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Optimization of Culture Medium for Novel Cell-Associated Tannase Production from *Bacillus massiliensis* Using Response Surface Methodology

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Naturally immobilized tannase (tannin acyl hydrolase, E.C. 3.1.1.20) has many advantages, as it avoids the expensive and laborious operation of isolation, purification, and immobilization, plus it is highly stable in adverse pH and temperature. However, in the case of cell-associated enzymes, since the enzyme is associated with the biomass, separation of the pure biomass is necessary. However, tannic acid, a known inducer of tannase, forms insoluble complexes with media proteins, making it difficult to separate pure biomass. Therefore, this study optimizes the production of cell-associated tannase using a "protein–tannin complex" free media. An exploratory study was first conducted in shake-flasks to select the inducer, carbon source, and nitrogen sources. As a result it was found that gallic acid induces tannase synthesis, a tryptose broth gives higher biomass, and lactose supplementation is beneficial. The medium was then optimized using response surface methodology based on the full factorial central composite design in a 3 l bioreactor. A 2^3 factorial design augmented by 7 axial points ($\alpha = 1.682$) and 2 replicates at the center point was implemented in 17 experiments. A mathematical model was also developed to show the effect of each medium component and their interactions on the production of cell-associated tannase. The validity of the proposed model was verified, and the optimized medium was shown to produce maximum cell-associated tannase activity of 9.65 U/l, which is 93.8% higher than the activity in the basal medium, after 12 h at pH 5.0, 30°C. The optimum medium consists of 38 g/l lactose, 50 g/l tryptose, and 2.8 g/l gallic acid.

Keywords: *Bacillus massiliensis*, bioreactor, cell-associated tannase, central composite design, response surface methodology

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly known as "tannase", catalyzes the hydrolysis of ester and depside bonds in tannins. Tannase is used in the production of gallic acid, which in turn is important for the manufacture of the antimalarial drug trimethoprim and the antioxidant propyl gallate. Tannase is also used as a clarifying agent in the manufacture of wines, fruit juices, and coffee-flavored drinks [4]. Treatment of fruit juices with tannase has been found to reduce the bitterness while improving quality due to the lower haze and slower deterioration of the juice [14]. Furthermore, a major application of tannase is in the production of instant tea [2], where tannase is used to solubilize tea cream that forms in the extract upon cooling. As a result, it reduces the flavor loss and turbidity and increases the cold-water solubility. However, since the enzyme needs to be subsequently denatured by heating to over 90°C, this leads to loss of flavor. As a solution, immobilized tannase can facilitate recovery, avoids the need to heat the tea, improves the flavor, and reduces the processing costs [7]. Tannase is usually immobilized by physical adsorption, covalent binding, and entrapment [4].

Most of the reported literature available is pertaining to extracellular tannase of fungal and bacterial origin [2]. There are quite a few reports of intracellular tannase activity in molds as well [11, 18]. However, Belur *et al.* [5] were the first to report cell-associated tannase (CAT) activity in several bacterial isolates. CAT represents a unique naturally immobilized form of tannase, which avoids the expensive and laborious operation of isolation, purification, and immobilization. In addition, natural immobilization has a higher recovery and yield when compared with chemical or physical immobilizations, plus naturally immobilized enzymes invariably exhibit very high stability against adverse pH and temperature compared with the free enzyme [5, 9, 10, 17]. For example, when studying the kinetics and thermodynamic aspects of propyl gallate synthesis, Yu and Li [21] reported that the mycelium-bound tannase of *Aspergillus niger* had a higher stability in organic solvents.

Tannins and other polyphenolic compounds (tannic acid) form insoluble complexes with proteins, which is irreversible [6]. This restricts the combined use of organic

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nitrogen sources and tannic acid in production media. In the case of CAT, since the tannase is associated with the biomass, separation of the pure biomass is necessary for its use. However, separation of the pure biomass from suspended particles including protein precipitates is difficult, necessitating the design of a productive non-turbid medium. Accordingly, this study used shake-flasks trials to design a non-turbid medium for enhancing cell-associated tannase activity. Based on the results, a response surface methodology using a full factorial central composite design was then applied to optimize the medium in a 3 l bioreactor.

