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Original Article

Production and Characterization of Tannase By *Bacillus subtilis* in Solid State Fermentation of Corn Leaves

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Abstract

Introduction: Tannase (tannin acyl hydrolase EC3.1.1.20) is an industrially important enzyme with extensive applications. The current study aimed to optimize the tannase production employing corn leaves as substrate and characterize tannase activity.

Materials and Methods: Tannase producing bacterial strains were isolated from *Catla catla* fish gut. The highest enzyme-producing bacterial strain was identified as *Bacillus subtilis* using 16S rDNA sequencing.

Results: Fermentation parameters and additional medium components were optimized with the application of one variable at a time and enhanced tannase was obtained with 50% substrate moisture, acetate buffer (pH 4.0) as enzyme extraction medium with 2 ml volume, 45 °C incubation temperature, pH 5, 2% inoculum size, 24 h incubation time, 150 rpm agitation, large-sized substrate particles (4.0 mm), enzyme extraction without centrifugation and medium components ($MgSO_4$, 4% tannic acid and yeast extract). The central composite design was employed to optimize the concentrations of optimal medium components, which were found as 4.0% tannic acid, 0.5% $MgSO_4$ and 1.5% yeast extract for the highest tannase production (211.97±0.08 U/ml). Tannase characterization revealed the maximum tannase activity at pH 8, 30 °C (with 30 min incubation period and 0.35% substrate concentration).

Conclusions: The results of the present study revealed the potential of utilization of agricultural resources (corn leaves) as a low-cost substrate to reduce the production cost of tannase.

Keywords: *Bacillus subtilis*, Tannase, Production, Central Composite Design, Characterization

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Introduction

Tannin acyl hydrolase (EC3.1.1.20), generally named tannase, catalyzes the breakdown of hydrolysable tannins, such as tannic acid by breaking ester depside bonds to yield glucose and gallic acid.[1,2] Tannase is an industrial enzyme with extensive applications in preparing instant tea, soft drinks, wine and reducing the bitterness and haze of juices. This enzyme is used to produce gallic acid which is used as a substrate to synthesize the trimethoprim and propyl gallate, which are the vital components of industries such as food and pharmaceutical. Furthermore, gallic acid has also been reported for its applications in adhesives, cosmetics and food industries.[3-6]

Despite extensive industrial use of tannase, the major limitations of its applications are expensive medium components, processes and use of pure tannic acid as an inducer.[7] On the other hand, Pakistan is an agricultural country. Therefore, interest in the use of agricultural resources has been increased remarkably due to their low cost and huge applications. Many reports have indicated agricultural wastes such as Jamun leaves, palm kernel cake, tamarind seed powder, sugarcane bagasse, rice straw powder, tea stalks, coffee husk, olive mill waste, cashew bagasse as a substrate for the production of tannase.[1,8-15] Corn is widely used as staple food for human consumption and is also used as fodder for animals. After harvesting the maize, most of its plant body is wasted or used for fodder. Due to its excess waste, an attempt was made to utilize the corn leaves for tannase production in this study. Tannase is produced by different organisms, but microorganisms play a leading role in tannase production. A variety of bacteria has been reported with the potential of tannase synthesis.[16-18] At the industrial level, both submerged fermentation (SmF) and Solid-State Fermentation (SSF) are applicable, but evidence supports the SSF to be the more appropriate for the utilization of agricultural wastes to produce industrially valuable enzymes.[19] The SSF has more manufacturing and commercial advantages with cost-effective downstream and upstream processes, yielding more products and less effluents.[20] Furthermore, fermentation conditions

like moisture, temperature, inoculum size may significantly influence the SSF process and consecutively affect the product formation.[21]

To develop economically efficient bioprocess, the optimization of various conditions of the fermentation process is essential. One Variable At a Time (OVAT) is a conventional optimization method where one variable's effect is determined by varying its values while keeping all the other variables constant.[22] The recent optimization approach is the Response Surface Methodology (RSM) that has application in designing experiments to optimize or improve the response or performance of a product or process. This technique combines the experimental design, regression analysis and methods in a strategy to optimize the response's expected value.[23] In the present report, the experiments have been designed to optimize the tannase production and activity from *Bacillus subtilis* under the SSF process using corn (*Zea mays*) leaves with the application of OVAT and RSM methodologies.

Materials and Methods

Isolation and Screening of Potential Bacteria

Bacterial strains with tannase producing potential were isolated from the gut of freshwater fish, *Catla catla*. The gut content was serially diluted in 0.9% saline and plated on sterile tannic acid medium (2.8% nutrient agar supplemented with 0.5% tannic acid) under sterilized conditions. After 24 h of incubation (37 °C), bacterial colonies appeared on the medium. The tannase producing bacteria were screened following Osawa and Walsh[24] based on the hydrolysis zone around their colonies by hydrolyzing the tannic acid. All zone producing strains were subjected to further screening employing tannase assay. Among all zone producers, the bacterial strain showing the maximum value of enzyme synthesis was selected and used during experiments to optimize the fermentation and medium components.

Identification of Bacterial Strain

The selected bacterial isolate with the highest tannase production was identified using 16S rDNA. Briefly, an overnight incubated pure culture of the bacterial isolate was used for DNA extraction. The DNA was amplified using universal primers, following Shakir.[26] PCR reaction mixture (50 µl) contained DNA template (5 µl), 10X PCR buffer (5 µl), 2.0 U/µl Taq polymerase (2 µl), 1 mM dNTPs (5 µl), 25 mM $MgCl_2$ (5 µl), dH_2O (18 µl), 10 pM forward primer (5 µl) and10 pM reverse primer (5 µl). DNA was amplified using thermocycler with conditions: initial denaturation at 95 ºC for 5 min, 35 cycles of the second denaturation for 45 sec at 94 ºC, extension for 1 min at 72 ºC, final extension for 7 min at 72 ºC. The PCR products were examined using 1% agarose gel electrophoresis and DNA was purified with a GenJET™ purification kit. Purified amplicons of 16S rDNA were commercially sequenced. The nucleotide sequence file was aligned using NCBI BLAST and the bacterial strain was identified based on the percentage similarities with already submitted sequences of classified bacteria in databases. The neighbor-joining (unrooted tree) was constructed using the NCBI BLAST Tree method.

Substrate

Corn leaves were obtained from district Chakwal, Pakistan, cut into small pieces, properly dried and then ground for fine powder to be used as a substrate in optimizing experiments of tannase under SSF.

Tannase Production

For each tannic acid degrading strain, 100 ml production medium (tannic acid 0.5%, yeast extract 0.275% and $CaCl_2$ 0.1%) was taken in each 250 ml Erlenmeyer flask and autoclaved for 15-20 min at 121 °C and 15 psi. After that flask was inoculated (1%) with 24 h old culture cells and after 24 h incubation (37 °C), the fermentation medium was centrifuged at 8000 rpm for 15 min at 4 °C. After centrifugation, the supernatant was used as a source of crude tannase enzyme.

Tannase Assay

Tannase assay was performed by the Miller[25] method. Tannic acid (0.5%) prepared in 0.1 M acetate buffer (pH 5.0) was used as substrate. Crude tannase (0.5 ml) was incubated with substrate (0.5 ml) for 30 min at 37 °C. After incubation, 3 ml of di-nitro-salicylic acid was added to terminate the reaction. The solution was boiled in the water bath for 15 min, followed by the dilution with 10 ml distilled water and optical density was taken at 540 nm using spectrophotometer against blank. The glucose was used as standard. One tannase-unit was defined as the amount of tannase used to hydrolyze 1 mM of tannic acid as substrate in one min under standard tannase assay conditions.

Optimization of Physico-chemical Parameters

The effect of various parameters, i.e., moisture contents of the substrate (50-90%), different tannase extraction mediums (distilled water, tap water, NaCl 1%, acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0 and 7.0), Tris-HCl buffer (pH 8.0 and 9.0) and glycine NaOH buffer (pH 10.0 and 11.0), volume of optimal extraction medium (1-6 ml), incubation temperature (37 °C, 40 °C, and 45 °C), medium pH (3, 5, 7, 9, and 11), inoculum size (1, 2 and 3%), incubation period (24, 48, 72, and 96 h), agitation effect (150 rpm shaking and non-shaking), substrate particle sizes (2.8, 3.4, 4.0 mm) and effect of centrifugation (8000 rpm speed at 4 ºC for 15 min) were optimized under SSF for tannase production using OVAT.

Optimization of Medium Components

The presence of additional salt, carbon and nitrogen source

has a vital role in SSF. To assess the role of these medium components on tannase production, the substrate (corn leaves) was supplemented with different salts (KCl, NaCl, K_2HPO_4, KH_2PO_4, $CaCl_2$, $MgSO_4$), concentrations of tannic acid (1.5, 2.0, 2.5, …, 4.0%) as carbon source and nitrogen sources (malt extract, yeast extract and peptone). These parameters were optimized based on the highest tannase production using OVAT under SSF.

Optimization of Concentration of Medium Components Using Central Composite Design

Concentrations of optimized medium components were optimized for optimal tannase production employing central composite design (CCD) of RSM. This study applied three medium components and a five-level face-centred cube design consisting of 17 experiments. Independent variables were tannic acid, $MgSO_4$ and yeast extract concentrations, while the response was tannase production (U/ml). Each variable was investigated at five levels (-2. -1, 0, +1, +2) as described in Table 1.

