *P*-values (Table 4). *P*-values are used to check the significance of each coefficient, and also to indicate the strength of the interaction between each independent variable (i.e., the smaller the *p*-value, the more significant the corresponding coefficient). In this study, the *p*-values of X_1, X_2, X_3, X_4, X_1X_3, X_2X_3, X_2X_4, X_3X_4, X_1^2, X_2^2, X_3^2 and X_4^2 are highly significant ($P < 0.005$). The high significance of model terms indicates that it can act as a limiting factor, with even small variations substantially altering the yield of gallic acid production. Significant interactions between variables means that change in level of one variable affects the level of other, therefore, treating them separately may not reflect their real influence on the gallic acid production.

The quadratic mathematical model, which included all terms regardless of their significance level, can be given as Eq. (3):

$$Y_p = +24.38 +3.55X_1 -1.61X_2 +0.30X_3 +1.42X_4 - 0.015X_1X_2 +0.39X_1X_3 -0.011X_1X_4 +0.63X_2X_3 +0.54X_2X_4 -0.27X_3X_4 -2.16\ X_1^2 -3.55X_2^2 -2.48X_3^2 -3.09X_4^2 \quad \ldots\ldots (3)$$

Where, Y_p is the predicted response (gallic acid production); X_1, X_2, X_3 and X_4 are the coded values of incubation time, incubating temperature; pH and concentration of tannic acid, respectively.

Table 4 *Significance of regression coefficients*

Factor	Coefficient Estimate	Standard Error	*p*-value *
Intercept	24.377	0.095	< 0.0001
X_1	3.546	0.048	< 0.0001
X_2	-1.609	0.048	< 0.0001
X_3	0.299	0.048	< 0.0001
X_4	1.416	0.048	< 0.0001
X_1^2	-2.162	0.045	< 0.0001
X_2^2	-3.552	0.045	< 0.0001
X_3^2	-2.479	0.045	< 0.0001
X_4^2	-3.091	0.045	< 0.0001
X_1X_2	-0.015	0.058	0.8004
X_1X_3	0.395	0.058	< 0.0001
X_1X_4	-0.011	0.058	0.8496
X_2X_3	0.628	0.058	< 0.0001
X_2X_4	0.541	0.058	< 0.0001
X_3X_4	-0.271	0.058	0.0003

* Values of *P*-value less than 0.05 indicate significant

This regression equation is solved by the method of Myers and Montgomery (2002). Maximum gallic acid production of 25.281µg/mL is predicted to be obtained at the following conditions; 30.4 min time incubating, 51°C incubating temperature, pH 5.6 and 25.3 mg/mL tannic acid concentration. The maximal liberated gallic acid obtained experimentally is 24.70µg/mL and very close to predicted response obtained at centre points (average of six centre points was 24.38µg/mL).

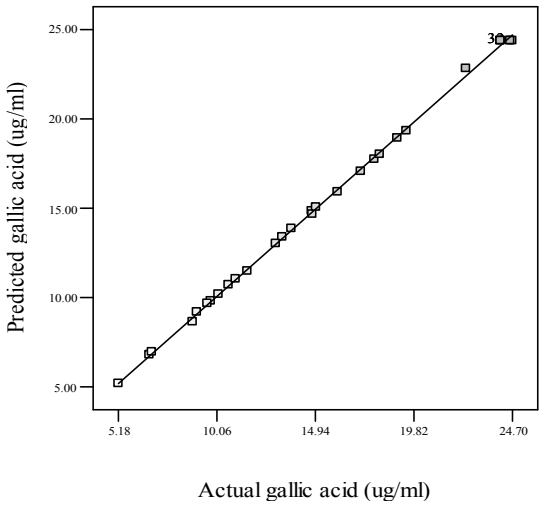

Figure 1 *Observed vs. the predicted gallic acid under optimum conditions*

Regression model can be use to predict future observations to the response of gallic acid formation corresponding to particular values of variables. In predicting new observations and in estimating the mean response at a given point, one must be careful about extrapolating beyond the region containing the original observations. It is very possible that a model that fits well in the region of the original data will no longer fit well outside the region. Fig. 1 shows observed gallic acid (the response) versus those from the empirical model. The figure proves the predicted data of the response from the empirical model is in agreement with the observed ones in the range of the operating variables.

The effect of independent variables on the yield of gallic acid by the effect of tannase is shown in the perturbation graph (Fig. 2). The plot revealed that gallic acid is sensitive to changes in all variables. The yield of gallic acid increased with increase the time incubation and decreased with higher incubating temperature. On the other hand, pH and concentration of tannic acid increased the yield of gallic acid linearly up to center value; then, there is significantly no discernable effect with their further increase.