Materials and Methods

Microorganism and Culture Media

The cell-associated tannase (CAT)-producing bacterium was isolated by Belur *et al*. [5] from the rotting grape must of a local vinery (Bangalore, India). The isolate was deposited and identified in the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH Chandigarh, India), bearing the number MTCC 8930. The organism was identified based on 16S rDNA sequence analysis as *Bacillus massiliensis* and the Gene Bank accession number for the nucleotide sequence is AB633324.

The culture was maintained on nutrient agar slants at 4°C. The unoptimized medium used for growth and CAT production, called the MPSM medium, contained the following: Tannic acid (Sigma-Aldrich) 5 g/l; K_2HPO_4, 0.5 g/l; KH_2PO_4, 0.5 g/l; NH_4NO_3, 3 g/l; casein hydrolysate (Fluka), 3 g/l; and 1 ml of salt solution. The salt solution contained the following: $(NH_4)_6MoO_2{\cdot}4H_2O$, 0.1 g/l; $MnCl_2{\cdot}4H_2O$, 2 g/l; $CuSO_4{\cdot}5H_2O$, 0.2 g/l; and $CoSO_4{\cdot}5H_2O$, 2.8 g/l. The tannic acid was filter sterilized and added to the steam-sterilized medium aseptically. The initial pH of the MPSM medium was 6.5. For the inoculum development, one loopful of culture, maintained on a nutrient agar slant, was transferred to 100 ml of the MPSM medium and cultivated for 24 h at 150 rpm and 30°C on a rotary shaker. A 2% inoculum was used for all the shake-flask trials.

The cell densities were determined by reading the optical densities at 600 nm in a UV–VIS spectrophotometer (LABOMED Inc.). The biomass was determined by drying the pellet obtained after centrifugation (5,500 ×*g*, 15 min) at 80°C for 36 h until it reached equilibrium weight. The optical density (Y) was found to be related to the dry cell weight (X) based on the equation Y=3.116X.

Tannase Activity Determination

The tannase activity was estimated using the modified method given by Van de Lagemaat and Pyle [19]. According to this procedure, the culture broth was centrifuged at 5,500 ×*g* for 20 min, and then the cell pellet was washed and resuspended in a 0.1 M citrate buffer of pH 3.5. Next, 50 µl of this solution was added to 1 ml of 0.3 mM tannic acid in a 0.1 M citrate buffer (pH 3.5). After incubation at 30°C for 30 min, the reaction was stopped by the addition of 0.2 ml of HCl (2 M). Blanks were produced by adding 50 µl of the enzyme solution to the same reaction mixture already containing HCl. The quantity of gallic acid released during the hydrolysis of tannic acid was used to represent the tannase activity. Gallic acid was measured using the rhodanine reaction, and the sensitivity of the assay was 36 $\times 10^{-6}$ U/ml. One unit of enzyme activity was defined as 1 µmole of gallic acid released per minute under the assay conditions.

Shake-Flask Experiments

All the shake-flask trials were conducted in 250 ml Erlenmeyer flasks containing 100 ml of various media, and the flasks were incubated at 30°C in an incubator shaker at 250 rpm (Scigenics, India). To determine the effect of induction on CAT activity, the tannic acid in the MPSM medium was replaced with gallic acid (5 g/l), methyl gallate (5 g/l), or glucose (5 g/l).

Several common bacteriological media, namely a nutrient broth, tryptose broth, and brain heart infusion broth (HiMedia, India), were used without any supplementation of gallic acid or tannic acid, and the objective was to select the medium that produced highest biomass in the shortest time. Next, a simple sugar, namely glucose, fructose, sucrose, or lactose, was added to the selected bacteriological medium containing the tannase inducer. As such, the medium contained 52 g/l tryptose (HiMedia, India), 5 g/l gallic acid (Sigma), and one sugar (20 g/l), where the objective was to evaluate the effect of sugar supplementation on both the tannase and biomass production.

The tannase induction patterns were studied in a medium having tryptose as the nitrogen source (52 g/l) and lactose as the carbon source (35 g/l) and gallic acid as the inducer (0.5–6.0 g/l) at an initial medium pH of 5.0. Meanwhile, the tannase repression studies were carried out in a medium having tryptose as the nitrogen source (52 g/l), 2 g/l of gallic acid as the inducer, and several lactose concentrations (10–60 g/l) as a potential catabolic repressor.