ANOVA was applied to assess the significance of the model and regression coefficients. The regression analysis predicted the response (tannase production) by the application of second-order polynomial equation.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=0}^{k} \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j \quad (1)$$

Y is the response of tannase production (U/ml), Xi, Xj are the independent variables, whereas k represents the total number of applied variables. β_o, β_{ij} and β_{ii} are coefficients of intercept, interaction and quadrate, respectively.

Table 1. Levels of Medium Components (Independent Variables)

Medium components	Code	Levels				
		-2	-1	0	+1	+2
Tannic acid (%)	A	3.00	3.50	4.00	4.50	5.00
$MgSO_4$ (%)	B	0.10	0.25	0.50	0.75	1.00
Yeast Extract (%)	C	0.50	0.75	1.00	1.25	1.50

Characterization of Tannase

Effect of pH

To evaluate the pH (4-11) effect on crude tannase activity, different buffers such as acetate (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0), tris-HCl (pH 8.0 and 9.0) and glycine-NaOH (pH 10.0 and 11.0) were used. The pH value related to maximum activity was recorded as optimal.

Effect of Temperature

To determine optimum temperature, tannase activity was checked at temperatures varying from 20 °C to 90 °C at optimum pH. The temperature value showing the highest enzyme activity was considered as temperature optima.

Effect of the Incubation Period

Crude tannase with optimal temperature and pH was incubated for different time periods (15, 30, 45…, 75 min). The incubation period corresponding to the best activity was recorded as optimum.

Effect of Substrate Concentration

Different concentrations (0.25, 0.30, 0.35…, 0.60%) of tannic acid were applied with all previously optimized factors to assess the optimum concentration with the highest tannase activity.

Statistical Analysis

Experiments were conducted in triplicates and the results were presented in the form of mean value and standard deviation. Statistical significance was analyzed using t-test and one-way ANOVA followed by Tukey's test (p<0.05) by SPSS Statistics software 20 and Microsoft Excel 2019. Statistics for RSM was applied using STATISTICA (99th edition) software.

Results

Screening and Identification of Bacteria

In the present study, three bacterial isolates showed a greenish zone of hydrolysis on tannic acid incorporated medium after 24 h incubation and they were coded as B1, B2 and B3. The tannase assay showed that B3 produced significantly highest tannase with an enzyme value of 2.65 ± 0.35 U/ml (Figure 1). The *16S rDNA* gene sequence revealed bacterial strain (B3) as *Bacillus subtilis*. The phylogenetic tree with neighbor joining (unrooted tree) method has been demonstrated in Figure 2.

Optimization of Physico-chemical Parameters

Effect of Substrate Moisture

In SSF, the value of moisture content in corn substrate varied from 50% to 90%. The optimal tannase production (48.69 ± 0.64 U/ml) was achieved with 50% moisture content (distilled water) in the substrate. With the increase in moisture, the tannase was observed to be decreased (Figure 3).

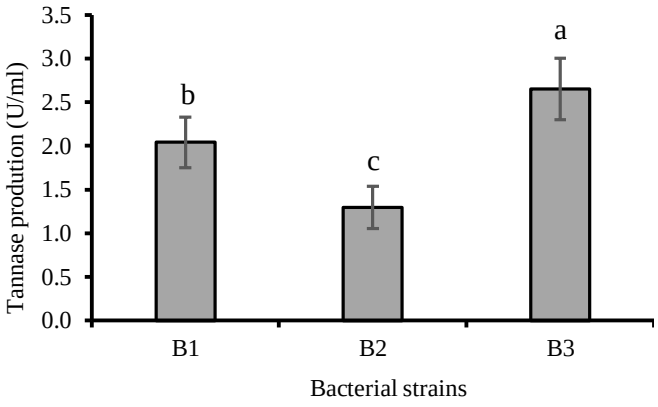

Figure 1. Enzyme Production by Bacterial Strains as The Result of Tannase Assay. Different alphabets on bars represent them significantly different.

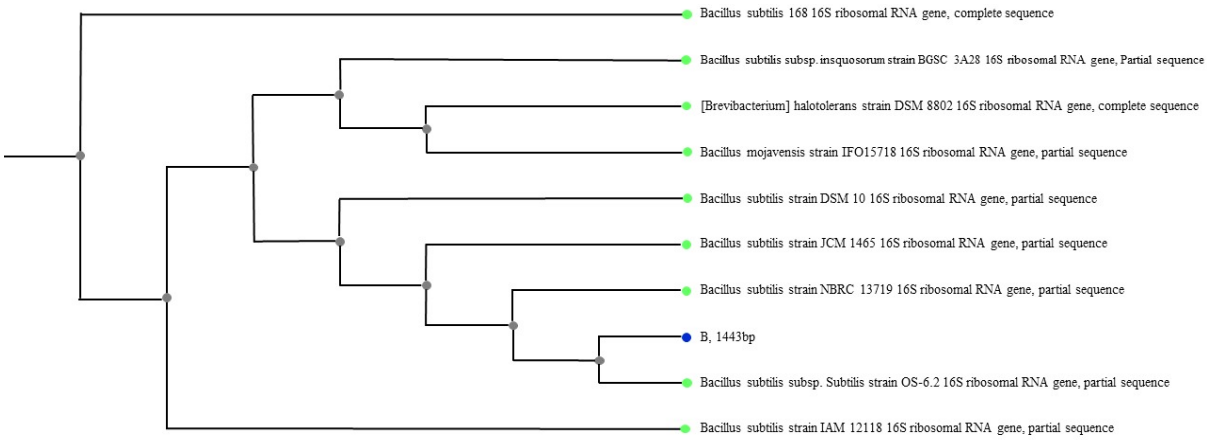

Figure 2. Phylogenetic Tree – Neighbor Joining (Unrooted Tree) for *B. subtilis* by NCBI BLAST Tree Method.

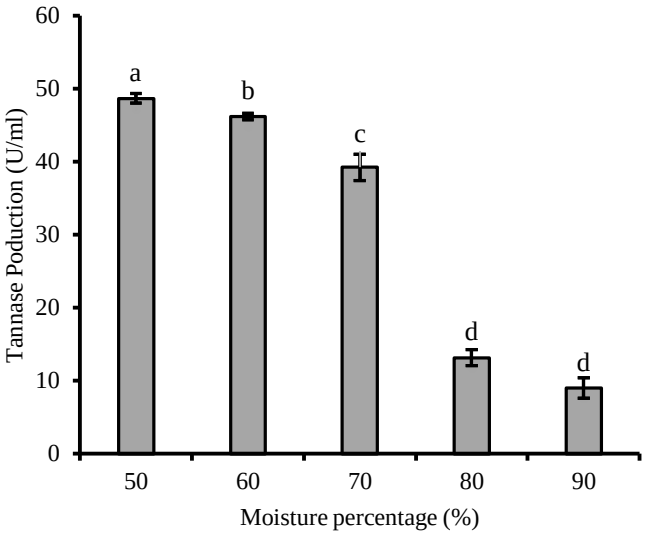

Figure 3. Tannase Production at Various Levels of Substrate Moisture Content (%). Different alphabets on bars represent them significantly different.

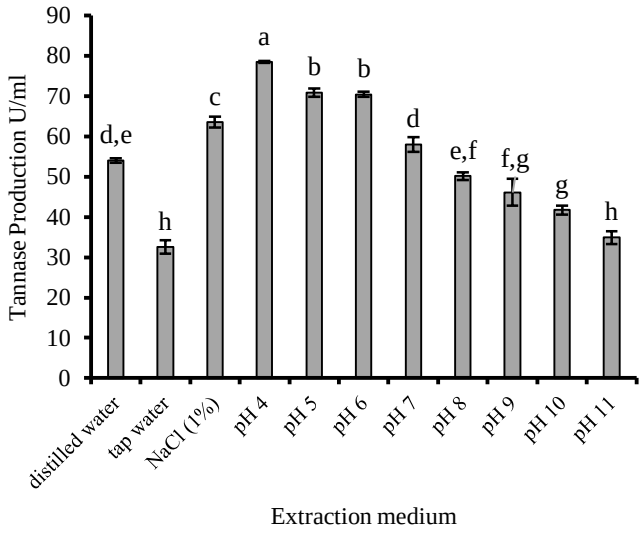

Figure 4. Tannase Production with Different Enzyme Extraction Mediums. Bars having no alphabets are significantly different.

Effect of Enzyme Extraction Medium

For tannase extraction, different medium (distilled water, tap water, NaCl 1%, acetate buffer with pH 4.0 and 5.0, phosphate buffer with pH 6.0 and 7.0, Tris-HCl buffer with pH 8.0 and 9.0 and glycine-NaOH buffer of 10.0 and 11.0 pH) were used. The highest enzyme (78.44 ± 0.25 U/ml) was produced using an acetate buffer of pH 4.0, whereas tap water was the least feasible extraction medium (Figure 4).

Effect of Volume of Enzyme Extraction Medium

Different volumes of optimum enzyme extraction medium (acetate buffer pH 4.0) were used during fermentation. Results in Figure 5 showed that tannase production (81.59 ± 0.64 U/ml) was significantly higher with 2 ml volume of acetate buffer pH 4.0, which was observed to be decreased with further increase in volume. However, no enzyme was produced with minimum applied volume (1 ml) of the extraction medium.