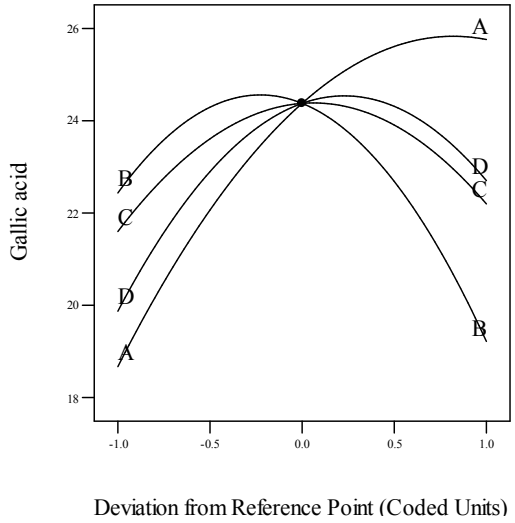

Figure 2 *Perturbation graph showing the effect of incubation period, temperature, pH and tannic acid concentration on the yield of gallic acid (Where, A is X_1; B is X_2; C is X_3 and D is X_4)*

The 3D response surfaces for variables terms are plotted to study interaction among various factors and their optimum values attaining the maximal yield of gallic acid production. The plots were generated by plotting the response (gallic acid) on z-axis against two independent variables while keeping the other independent variables at their 0-level. The significant interaction terms (X_1X_3, X_2X_3, X_2X_4 and X_3X_4) are the only plotted here as shown in Fig. 3 A, B, C and D.

Relative effect of two variables X_1X_3 (incubation period and pH) is very high significant effects on the rate of gallic acid when temperature and concentration of tannic acid were kept at their central levels (50°C and 25mg/mL tannic acid) as depicted in the response surface plot of Fig. 3A. Maximum yield of gallic acid (24.7µg/mL) is observed beyond 25 min and pH 5.5. Increasing the incubation time more than 25 min led to increase the value of gallic acid liberation. Also, pH value beyond pH 5.5 resulted in decline the yield of gallic acid production.

By solving the inverse matrix and analysis of Fig. 3A according to (Eq. (3)) using Design-Expert Software, at incubation time (5-35 min), pH levels (pH 4.5-6.5) and 0-levels of temperature and tannic acid concentrations. The predicted result reveals that 28.8 min incubation and pH 5.6 are optimum for the maximal value of gallic acid (25.43µg/mL).

On the other hand, the minimal value of gallic acid (16.15µgmL) is predicted at pH 6.5 after 15.1 min incubation. The results had been suggested that increasing long incubation period at pH range (5.0-6.0) is beneficial to increase the yield of gallic acid. Tannase exhibited the optimum activity in the acidic pH range, and its activity decreases in the alkaline range (Rodríguez *et al.,* 2008; Raghuwanshi *et al.,* 2011). A similar optimal pH 5.5 has been reported for tannase from *Paecilomyces variotii* (Battestin and Macedo, 2007). The effect of pH on the activity of tannase may be attributed to its amino acids and active site which undergoes protonation or deprotonation; also, it may attributed to the conformational changes induced by amino acids ionization (Sabu *et al.,* 2005).

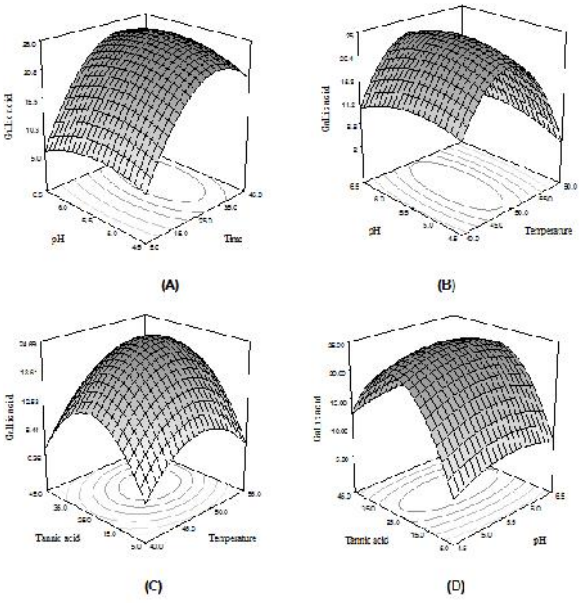

Figure 3 *Response surface plots of the combined effects of (A) incubation period and pH, (B) temperature and pH temperature, (C) temperature and tannic acid concentration, and (D) pH and tannic acid concentration on gallic acid production by tannase*

The relation between incubating temperature and pH is also significant in their effects on the yield of gallic acid (Fig. 3B). Maximal rate of gallic acid (24.7µg mL^{-1}) is observed beyond 50°C and pH 5.5, when incubation period and concentration of tannic acid are at their central values. By the analysis of Fig. 3B using the Design-Expert software, the maximum predicted gallic acid is

24.56µg/mL. Under this circumstance, the optimum temperature and pH values are 48.9°C and pH 5.5, respectively. 50°C was reported as the optimum for maximal tannase activity and the hydrolysis of tannins (Battestin and Macedo, 2007; Raghuwanshi *et al.,* 2011). In contrast, 40ºC have been reported as the optimum temperature for the activity of *Aspergillus caespitosum*, *Peniccilium charlesii*, *P. crustosum* and *P. restrictum* tannases (Batra and Saxena, 2005).