Bioreactor Experiments

The batch fermentations were carried out in a 3 l *in situ* sterilizable stirred tank bioreactor (Scigenics, India) with a sterilizable dissolved oxygen (DO) probe (Mettler Toledo) and pH probe (Mettler Toledo). The DO and pH probes were both calibrated using standard procedures before every batch, and the trials were conducted under the following conditions: medium volume 1.7 l, inoculum volume 5% (v/v), and temperature 30°C. The aeration and agitation were controlled to keep the dissolved oxygen level above 20% of the saturation, pH was maintained by adding the appropriate amount of 2 N HCl and 2 N NaOH, and the temperature was maintained at 30°C by circulating hot water/cold water through the jacket during the fermentation. The sampling was performed aseptically every two hours, and 0.2% (v/v) polypropylene glycol was used as the antifoaming agent.

The inoculum was developed in shake-flasks, and the inoculation medium used for fermentation studies consisted of (g/l) glucose, 10; lactose, 10; tryptose broth, 60; and gallic acid, 2; at medium initial pH 5 and inoculum age of 12 h.

Response surface methodology using central composite design was applied to model the CAT activity. This approach involved a full factorial search by examining simultaneous, systematic, and efficient variations of significant components (lactose, tryptose, and gallic acid), identifying possible interactions and higher order effects, and determining the optimum operational conditions. A 2^3 factorial design augmented by 7 axial points ($\alpha = 1.682$) and 2 replicates at the center point was implemented in 17 experiments, where the effect of each component on the CAT activity was taken as a response. Five levels of variation were selected for each variable as

shown in Table 2, and to simplify the calculations, the independent variables were coded as Z:

$$Z = (X - Xo)/\Delta X \quad (1)$$

where X is the corresponding natural value, Xo is the natural value in the center of the domain, and ΔX is the increment of X corresponding to one unit of Z.

A second-order modal of response surface was used to calculate the predicted response.

$$Y = \beta 0 + \Sigma\ \beta i\ Xi + \Sigma\ \beta ii\ Xi^2 + \Sigma\ \beta ij\ XiXj \quad (2)$$

where Y represents the response variable, βo is the interception coefficient, βi is the coefficient of the linear effect, βii is the coefficient of the quadratic effect, and βij is the coefficient of the interaction effect. The responses for each run were subjected to multiple nonlinear regressions using the software Design-Expert (Stat-Ease Inc, Minneapolis, USA) to obtain the coefficients of the polynomial equation. The F-test was employed to evaluate the statistical significance of the quadratic polynomial model (Eq. 2), and the quality of the fit of the model was expressed by the coefficient of the determination of correlation R^2 and adjusted R^2. The significance of regression coefficient was tested using a t-test, where the level of significance was given as the values of prob>F less than 0.05. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the significant variables on the CAT activity. The global optimum levels of the selected variables were obtained by solving the regression equation and using desirability charts. Experimental validation of the predicted model was also performed.

RESULTS AND DISCUSSION

Induction Effect

CAT activity was noticed in the flasks with the MPSM medium (using tannic acid as the inducer) and also in the flasks where tannic acid was replaced with gallic acid and methyl gallate (Fig. 1). The highest tannase activity was recorded within 24 h of incubation in the gallic acid- and methyl gallate-containing media. However, in the medium using tannic acid as the inducer, highest activity was recorded after 24 h incubation, which shows the scope for reducing the fermentation time when using gallic acid as the inducer. In the flask that had glucose instead of tannic acid in the MPSM medium, no activity was noticed, even after 24 h of incubation. It should be noted that these findings are significant, since they facilitate the design of a non-turbid medium. Essentially, gallic acid can be used instead of tannic acid as an inducer of tannase activity, and since gallic acid does not form complexes with proteins, unlike tannins, this allows proteinaceous medium ingredients to be used when designing the medium.

The results clearly demonstrated that the presence of an inducer was essential for the production of CAT, which concurs with the earlier reports that tannase is an inducible enzyme produced in the presence of certain tannins or their degradation products [11]. However, in the case of fungi, low constitutive levels of the enzyme have been measured even in the absence of tannic acid [1, 8]. Lekha and Lonsane [11] questioned the inductive role of tannic acid because of its large molecular size and reactivity, which prevent the uptake of the molecule through the cell membrane. The reaction of tannic acid with the cell wall has also been reported to impair permeability. Therefore, these facts suggest that tannic acid cannot be an inducing agent. Thus, the probable mechanism of induction is that the microbes produce a basic constitutive amount of tannase that hydrolyzes tannic acid to glucose and gallic acid. These smaller molecules then enter the microbial cell and function as the inducer [11]. Notwithstanding, other researchers have demonstrated that gallic acid does not induce tannase production and in some cases even show end-product repression [1, 8]. Meanwhile, Bajpai and Patil [3] showed that tannic acid, gallic acid, and methyl gallate possess inductive properties. As such, these contradictions would seem to indicate that the regulatory mechanism may vary for different organisms [20]. However, no reports have yet been published on the inductive role of gallic acid in bacterial tannase production.