Effect of Incubation Temperature

Incubation temperature plays a role during enzyme production. Experiments were performed at different temperatures of 30 °C, 37 °C and 45 °C. Results represented that enzyme production (86.53 ± 0.17 U/ml) was significantly higher at 45 °C than other applied temperatures (Figure 6).

Effect of Inoculum Size

Sufficient inoculum is necessary for proper biomass growth and enzyme production. Three different inoculum sizes like 1, 2 and 3% were used and the results showed that 2% inoculum gave the highest tannase production (88.76 ± 1.78 U/ml). While with 1% inoculum, the lowest enzyme (70.70 ± 0.58 U/ml) production was observed (Figure 6).

Effect of the Incubation Period

To determine the optimal incubation period for tannase production, results were noted for different incubation periods at an optimum temperature of 45 °C. The maximum tannase

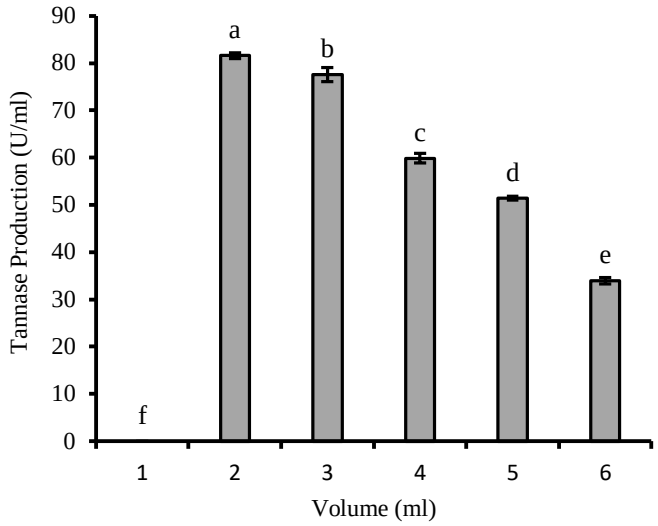

Figure 5. Tannase Production with Different Volumes of Optimal Enzyme Extraction Medium. Different alphabets on bars represent them significantly different.

(89.70 ± 0.24 U/ml) was recorded at 24 h incubation, while the production was noted to decrease with increasing time period (Figure 6).

Effect of Agitation

A significantly higher enzyme value (89.98 ± 0.29 U/ml) was obtained when agitation was applied rather than the static condition (70.37 ± 2.22 U/ml) in the experiment (Figure 6).

Effect of Substrate Size

Substrate particles size is an essential parameter for bacterial action. The maximum tannase (118.35 ± 2.12 U/ml) was produced with the larger substrate particle size (4.0 mm). With medium-sized particles (3.4 mm), the lowest enzyme production (81.77 ± 1.94 U/ml) was observed (Figure 7).

Effect of Centrifugation

It was established that higher tannase production (118.35 ± 2.12 U/ml) from *B. subtilis* was achieved without centrifugation condition (Figure 8) after termination of fermentation time.

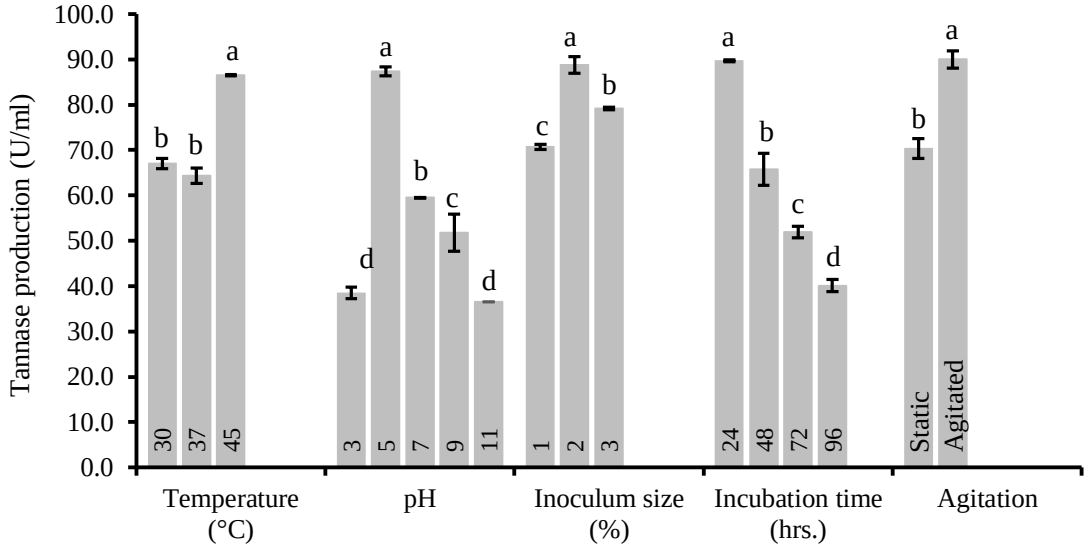

Figure 6. Effect of Different Parameters on Tannase Production from *B. subtilis*. Different alphabets on bars represent them significantly different.

Additional Medium Components

Effect of Salts

When different salts were applied in the fermentation medium, the highest tannase synthesis (120.89 ± 2.31 U/ml) was achieved with $MgSO_4$, while NaCl, KCl and $CaCl_2$ had the least effect on enzyme production (Figure 9).

Effect of Tannic Acid

Concentrations of tannic acids varying from 1.5% to 4.0% were incorporated in the substrate as an additional carbon source. The highest enzyme production (122.47 ± 1.85 U/ml) was recorded when 4.0% tannic acid was used, whereas 1.5% showed the minimum production (47.52 ± 0.78 U/ml) as described in Figure 10.

Effect of Organic Nitrogen Source

Nitrogen source plays a vital role during enzyme productivity. On the application of yeast extract, malt extract and peptone in the substrate, optimal tannase synthesis (125.28 ± 0.89 U/ml) was achieved with yeast extract, while malt extract yielded the lowest enzyme production (94.22 ± 1.09 U/ml) (Figure 11).

Central Composite Design (CCD) of RSM

With the application of CCD of RSM, concentrations of optimal salt, carbon and nitrogen sources were optimized under SSF. The highest tannase production (211.97 ± 0.08 U/ml) by *B. subtilis* was achieved with 4.0% tannic acid,

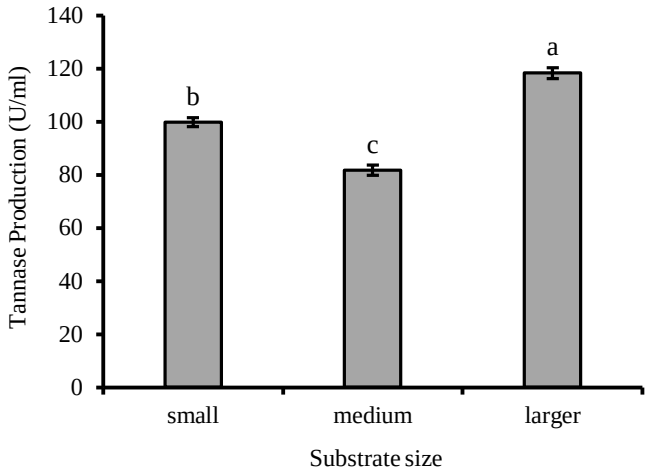

Figure 7. Tannase Production with Different Substrate Particle Sizes. Different alphabets on bars represent them significantly different.

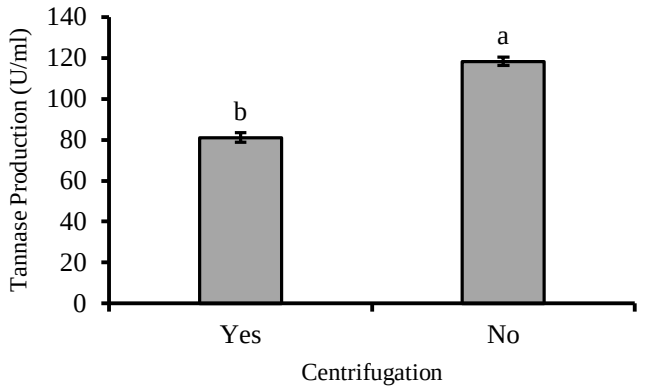

Figure 8. Tannase Production with and without Centrifugation. Different alphabets on bars represent them significantly different.

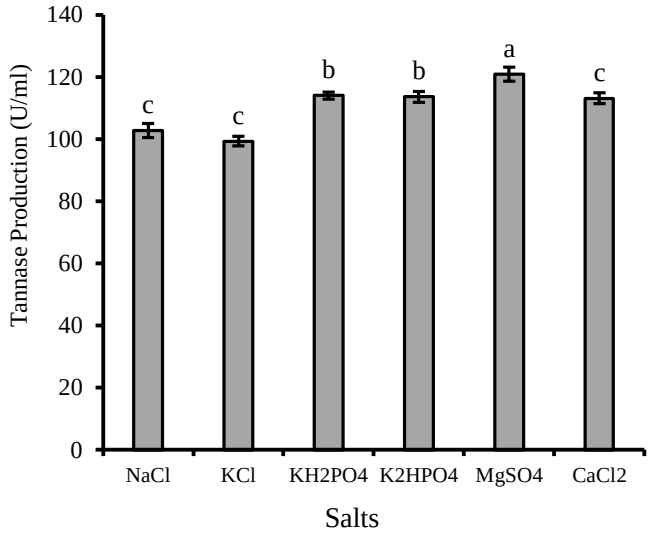

Figure 9. Tannase Production with Various Salts. Different alphabets on bars represent them significantly different.