Fig. 3C shows significant interaction between concentration of tannic acid and incubating temperatures on gallic acid production when pH and incubation period were kept at their central levels. By analysis of Figure 3C using the Design-Expert software, the higher amount of gallic acid (24.7µg/mL) is predicted; where, 49°C and 27.2 mg/mL tannic acid as the optimum. However, minimum gallic acid (13.4µg/ml) is predicted when the temperature and tannic acid are 55°C and 15 mg/mL, respectively. Decreasing the yield of gallic acid at higher concentrations of tannic acid (30-45 mg/mL) may be attributed to the complexes with enzyme protein thereby tannase activity is slightly inhibited. Similar observations were reported where, increasing the concentration of tannic acid beyond 3.5% led to abrupt inhibition of *Bacillus sphaericus* tannase activity (Raghuwanshi *et al.,* 2011).

Further, interaction between pH value and tannic acid concentrations is significantly effect the rate of gallic acid as shown in Fig. 3D. By analysis of this figure, the higher predicted gallic acid (24.6µg/mL) is obtained at pH 5.5 and 27.3 mg mL^{-1} tannic acid.

From Fig. 3 A, B, C and D, 30.4 min time incubating, 51°C incubating temperature, pH 5.6 and 25.3 mg mL^{-1} tannic acid are the adequate assay conditions for attaining maximum gallic acid (25.281µg/mL). Under the optimized conditions, it clearly indicates that this statistical design is considered as a potential method for improvement and overproduction of gallic acid by this tannase, and it is able to apply industrially.

Usually, it is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy (Myers and Montgomery, 2002).

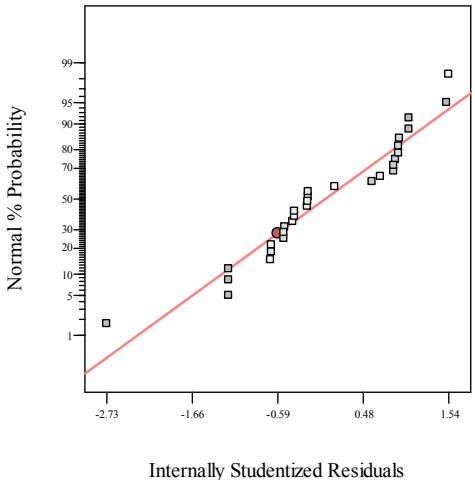

Figure 4 *Normal probability plot for gallic acid model*

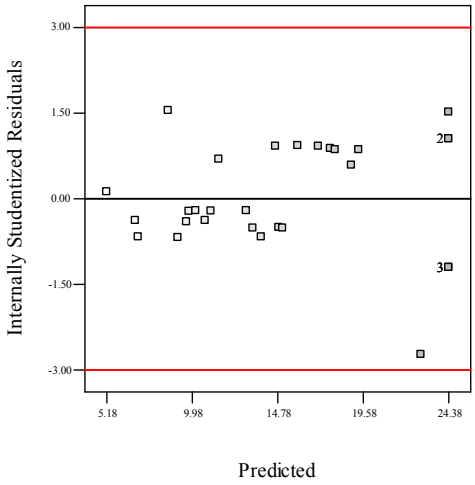

Figure 5 *Residuals vs. predicted plot for gallic acid model*

By constructing a normal probability plot of the residuals, a check is made for the normality assumption, as given in Fig. 4. The normality assumption is satisfied as the residual plot approximated along a straight line. Fig. 5 presents a plot of residuals versus the predicted response. The general impression is that the residuals scatter randomly on the display, suggesting that the variance of the original observation is constant for

all values of gallic acid. The plots (Figs. 4 and 5) are satisfactory, so it conclude that the empirical model is adequate to describe the gallic acid production by response surface.

Conclusion

The present paper studied the interaction of different assay conditions on tannic acid hydrolysis for maximization the amount of gallic acid production by the effect of *Aspergillus awamori* tannase. Rare reports for statistical optimization and modeling are available in literature regarding the production of gallic acid due to tannase hydrolysis. However, response surface analysis is a common practice in biotechnology, and various researchers have applied this technique for different optimization. In this study, the response surface methodology resulted in a 4.75 fold increase (from 5.20 to 24.7µg/mL) of gallic acid by tannase. The central points for the production of gallic acid by tannase using RSM are 25 min, pH 5.5, 50°C and 25 mg/mL tannic acid. The statistical analysis of obtained data gave an understanding the interaction between different physiological factors that lead to maximal gallic acid production. The high gallic acid productivity and low production cost may further broaden this application to the industrial scale. In addition, the applicability of the statistical approach has proved a useful and powerful tool for the development of optimal conditions and to gather sufficient information with a minimum number of experiment trials.

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