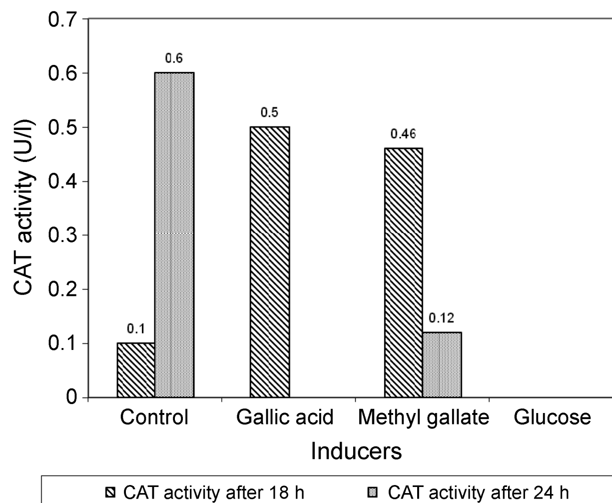

Fig. 1. Effect of inducers on CAT production. MPSM medium with tannic acid was used as control.
Tannic acid was replaced with gallic acid, methyl gallate and glucose in flasks, and incubated at 30°C, 250 rpm.

Medium Selection

The results of the shake-flask trials using various general bacteriological media are given in Table 1. Double-strength tryptose broth showed maximum biomass content compared with other media. Hence, double-strength tryptose broth (52 g/l) was used in subsequent trials.

Table 1. Selection of medium.

Medium	Concentration (g/l)	Max. biomass (g/l)	Incubation time (h)
Nutrient broth	39	2.18	42
Nutrient broth	52	3.40	42
Tryptose broth	26	3.63	48
Tryptose broth	52	5.23	48
Brain heart infusion broth	37	3.43	30
MPSM medium	13	3.01	24

Incubation at 30°C, 250 rpm was continued up to the beginning of the stationary phase. Maximum dry cell weight achieved is shown.

Effect of Sugar Supplementation

The results are presented in Fig. 2. Since glucose is an easily metabolizable sugar and also the hydrolysis product of tannic acid, the inhibition of tannase formation by catabolite repression or feedback inhibition was expected. However, contrary to those expectations, reasonably good CAT activity was noticed in the medium supplemented with glucose, whereas the medium supplemented with lactose showed even better CAT activity compared with the other sugars. Very few reports are available on the effect of carbon supplementation in the case of bacterial tannase. Mondal and Pati [13] reported catabolite induction by glucose in the tannic acid-containing medium, while Mondal *et al*. [12] noted the induction of tannase production by glucose, lactose, and sucrose at lower concentration (1 g/l), yet repression at higher concentrations in tannic acid-containing media, where lactose at 5 g/l concentration completely repressed the tannase production in the case of *Bacillus licheniformis*. Selwal *et al*. [16] also reported repression of tannase production when a vegetable tannin-containing medium was supplemented with 2 g/l of simple sugars. However, in the case of fungal tannase, there are conflicting reports on the effect of simple sugar supplementation [2]. Therefore, a detailed study on induction and repression was necessary to select the range of gallic acid and lactose concentrations for media optimization trials.

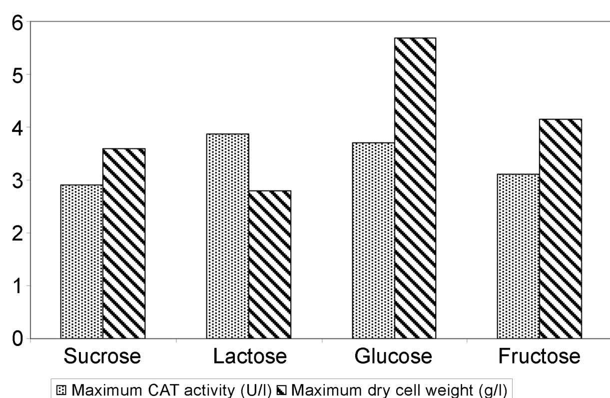

Fig. 2. Effect of simple sugar supplementation.
The medium contained 52 g/l tryptose, 5 g/l gallic acid, and one sugar (20 g/l).