0.5% $MgSO_4$ and 1.5% yeast extract during the 11th run (Table 2). The response of the CCD design was estimated by second-order polynomial regression equation (2).

$$Y = 375.727 + 10.885A + 284.144B - 782.118C - 13.070A^2 + 54.846B^2 + 103.997C^2 - 90AB + 157.330AC - 50.519BC \quad (2)$$

Where Y is tannase production (response), A is tannic acid (%), B is $MgSO_4$ (%) and C is yeast extract (%).

ANOVA for the CCD model was statistically significant with F-value of 196.2873 and $p<0.01$ (Table 3). The concentrations of $MgSO_4$ and yeast extract were observed as significant ($p<0.05$). The coefficient of determination (R^2) was found 0.9660, which describes 96.60% sample variations and only lower than 4% of the variance. The regression model showing the value of R^2 more than 0.95 depicts the high correlation. Furthermore, the high similarity between the observed and predicted values (Table 2, Figure 12) indicated that the model was statistically robust. The contour plots and desirability chart resulting from RSM have been depicted in Figures 13 & 14.

Tannase Characterization

Effect of pH

For the assessment of the pH effect on crude tannase activity, experiments were performed with different pH ranging from 4.0 to 11.0. The maximum tannase activity 218.38 ± 0.90 U/ml was achieved at pH 8.0, while the minimum activity was observed in 103.81 ± 2.33 U/ml at 4.0 (Figure 15).

Effect of Temperature

Results in Figure 15 depicted the tannase activity at different incubation temperatures (20-90 °C). The enzyme activity was significantly highest (219.78 ± 0.64 U/ml) at 30 °C while at high temperature (90 °C), the lowest value (125.10 ± 1.36 U/ml) was recorded.

Effect of Incubation

The crude enzyme was incubated for different incubation times at optimal temperatures and the highest tannase activity (233.34 ± 0.67 U/ml) was achieved after 30 min. While by further increasing the incubation duration, a decrease in the enzyme activity was seen up to 135 min (Figure 15).

Effect of Substrate Concentration

The results for the effect of concentration of tannic acid on tannase activity depicted the highest activity (234.34 ± 1.44 U/ml) with 0.35% tannic acid (Figure 15). After optimal value, the enzyme activity tended to decrease with increasing concentrations of substrate.

Table 2. Experimental Design and Responses of CCD for Tannase Production from *B. subtilis* in SSF

Exp. No.	Tannic Acid (%)	$MgSO_4$ (%)	Yeast Extract (%)	Tannase production (U/ml)		
				Observed	Predicted	Residue Value
1	4.00	0.10	1.00	160.9865	159.1957	1.79079
2	4.00	0.50	0.50	174.9611	172.5704	2.39078
3	4.50	0.75	1.25	196.7023	197.1600	-0.45774
4	4.00	1.00	1.00	189.8559	189.1520	0.70381
5	5.00	0.50	1.00	168.3774	167.0442	1.33318
6	4.50	0.25	1.25	211.7822	212.1168	-0.33458
7	4.00	0.50	1.00	164.0244	161.5404	2.48398
8	3.00	0.50	1.00	130.4139	129.8971	0.51673
9	3.50	0.75	0.75	190.9587	192.4740	-1.51533
10	3.50	0.25	0.75	148.3249	149.7171	-1.39217
11	4.00	0.50	1.50	201.9681	202.5089	-0.54086
12	4.50	0.75	0.75	147.7370	149.1730	-1.43598
13	3.50	0.75	1.25	162.2339	161.7958	0.43806
14	3.50	0.25	1.25	131.2547	131.6686	-0.41393
15	4.50	0.25	0.75	149.2120	151.5000	-2.28797
16	4.00	0.50	1.00	162.4524	161.5404	0.91205
17	4.00	0.50	1.00	159.3496	161.5404	-2.19080

Table 3. ANOVA Values for Regression Model Obtained from CCD Applied for Medium Component Optimization for Tannase Production from *B. subtilis* in SSF

Effect	SS	DF	MS	F-value	P-value
Model	9027.899	9	1003.099	196.2873	0.000000
A (tannic acid)	1.902	1	1.902	0.3722	0.561106
B ($MgSO_4$)	810.790	1	810.790	158.6562	0.000005
C (yeast extract)	3235.762	1	3235.762	633.1770	0.000000
A^2	210.050	1	210.050	41.1029	0.000363
B^2	160.819	1	160.819	31.4692	0.000808
C^2	831.215	1	831.215	162.6529	0.000004
AB	1016.280	1	1016.280	198.8667	0.000002
AC	3094.110	1	3094.110	605.4584	0.000000
BC	79.756	1	79.756	15.6067	0.005528
Error	35.773	7	5.110		

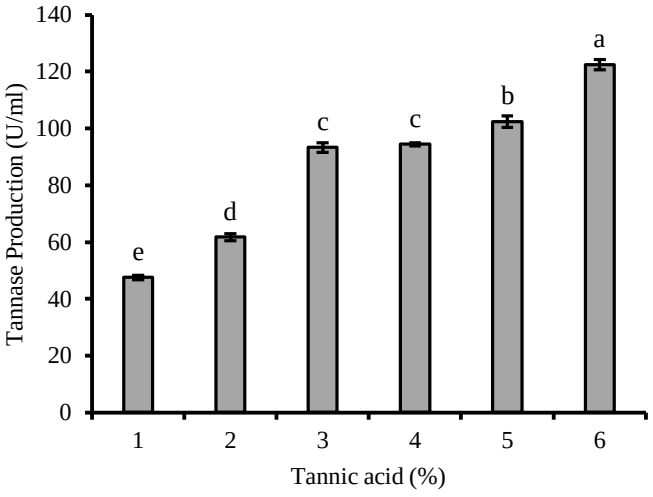

Figure 10. Tannase Production with Various Concentrations of Tannic Acid. Different alphabets on bars represent them significantly different.

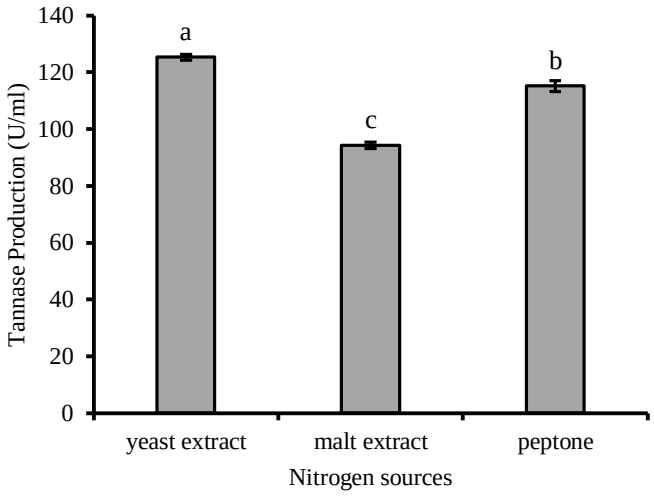

Figure 11. Tannase Production with Different Organic Nitrogen Sources. Different alphabets on bars represent them significantly different.

Discussion

Tannic acid is usually considered toxic for bacteria, but many tannic acid-resistant bacteria use it as carbon and energy sources.[8] Tannin degrading bacteria capable of tannase synthesis could be isolated from many sources. In the present study, *Bacillus subtilis* with tannase producing potential was isolated from the gut content of freshwater fish, *Catla catla*. Although many reports are available on the occurrence of microbes with tannase producing capacity in the digestive tract of herbivores (ruminant and non-ruminant),[27-29] but few investigations have been carried out previously to isolate the tannase producing microbes from the fish gut.[30] Similar to the present study, Talukdar et al.,[31]

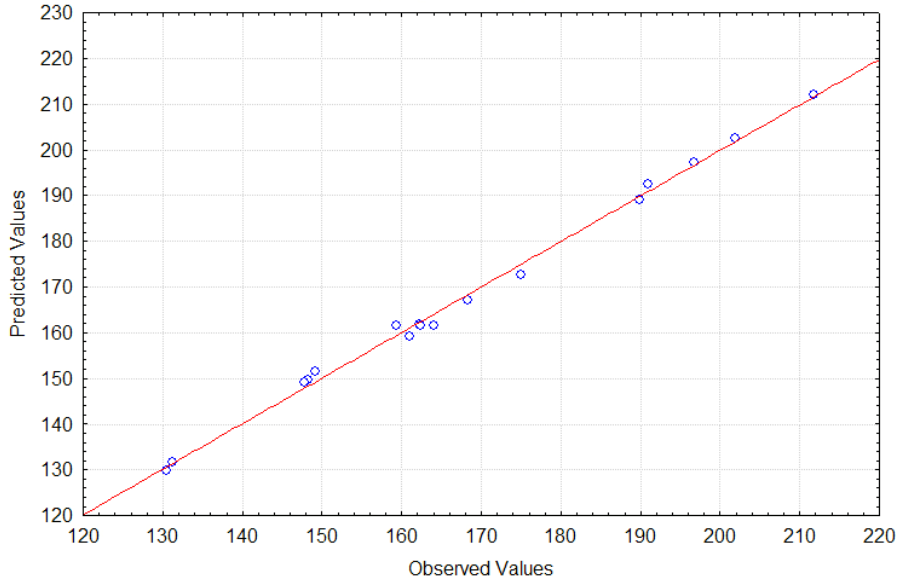

Figure 11. Residual Plot Showing Observed Versus Predicted Values from CCD for Tannase Production by *B. subtilis* in SSF.

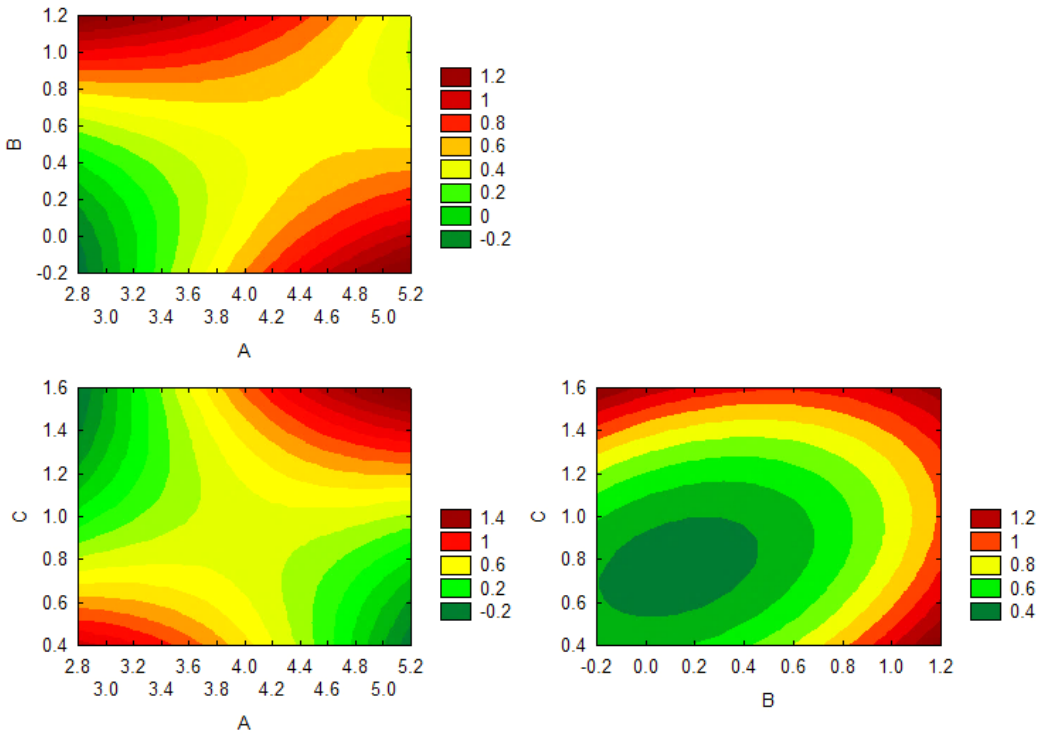

Figure 12. Contour Plots Showing the Interaction of A: Tannic Acid (%), B: $MgSO_4$ (%) and C: Yeast Extract (%) for Tannase Production by *B. subtilis* During CCD in SSF.

isolated tannase producing *B. subtilis* from the gut of freshwater fish. Mandal and Ghosh[30] have reported the isolation of several microbes capable of tannase production from *Catla catla* and other fresh water fishes. Tannin in plants inhibits the productivity and growth of animals on them[29] and feed containing a high level of tannin has drastic effects on omnivorous and herbivorous species of fish.[32,33] The presence of tannin degrading bacteria by producing tannase in the gut might result from co-evolution for tannin-like compounds and such fish species.[30] Besides fish gut content, potential microbes capable of tannase production have been isolated from many other resources. Tannase producers *Citrobacter freundii* and *Enterobacter cloacae* have been reported from tannery effluent and soil, respectively.[34,35] In the following study, the optimization

of various fermentation conditions was performed under SSF for maximal tannase production. In SSF, proper moisture is a significant factor for substrate bulging, microbial expansion and products formation and its value depends on the substrate and the used microbe.[36] In our study, with an increase of moisture content (distilled water) in the solid substrate, the enzyme production enhanced up to an optimal level of 50% moisture, whereas further increase of moisture lowered the enzyme synthesis. Various reports about tannase production in SSF indicated that the optimum moisture ranged from 40% to 70%.[37] Similar results to our study were presented by Sabu et al.,[38] for optimal tannase synthesis from *Lactobacillus* sp. ASR-S1 using coffee husk as substrate with 50% moisture, hereafter the tannase value decreased. Sabu et al.,[9] reported moisture level of 53.5% for maximal tannase synthesis in SSF from *Aspergillus niger* ATCC 16620. The decrease of enzyme productivity after optimal level could be due to reduced availability of oxygen for microbes, deduced spaces among particles and varied particles structure with the increase of moisture.[1] Mandal and Ghosh[39] reported the highest tannase production at 60% using groundnut oil cake as substrate and enzyme value decreased with higher moisture. The lesser enzyme production at high or lower moisture level may also be due to decrease of total organic material decomposition that affects the tannase synthesis.[19,40] So, a particular range of optimal moisture is required to be maintained in SSF.[38] However, for *Aspergillus foetidus*, 80% moisture yielded the highest tannase production.[41]

Tannase, in general, is an extracellular enzyme that is extracted by using water or buffer.[5] In our study, 2 ml of acetate buffer with pH 4.0 was the most suitable solvent for tannase extraction among all applied extraction mediums. Mondal et al.,[42] used 0.5 ml acetate buffer (pH 5.25) for tannase extraction, while acetate buffer (pH 5.0) was reported by Ferreira et al.[43] Also, Sabu et al.,[38] reported 0.05 M citrate buffer (50 ml) with pH 5.0 to extract the produced tannase. In contrast, water was used for the extraction of the tannase enzyme by Chatterjee et al.[44]

Temperature is a vital parameter for enzyme production that may cause inhibition and denaturation of enzymes and even cell death.[38] In this study, with the increase of incubation temperature, the enzyme production also increased while enhanced tannase productivity was found optima of 45 °C. Very few reports are available for tannase production at a higher temperature. Aftab et al,.[45] observed the elevation of tannase synthesis with temperature increase, whereas maximal synthesis was found at 41 °C for *Bacillus subtilis*. Govindarajan et al.,[46] reported the optimal tannase production from *Enterobacter cloacae* strain 41 at 50 °C. Lesser enzyme synthesis at lower temperature could be related to less substrate transportation across the cell. With the rise of temperature, the molecule kinetic energy increases,

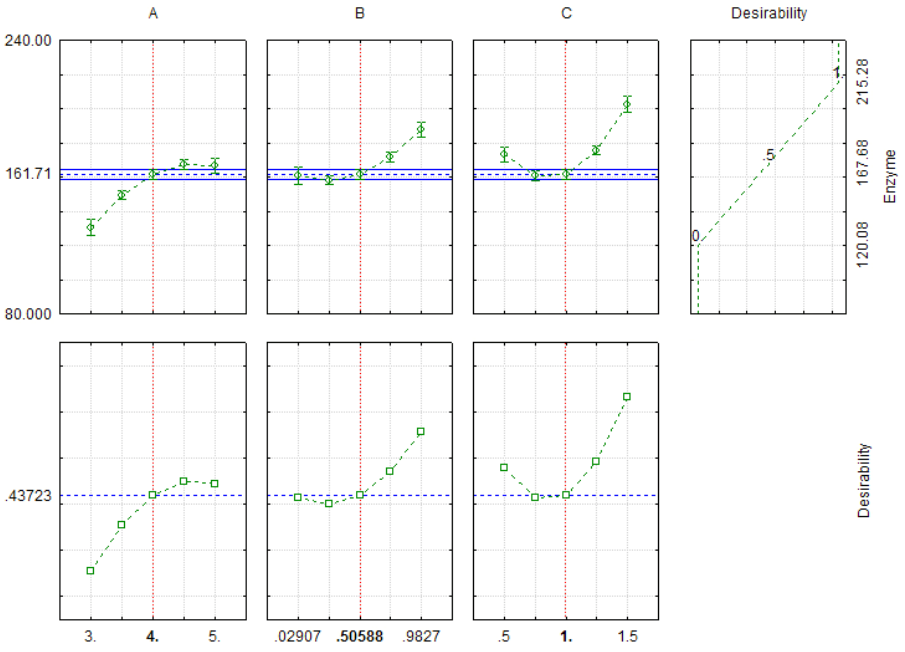

Figure 13. Desirability Chart for *B. subtilis* Tannase Production During CCD in SSF.