The CAT activity per liter and CAT activity per gram of biomass were the highest in the case of the lactose-supplemented medium. Even though the biomass content in medium supplemented with glucose was the highest (5.68 g/l) compared with the other media, the CAT activity per liter was less, demonstrating that achieving a higher biomass alone may not lead to a higher enzyme activity.

Induction and Repression Studies

Fig. 3A shows that the gallic acid concentration was an important factor affecting CAT production. The maximum

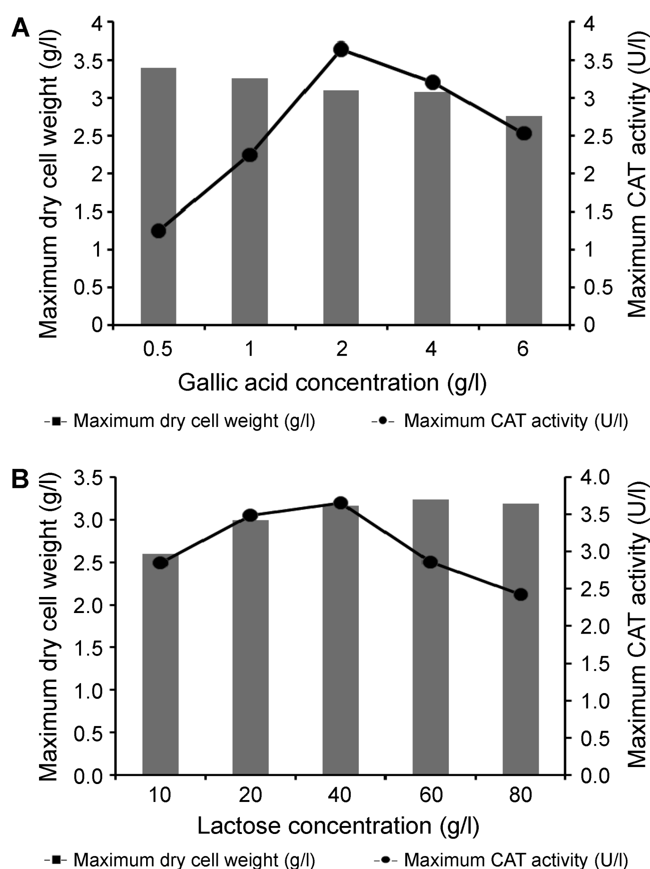

Fig. 3. Induction and repression studies.
(**A**) The medium consisted of 52 g/l tryptose, 35 g/l lactose, and 0.5−6 g/l gallic acid. (**B**) The medium consisted of 52 g/l tryptose, 2 g/l gallic acid, and 10−60 g/l lactose.

CAT activity (3.6 U/l) was obtained with a gallic acid concentration of 2.0 g/l, while a further increase in the gallic acid concentration led to a repression of CAT production. A gallic acid concentration of 6 g/l produced about 69% of the peak activity, along with a marginal reduction (15.5%) in the biomass content.

The tannase repression studies were carried out using 2 g/l of gallic acid as the inducer and several lactose concentrations (10−60 g/l) as a potential catabolic repressor. Fig. 3B shows that the CAT activity increased with lactose concentrations of up to 40 g/l, and then decreased with any further increase in the lactose concentration, possibly due to repression effect. Since, lactose is made out of galactose and glucose, and glucose is the product of tannin hydrolysis, it is reasonable to believe that the higher concentration of lactose caused catabolite repression. Moreover, no further increase in the biomass content was observed with more than 40 g/l of lactose, perhaps due to the nitrogen source limitation. Similarly, Sabu *et al*. [15] described the inhibition of tannase activity with 10 g/l of simple carbon sources in *Lactobacillus* sp. *ASR-S*, while Selwal *et al*. [16] noted suppressed extracellular tannase yield due to the presence of 2 g/l of additional carbon sources in *Pseudomonas aeruginosa* IIIB 8914.

Optimization of Medium in Bioreactor

From the shake-flask trials, it was evident that gallic acid can be used as an inducer in place of tannic acid. In addition, double-strength tryptose showed a higher biomass production compared with the other nitrogen sources, whereas the supplementation of medium with lactose was found to be more productive compared with the other simple sugars. The induction and repression studies showed that the gallic acid concentration was an important variable, where a higher concentration (above 2 g/l) caused catabolite repression. Similarly, lactose showed repression when used above 40 g/l. Thus, the medium was optimized using a central composite design for three selected variables; lactose as the carbon source, tryptose as the organic nitrogen source, and gallic acid as the inducer. Based on the results of the shake-flask study, the variables settings were selected as shown in Table 2.

Table 2. Levels of different medium components used in central composite design.

Coded values	Actual values		
Z^a	X_L Lactose (g/l)	X_T Tryptose (g/l)	X_G Gallic acid (g/l)
-1.628	6.36	19.77	0.3065
-1	20	30	1.5
0	40	45	3.25
1	60	60	5
1.628	73.64	70.23	6.1935

[a]Conversion of natural values (uncoded form) into coded form using following formula: $Z = (X - Xo)/\Delta X$, where X is the corresponding natural value, Xo is the natural value in the centre of the domain, and ΔX is the increment of X corresponding to one unit of Z.

The experimental data for response variable Y (CAT activity) are given in Table 3. The design matrix and the fitness of each term were analyzed using ANOVA and the results are presented in Table 4. The regression coefficients for each term are given in Table 5. The second-order

Table 3. Full factorial central composite design matrix using experimental and predicted values of cell-associated tannase activity.

Run order	(A) Lactose (g/l)	(B) Tryptose (g/l)	(C) Gallic acid (g/l)	CAT activity (U/l)		Residual value
				Actual	Predicted	
1	20.00	30.00	1.50	4.17	3.76	0.41
2	60.00	30.00	1.50	5.53	4.82	0.71
3	20.00	60.00	1.50	7.72	7.24	0.48
4	60.00	60.00	1.50	6.25	5.89	0.36
5	20.00	30.00	5.00	3.03	2.56	0.47
6	60.00	30.00	5.00	4.50	4.15	0.35
7	20.00	60.00	5.00	5.47	5.36	0.12
8	60.00	60.00	5.00	4.94	4.53	0.41
9	6.36	45.00	3.25	3.74	4.22	-0.48
10	73.64	45.00	3.25	3.72	4.41	-0.69
11	40.00	19.77	3.25	3.22	3.98	-0.76
12	40.00	70.23	3.25	6.81	7.23	-0.41
13	40.00	45.00	0.31	5.57	6.34	-0.77
14	40.00	45.00	6.19	3.78	4.19	-0.40
15	40.00	45.00	3.25	9.83	9.55	0.28
16	40.00	45.00	3.25	9.60	9.55	0.05
17	40.00	45.00	3.25	9.42	9.55	-0.13

Table 4. Analysis of variance for response surface quadratic model.

Source	Sum of squares	Degree of freedom	Mean square	F-value	p-value Prob > F
Model	77.04	9	8.56	15.5	0.0008 significant
A-Lactose	0.045	1	0.045	0.082	0.7829
B-Tryptose	12.74	1	12.74	23.07	0.002
C-Gallic acid	5.58	1	5.58	10.11	0.0155
AB	2.92	1	2.92	5.28	0.0551
AC	0.14	1	0.14	0.25	0.6325
BC	0.24	1	0.24	0.44	0.5298
A^2	38.6	1	38.6	69.88	< 0.0001
B^2	21.98	1	21.98	39.8	0.0004
C^2	25.93	1	25.93	46.95	0.0002
Residual	3.87	7	0.55		
Lack of fit	3.78	5	0.76	17.63	0.0545not significant
Pure error	0.086	2	0.043		
Cor. total	80.9	16			

R-Sq = 95.23% , R-Sq(pred) = 64.39%, R-Sq(adj) = 89.10%.

polynomial equation in coded units for the tannase activity was found to be

$$Y = 9.55108 + 0.0578X_L + 0.96593X_T - 0.63942X_G - 1.85023X_L^2 - 1.39649X_T^2 - 1.51665X_G^2 - 0.60386X_LX_T + 0.13136X_LX_G - 0.17364\, X_GX_T$$