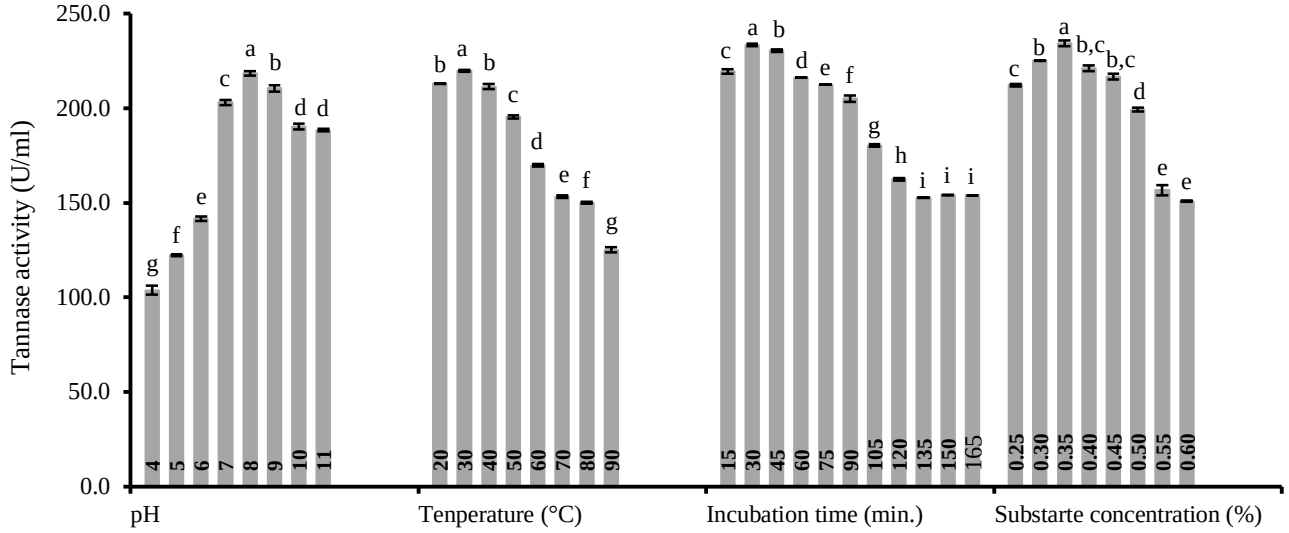

Figure 14. Effect of Different Parameters On the Tannase Activity. Bars having no common alphabets are significantly different (p> 0.05).

With the rise of temperature, the molecule kinetic energy increases, which results in the acceleration of the reaction.[47] However, Aharwar and Parihar[37] reported the general range of 25-35 °C for tannase production in SSF. The temperature of 30 °C and 37 °C was assessed as optima for tannase synthesis.[48,49] Such variations could depend on the type of microbe, its nature and surrounding conditions.[50] pH is also considered as an important parameter playing a vital role in metabolite synthesis.[51] The pH range of 3-11 was applied and the highest tannase production was observed at pH 5.0. While a further increase in pH, decreased the enzyme value. In SSF, tannase production mostly showed optimum value in acidic range.[37] Aftab et al.,[45] also noted that *B. subtilis* produced the best enzyme at pH 5.0. Murad et al.,[52] reported the tannase synthesis at maximum from *Aspergillus niger* at pH 5.0. Kumar et al.,[1] recorded a similar trend of pH on tannase production with pH optimal 5.5 using Jamun leaves. Keeping in the view, enzymes are protein in nature, the ionic character of the carboxylic and amino group attached to protein surface could be affected by changing pH and consequently influencing the enzyme catalytic activity. High and very low pH levels could affect the fermentation process due to enzyme inactivation.[47] However, Talukdar et al.,[31] found neutral pH as optima for tannase production.

In SSF, an appropriate inoculum size is essential for metabolites synthesis, while a smaller inoculum size would not induce the growth of microbes and enzyme synthesis.[21,53] Our results showed the highest tannase production with 2% inoculum of *B. subtilis*. While lower tannase value was observed lower (1%) or with higher (3%) inoculum sizes. In accordance to our results, Banerjee et al.,[54] also reported 2% inoculum for optimal tannase synthesis for *Aureobasidium pullulans* DBS66. Similarly, Murad et al.,[52] observed the tannase production with 2% inoculum ratio. Lower inoculum would be insufficient for proper microbial growth and enzyme production, so an appropriate amount of inoculum is essential for optimal enzyme synthesis.[38,55] Beyond the optimal level, the decrease in enzyme synthesis could be due to enhanced microbial mass and nutrients depletion.[56] However, Beniwal et al.,[35] documented the highest tannase synthesis with 1% inoculum of *Enterobacter cloacae* MTCC 9125 in SSF. Results depicted the optimum incubation time of 24 h and beyond this period, the tannase production tended to decrease. The incubation period required during the fermentation depends on microbe and the pattern of enzyme production.[51] Aftab et al.,[45] also evaluated the optimal tannase synthesis from *B. subtilis* at 24 h of incubation. Sabu et al.,[9] recorded similar results for tannase production in the case of tamarind seed powder substrate and after 24 h, the enzyme synthesis declined. With increased incubation, the lower enzyme synthesis could result from enzyme inhibition or denaturation with time.[11,57] Moreover, the nutrients exhaustion and enhanced level of byproducts with the passage of time may lower the enzyme production. Moreover, Selvaraj and Vytla[58] mentioned the maximum tannase production at 32 h for *Bacillus*

gottheilii while Sabu et al.,[38] observed 48 h for *Lactobacillus* sp. ASR-S1 tannase synthesis in SSF. During incubation, agitation condition (150 rpm) positively affected tannase production compared to static condition. Optimal agitation speed ranges from 100 to 200 rpm for tannase productivity.[59] Purwanto et al.,[60] observed optimal tannase synthesis at 130 rpm agitation using *Aspergillus niger*. For *Lactobacillus plantarum* MTCC 1407, Natarajan and Rajendran[61] recorded maximum tannase production with agitation (125 rpm). Kumar et al.,[62] reported agitation at 103.34 rpm for high tannase synthesis. The highest tannase by *Enterobacter cloacae* strain 41 was mentioned with agitation at 100 rpm by Govindarajan et al.[46] Agitation helps in proper medium mixing, biomass transfer and heat and oxygen transfer.[63] Requirement of agitation condition depends on the nature of culture microorganism.[59] Sheela et al.,[64] observed optimal results for tannase production from *Serratia marcesans* at static conditions.

The highest tannase production was recorded with larger substrate particles, while the lowest enzyme was synthesized with small-sized particles, which was in agreement with Yee et al.,[65] for tananse synthesis in SSF. More enzyme with large-sized substrate particles could be due to higher aeation and respiration resulting in better reaction.[66] Small particles may also agglomerate that decrease the surface area and may reduce enzyme synthesis.[67] In contrast, Madeira Jr et al.,[68] reported the small size of substrate particles as optimum. However, Yee et al.,[65] suggested the combination of large-sized particles and small-sized particles for maximal tannase synthesis. The higher tannase was produced without centrifugation during enzyme extraction. Sabu et al.,[38] used Whatman filter paper No. 1 for separating the extracted tannase produced by *Lactobacillus* sp. ASR-S1 rather than centrifugation. This is while Subbalaxmi and Murty[69] applied centrifugation during enzyme extraction.

Tannase needs metallic ions for the expression of its proper catalytic activity.[51] In the present study, the maximum tannase was produced with the salt having Mg^{+2} in the medium. Jana et al.,[47] also reported Mg^{+2} for optimal tannase production from *B. subtilis*. In SSF, Mg^{2+} ($MgSO_4$) is used commonly to enhance tannase production.[13,14,70] Stimulation of tannase enzyme in the presence of Mg^{+2} (divalent metallic ion) could be because of the enzyme stabilization in its active conformation instead of being involved in catalysis. This ion probably acts as an ion or salt bridge to maintain the enzyme confirmation.[59]

Tannase is reported as an inducible enzyme that requires tannin or tannic acid for its production from microbes.[71] Tannic acid act as a source of carbon.[59] When tannic acid was applied in increasing concentration, the maximum value was recorded at the highest applied concentration, i.e., 4.0% tannic acid. The range for good tannase production has been reported as 1-12%.[53,54,72,73] Similar results were also reported for *Bacillus gottheilii* who produced the maximum tannase at 4% tannic acid.[58] Banerjee et al.,[54] reported tannic acid (5%) for optimal synthesis of tannase by *Aspergillus aculeatus* DBF9. However, Battestin and Macedo[10] observed the highest tannase productivity with 12% of tannic acid along with coffee husk and wheat bran using *Paecilomyces variotii*. Beside carbon source, nitrogen source in the medium is also vital for microbial growth and enzyme synthesis.[74] Yeast extract, malt extract and peptone were applied as an organic nitrogen source and maximum tannase was obtained with yeast extract. Wu et al.,[75] and Murad et al.,[52] also recorded the yeast extract as optimal results during tannase production. In contrast, the yeast extract was also observed for the negative effect on tannase synthesis.[46]

The CCD of RSM revealed that the maximum tannase (211.97 ± 0.08 U/ml) was obtained with the combination of 4.0% tannic acid, 0.5% $MgSO_4$ and 1.5% yeast extract. Lima et al.,[76] recorded maximal tannase with 3.5% tannic acid in RSM with Barbados cherry substrate. The highest tannase was obtained with 6% tannic acid as recorded by Madeira Jr, Macedo.[73] However, 0.68% tannic acid was statistically optimized by Lekshmi et al.,[77] using pomegranate peel for tannase synthesis. Wu et al.,[75] optimized the tannase with glucose 8.11%, tannin 7.49%, $(NH_4)_2SO_4$ 9.26% and yeast extract 2.25% using tea stalk in SSF with RSM.