The very low probability value (P model >F = 0.001) for the Fisher F-test and insignificant lack of fit demonstrated a very high significance for the regression model. The model F-value of 15.50 implied that the model was significant. Moreover, the goodness of fit of the model was checked by the determination coefficient (R^2), which was calculated to be 0.9523, implying that 95.23% of the experimental data of the CAT activity was compatible with the data predicted by the model (Table 3), whereas only 4.77% of the total variations was not explained by the model. The R^2 value was always between 0 and 1, and a value >0.75 indicated the aptness of the model. For a good statistical model, the R^2 value should be close to 1.0. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj $R^2 = 0.891$) was also high, advocating the high significance of the model. In the present case, the adjusted R^2 value was 0.891, which was less than the R^2 value of 0.9523. Additionally, the Pred R^2 of 0.65 was in reasonable agreement with the Adj R^2 of 0.891. Each actual response value was compared with the predicted value calculated from the model. A comparison of the residuals with the residual variance (0.982) indicated that none of individual residuals exceeded twice the square root of the residual variance. Furthermore, the normal probability plot (Fig. 4) of the residuals roughly followed a straight line, indicating a normal distribution of residuals. Thus, all of these considerations confirmed the adequacy of the regression model.

The significance of each coefficient was determined by Student's t-test and the p-values, as listed in Table 4. The

Table 5. Estimated regression coefficients for cell-associated tannase activity (U/l).

Term	Coeff.	SE Coeff.
Intercept	9.55	0.43
A-Lactose	0.058	0.20
B- Tryptose	0.97	0.20
C- Gallic Acid	-0.64	0.20
A^2 –lactose X lactose	-1.85	0.22
B^2 -Tryptose X Tryptose	-1.40	0.22
C^2 -Gallic acid X Gallic acid	-1.52	0.22
AB-Lactose X Tryptose	-0.60	0.26
AC-Lactose X Gallic acid	0.13	0.26
BC-Tryptose X Gallic acid	-0.17	0.26

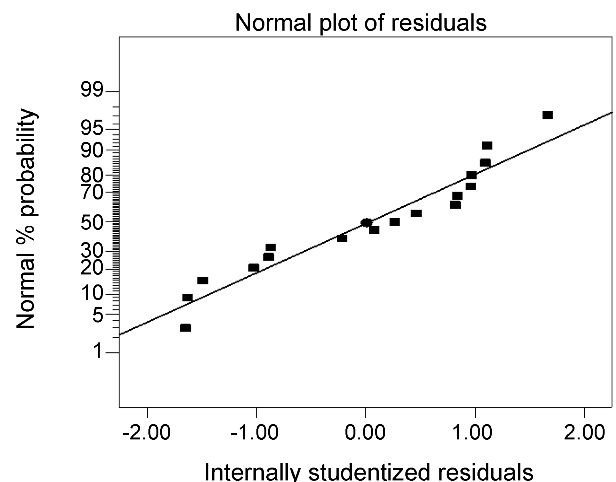

Fig. 4. Comparison between experimental values and predicted values of the RSM model.

larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient. Values of probability < 0.0500 indicated that the model terms were significant. In the present case, X_T, X_G, X_L^2, X_T^2, and X_G^2 were found to be significant model terms. Meanwhile, some model terms had values greater than 0.10, indicating that those are not significant. The positive coefficients for lactose and tryptose indicated a positive effect on the tannase activity at low concentrations, whereas at higher concentrations, they inhibited tannase activity, as evident from negative coefficients for the squared terms. In addition, the negative signs of squared coefficients indicated that the parabola would be open downwards (suggesting a maximum point). The interactive coefficient was not found to be significantly different from zero at a significance level of 0.05. Nevertheless, these coefficients were not removed as unnecessary in the regression equation.

The response surface plots as a function of two factors at a time, while maintaining the other factor at a fixed level, were more helpful in understanding the main and the interactive effects of each factor and determining the optimum level of each variable for the maximum response. The response surface curves for the tannase activity are shown in Fig. 5–7. In all figures, curves are convex in nature, suggesting well-defined optimum conditions; plus, as the variable ranges were appropriate, the optimum lies in the design space. The shapes of the contour plots indicated the nature and extent of the interactions. The prominent interaction of lactose and tryptose produced an elliptical contour plot in Fig. 5, whereas the less prominent or negligible interactions produced circular contour plots (Fig. 6 and 7). The response surface plots showed that increasing variable concentrations had a positive influence on the maximum CAT activity up to an optimum value, where high variable concentrations had a significantly negative influence on the response. Furthermore, the contour plots also revealed that the optimal variable values fell within the following ranges: lactose, 36–40 g/l; tryptose, 48–52 g/l; gallic acid, 2.6–3.2 g/l. The contour plots also revealed a rather broad plateau region in which the activities changed relatively little when the nutrient concentrations were varied, indicating that the optimal solution was able to accommodate small errors or variability in the experimental factors.

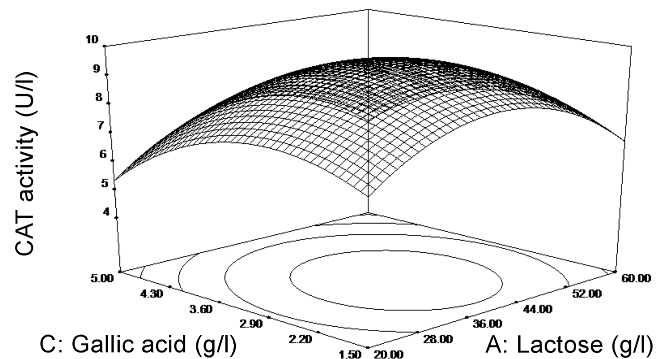

Fig. 6. Contour and surface plots of the cell-associated tannase (CAT) activity from the model equation. Effects of gallic acid and lactose.

The results from the response surface plots showed that the optimal concentrations of tryptose, lactose, and gallic acid were 50 g/l, 38 g/l, and 2.8 g/l, respectively. The maximum predicted value of enzyme activity was 9.71 U/l. To confirm this, two more trials were conducted using a medium consisting of the optimal points and the mean of the two trials was found to be 9.65 U/l. Thus, the good correlations between the predicted response and the experimental results (less than 5% difference) verify the validity of the response model and the existence of an optimal point. The optimization using the response surface methodology resulted in a 3.3-fold increase in CAT production when compared with that with the basal medium.

Thus, the statistical methods coupled with shake-flask experiments were successfully used to develop a medium that enhanced the novel cell-associated tannase activity.

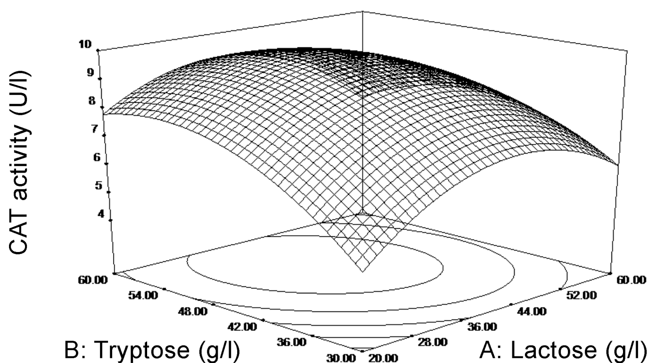

Fig. 5. Contour and surface plots of the cell-associated tannase (CAT) activity from the model equation. Effects of tryptose and lactose.

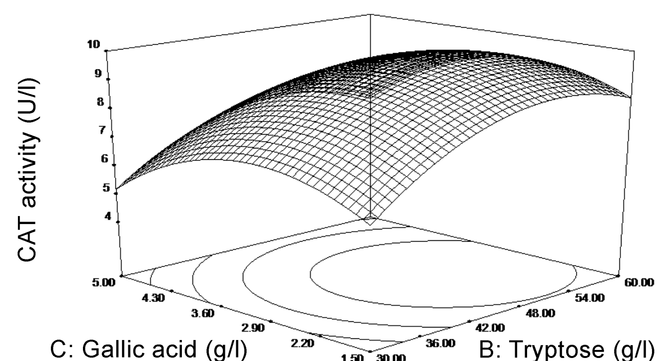

Fig. 7. Contour and surface plots of the cell-associated tannase (CAT) activity from the model equation. Effects of gallic acid and tryptose.

Moreover, the medium was devoid of any protein–tannin complex, allowing the separation of relatively pure biomass composed of tannase enzyme. Therefore, the results of this study are significant with regard to the intrinsic advantages of cell-associated tannase in several applications.

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