Optimal tannase activity was determined by optimizing the effects of physical parameters like pH, temperature, incubation time and substrate concentration. The pH affects the enzyme reaction by influencing the ionization state of amino acids in enzymes.[59] The impact of pH on tannase activity showed that when a range of pH 4-11 was applied, the tannase activity increased with pH and at pH 8, maximum activity was recorded; hereafter, the activity was observed to decrease with an increase of pH. For tannase activity,

generally optimal pH falls in the acidic range, but many investigations were also reported in the alkaline range.[18] Similar to our investigation, Iwamoto et al.,[78] also reported that a maximum tannase activity by *Lactobacillus plantarum* ATCC 14917T was obtained in alkaline range with optima of pH 8.0. Generally, microbial tannases have temperature optima from 20 to 60 °C for their activity.[18] Our investigations found the highest activity at 30 °C after which the activity declined at higher temperatures. In agreement to our investigation, a similar trend was reported by Rodríguez et al.,[79] showing temperature optima of 30 °C for tannase activity from for *Lactobacillus plantarum* CECT 748^T. Mahmoud et al.,[80] mentioned *Kluyveromyces marxianus* tannase activity at 35 °C while Iwamoto et al.,[78] reported it as 40 °C. An increase of temperature elevates the kinetic energy of enzymes which facilitates the enzyme reaction; however, after a limit, the potential energy becomes so high that it breaks the weak bonds in three-dimensional structure, which consequently denatures and inactivates the enzyme or substrate structure.[81] However, the optimum tannase activity from *Penicillium montanense* was observed at 50 °C.[76] Maximum tannase was shown after incubation of 30 min with the decrease of activity by increasing further incubation. Our results are parallel with the investigation of El-Fouly et al.[82] in which similar results were reported. The lower activity with prolonged time could be due to the enzyme denaturation with time.[57] For *Trichoderma harzianum* MTCC10841 optimum tannase activity was reported after incubating for 20 min.[83] Rana and Bhat[84] reported the highest tannase activity after 15 min. While 5 min of incubation as optima was reported by Mukherjee and Banerjee.[81] In our report, 0.35% of tannic acid in medium resulted the highest tannase activity. Further increase in concentration caused the enzyme activity to be decreased, which may result from the saturation of all enzyme active sites by tannic acid substrate.

Conclusion

Agricultural resources are generally immensely nutritious and rich in carbon and nitrogen, supporting microbial growth leading to enhanced enzyme production. The use of such resources as a potential substrate for the production of invaluable industrial enzyme is a practical approach. Agricultural materials contain tannin, which could be used as a cost-effective substrate for tannase synthesis instead of costly tannic acid in its pure form. The optimization of fermentation parameters and medium components with the application of OVAT followed by CCD confirmed the use of inexpensive corn leaves as a substrate in SSF for enhanced tannase production by *B. subtilis* in solid state fermentation. The crude tannase enzyme was optimally active at pH 8 and 30 ºC.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

References

1. Kumar R, Sharma J, Singh R. Production of tannase from *Aspergillus* ruber under solid-state fermentation using jamun (*Syzygium cumini*) leaves. Microbiol Res. 2007; 162(4):384-90. doi:10.1016/j.micres.2006.06.012
2. Beena P, Basheer SM, Bhat SG, Bahkali AH, Chandrasekaran M. Propyl gallate synthesis using acidophilic tannase and simultaneous production of tannase and gallic acid by marine *Aspergillus awamori* BTMFW032. Appl Biochem Biotechnol. 2011;164(5):612-28. doi:10.1007/s12010-011-9162-x
3. Lekha P, Lonsane B. Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid-state, liquid surface an submerged fermentations. Process Biochem. 1994;29(6): 497-503. doi:10.1016/0032-9592(94)85019-4
4. Mohapatra PD, Mondal KC, Pati BR. Production of tannase through submerged fermentation of tannin-containing plant extracts by *Bacillus licheniformis* KBR6. J Microbiol. 2006;55:297-301. doi:10.1111/j.1365-2672.2006.03207.x
5. Aguilar CN, Rodriguez R, Gutierrez-Sanchez G, Augur C, Favela-Torres E, Prado-Barragan LA, et al. Microbial tannases: advances and perspectives. Appl Microbiol Biotechnol. 2007;76(1):47-59. doi:10.1007/s00253-007-1000-2
6. Aithal M, Belur PD. Enhancement of propyl gallate yield in nonaqueous medium using novel cell-associated tannase of *Bacillus massiliensis*. Prep Biochem Biotechnol. 2013;43(5):445-55. doi:10.1080/10826068.2012.745873
7. Meena P, Tripathi AD, Srivastava S, Jha A. Utilization of agro-industrial waste (wheat bran) for alkaline protease production by *Pseudomonas aeruginosa* in SSF using Taguchi (DOE) methodology. Biocatal Agric Biotechnol. 2013;2(3):210-6. doi:10.1016/j.bcab.2013.05.003
8. Aissam H, Errachidi F, Penninckx M, Merzouki M, Benlemlih M. Production of tannase by *Aspergillus niger* HA37 growing on tannic acid and olive mill waste waters. World J Microbiol Biotechnol. 2005;21(4):609-14. doi:10.1007/s11274-004-3554-9
9. Sabu A, Pandey A, Daud MJ, Szakacs G. Tamarind seed powder and palm kernel cake: two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. Bioresour Technol. 2005;96(11):1223-8. doi:10.1016/j.biortech.2004.11.002
10. Battestin V, Macedo GA. Tannase production by *Paecilomyces variotii*. Bioresour Technol. 2007;98(9): 1832-7. doi:10.1016/j.biortech.2006.06.031
11. Paranthaman R, Vidyalakshmi R, Murugesh S, Singaravadivel K. Manipulation of fermentation conditions on production of tannase from agricultural by-products with *Aspergillus oryzae*. Afr J Microbiol Res. 2010;4(13):1440-5. doi:10.5897/AJMR.9000479
12. Wang F, Ni H, Cai H-N, Xiao A-F. Tea stalks–a novel

agro-residue for the production of tannase under solid state fermentation by *Aspergillus niger* JMU-TS528. Ann Microbiol. 2013;63(3):897-904. doi:10.1007/s13213-012-0541-5
13. Xiao A, Huang Y, Ni H, Cai H, Yang Q. Statistical optimization for tannase production by *Aspergillus tubingensis* in solid-state fermentation using tea stalks. Electron J Biotechnol. 2015;18(3):143-7. doi:10.1016/j.ejbt.2015.02.001
14. Bhoite RN, Murthy PS. Biodegradation of coffee pulp tannin by *Penicillium verrucosum* for production of tannase, statistical optimization and its application. Food Bioprod Process. 2015;94:727-5. doi:10.1016/j.fbp.2014.10.007
15. Liu TP, Porto TS, Moreira KA, Takaki GM, Brandro-Costa R, Herculano PN, et al. Tannase production by *Aspergillus* spp. UCP1284 using cashew bagasse under solid state fermentation. Afr J Microbiol Res. 2016;10(16):565-71. doi:10.5897/ajmr2016.7924
16. Aguilar C, Gutiйrrez-Sбnchez G. Sources, properties, applications and potential uses of tannin acyl hydrolase. Food Sci Technol Int. 2001;7(5):373-82. doi:10.1177/108201301772660411
17. Ayed L, Hamdi M. Culture conditions of tannase production by *Lactobacillus plantarum*. Biotechnol Lett. 2002;24(21):1763-5. doi:10.1023/A:1020696801584
18. Yao J, Guo GS, Ren GH, Liu YH. Production, characterization and applications of tannase. Mol Catal B Enzym. 2014;101:137-47. doi:10.1016/j.molcatb.2013.11.018
19. Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam PS-N. "Solid State Fermentation in Biotechnology: Fundamentals and Applications" Reference Book. Asiatech Publishers, Inc, 2001.
20. Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation: I-bioprocesses and products. Process Biochem. 2000;35(10):1153-69. doi:10.1016/S0032-9592(00)00152-7
21. Pandey A, Selvakumar P, Soccol CR, Nigam P. Solid state fermentation for the production of industrial enzymes. Curr Sci. 1999;77(1):149-62.
22. Singh SK, Singh SK, Tripathi VR, Khare SK, Garg SK. Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic *Pseudomonas putida* SKG-1 isolate. Microbial cell factories. 2011;10(1):114. doi:10.1186/1475-2859-10-114
23. Baş D, Boyacı IH. Modeling and optimization I: Usability of response surface methodology. J Food Eng. 2007;78(3):836-45. doi:10.1016/j.jfoodeng.2005.11.024
24. Osawa R, Walsh T. Visual reading method for detection of bacterial tannase. Appl Environ Microbiol. 1993;59(4):1251-2. doi:10.1128/aem.59.4.1251-1252.1993
25. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426-8. doi:10.1021/ac60147a030
26. Shakir HA. Enteric bacterial and heavy metals'load and health status of fishes from river ravi, pakistan: University of the Punjab, Lahore; 2012.
27. Bhat TK, Singh B, Sharma OP. Microbial degradation of tannins–a current perspective. Biodegradation. 1998;9(5):343-57. doi:10.1023/A:1008397506963
28. Odenyo A, McSweeney C, Palmer B, Negassa D, Osuji P. In vitro screening of rumen fluid samples from indigenous African ruminants provides evidence for rumen fluid with superior capacities to digest tannin-rich fodders. Aust J Agric Res. 1999;50(7):1147-57. doi:10.1071/AR98117
29. Goel G, Puniya A, Aguilar C, Singh K. Interaction of gut microflora with tannins in feeds. Naturwissenschaften. 2005;92(11):497-503. doi:10.1007/s00114-005-0040-7
30. Mandal S, Ghosh K. Isolation of tannase-producing microbiota from the gastrointestinal tracts of some freshwater fish. J Appl Ichthyol. 2013;29(1):145-53. doi:10.1111/j.1439-0426.2012.02054.x
31. Talukdar S, Ringш E, Ghosh K. Extracellular tannase-producing bacteria detected in the digestive tracts of freshwater fishes (Actinopterygii: Cyprinidae and Cichlidae). Acta Ichthyol Piscat. 2016;46(3):201-10. doi:10.3750/AIP2016.46.3.04
32. Al-Owafeir M. The effects of dietary saponin and tannin on growth performance and digestion in Oreochromis niloticus and Clarias gariepinus. University of Stirling (United Kingdom); 1999.
33. Becker K, Makkar H. Effects of dietary tannic acid and quebracho tannin on growth performance and metabolic rates of common carp (*Cyprinus carpio* L.). Aquaculture. 1999;175(3-4):327-35. doi:10.1016/S0044-8486(99)00106-4
34. Kumar RA, Gunasekaran P, Lakshmanan M. Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent. J Basic Microbiol. 1999;39(3):161-8. doi:10.1002/(SICI)1521-4028(199906)39:3<161::AID-JOBM161>3.0.CO;2-U
35. Beniwal V, Chhokar V, Singh N, Sharma J. Optimization of process parameters for the production of tannase and gallic acid by *Enterobacter cloacae* MTCC 9125. J Am Sci. 2010;6(8):389-97.
36. Kalogeris E, Iniotaki F, Topakas E, Christakopoulos P, Kekos D, Macris B. Performance of an intermittent agitation rotating drum type bioreactor for solid-state fermentation of wheat straw. Bioresour Technol. 2003;86(3):207-13. doi:10.1016/S0960-8524(02)00175-X
37. Aharwar A, Parihar DK. Tannases: production, properties, applications. Biocatal Agric Biotechnol. 2018;15:322-34. doi:10.1016/j.bcab.2018.07.005
38. Sabu A, Augur C, Swati C, Pandey A. Tannase production by *Lactobacillus* sp. ASR-S1 under solid-state fermentation. Process Biochem. 2006;41(3):575-80. doi:10.1016/j.procbio.2005.05.011
39. Mandal S, Ghosh K. Optimization of tannase production and improvement of nutritional quality of two potential low-priced plant feedstuffs under solid state fermentation by *Pichia kudriavzevii* isolated from fish gut. Food Biotechnol. 2013;27(1):86-103. doi:10.1080/08905436.2012.755929
40. Pandey A. Solid state fermentation-an overview. Solid-state fermentation. 1994:3-10.
41. Mukherjee G, Banerjee R. Biosynthesis of tannase and gallic acid from tannin rich substrates by Rhizopus oryzae and *Aspergillus foetidus*. J. Basic Microbiol. 2004;44(1):42-8. doi:10.1002/jobm.200310317
42. Mondal KC, Banerjee R, Pati BR. Tannase production by *Bacillus licheniformis*. Biotechnol Lett. 2000;22(9):767-9. doi:10.1023/A:1005638630782
43. Ferreira LR, Macedo JA, Ribeiro ML, Macedo GA. Improving the chemopreventive potential of orange juice by enzymatic biotransformation. Food Res Int. 2013;51(2):526-35. doi:10.1016/j.foodres.2013.01.018
44. Chatterjee R, Dutta A, Banerjee R, Bhattacharyya B. Production of tannase by solid-state fermentation. Bioprocess Eng. 1996;14(3):159-62. doi:10.1007/s004490050199
45. Aftab MN, Mukhtar H, Haq I. Production and Characterization of Tannase from a newly isolated *Bacillus subtilis*. Pak J Bot. 2016;48(3):1263-71.
46. Govindarajan R, Krishnamurthy M, Neelamegam R, Shyu DJ, Muthukalingan K, Nagarajan K. Purification, structural

characterization and biotechnological potential of tannase enzyme produced by *Enterobacter cloacae* strain 41. Process Biochem. 2019;77:37-47. doi:10.1016/j.procbio.2018.10.013
47. Jana A, Maity C, Halder SK, Das A, Pati BR, Mondal KC, et al. Structural characterization of thermostable, solvent tolerant, cytosafe tannase from *Bacillus subtilis* PAB2. Biochem Eng J. 2013;77:161-70. doi:10.1016/j.bej.2013.06.002
48. Raghuwanshi S, Dutt K, Gupta P, Misra S, Saxena RK. *Bacillus sphaericus*: The highest bacterial tannase producer with potential for gallic acid synthesis. J Biosci Bioeng. 2011;111(6):635-40. doi:10.1016/j.jbiosc.2011.02.008
49. Muslim SN, Mahammed AN, Musafer HK, AL_Kadmy IM, Shafiq SA, Muslim SN. Detection of the optimal conditions for tannase productivity and activity by *Erwinia carotovora*. J Med Bioeng. 2015;4(3):198-205. doi:10.12720/jomb.4.3.198-205
50. Irfan M, Nadeem M, Syed Q. One-factor-at-a-time (OFAT) optimization of xylanase production from *Trichoderma viride*-IR05 in solid-state fermentation. J Radiat Res Appl Sci. 2014;7(3):317-26. doi:10.1016/j.jrras.2014.04.004
51. Selwal MK, Yadav A, Selwal KK, Aggarwal N, Gupta R, Gautam S. Optimization of cultural conditions for tannase production by *Pseudomonas aeruginosa* IIIB 8914 under submerged fermentation. World J Microbiol Biotechnol. 2010;26(4):599-605. doi:10.1007/s11274-009-0209-x
52. Murad H, Abd El Tawab A, Kholif A, El-Nor SA, Matloup O, Khorshed M, et al. Production of tannase by *Aspergillus niger* from palm kernel. Biotechnology. 2014;13(2):68-73. doi:10.3923/biotech.2014.68.73
53. Rodrigues TH, Pinto GA, Goncalves LR. Effects of inoculum concentration, temperature, and carbon sources on tannase production during solid state fermentation of cashew apple bagasse. Biotechnol Bioprocess Eng. 2008;13(5):571-6. doi:10.1007/s12257-008-0014-7
54. Banerjee D, Mondal K, Pati B. Tannase production by *Aspergillus aculeatus* DBF9 through solid-state fermentation. Acta Microbiol Immunol Hung. 2007;54(2):159-66. doi:10.1556/AMicr.54.2007.2.6
55. Kashyap P, Sabu A, Pandey A, Szakacs G, Soccol CR. Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. Process Biochem. 2002;38(3):307-12. doi:10.1016/S0032-9592(02)00060-2
56. Ramirez-Coronel A, Darvill A, Viniegra-Gonzalez G, Augur C. Characterization of a bifunctional tannase from *Aspergillus niger*. Microbiology. 2003;149(10):2941-6.
57. Gautam P, Sabu A, Pandey A, Szakacs G, Soccol CR. Microbial production of extra-cellular phytase using polystyrene as inert solid support. Bioresour Technol. 2002;83(3):229-33. doi:10.1016/S0960-8524(01)00215-2
58. Selvaraj S, Vytla RM. Evaluation of model parameters for growth, tannic acid utilization and tannase production in *Bacillus gottheilii* M2S2 using polyurethane foam blocks as support. 3 Biotech. 2017;7(5):275. doi:10.1007/s13205-017-0909-0
59. Jana A, Halder SK, Banerjee A, Paul T, Pati BR, Mondal KC, et al. Biosynthesis, structural architecture and biotechnological potential of bacterial tannase: a molecular advancement. Bioresour Technol. 2014;157:327-40. doi:10.1016/j.biortech.2014.02.017
60. Purwanto L, Ibrahim D, Sudrajat H. Effect of agitation speed on morphological changes in *Aspergillus niger* hyphae during production of tannase. World J Chem. 2009;4(1):34-8. doi:10.4331/wjbc.v6.i3.265
61. Natarajan K, Rajendran A. Effect of fermentation parameters on extra cellular tannase production by *Lactobacillus plantarum* MTCC 1407. E-J Chem. 2009;6(4):979-84. doi:10.1155/2009/505087
62. Kumar M, Rana S, Beniwal V, Salar RK. Optimization of tannase production by a novel *Klebsiella pneumoniae* KP715242 using central composite design. Biotechnol Rep. 2015;7:128-34. doi:10.1016/j.btre.2015.06.002
63. Darah I, Sumathi G, Jain K, Lim S. Influence of agitation speed on tannase production and morphology of *Aspergillus niger* FETL FT3 in submerged fermentation. Appl Biochem Biotechnol. 2011;165(7-8):1682-90. doi:10.1007/s12010-011-9387-8
64. Sheela S, Smita V, Dipak V. Optimization of parameters for enhanced tannase production from a novel bacterial producer. World J Pharm Res. 2016;5(6):2131-9. doi:10.20959/wjpr20166-6455
65. Yee TW, Prabhu NG, Jain K, Ibrahim D. Process parameters influencing tannase production by *Aspergillus niger* using mangrove (*Rhizophora apiculata*) bark in solid substrate fermentation. Afr J Biotechnol. 2011;10(61):13147-54.
66. John RP, Nampoothiri KM, Pandey A. Solid-state fermentation for L-lactic acid production from agro wastes using *Lactobacillus delbrueckii*. Process Biochem. 2006;41(4):759-63. doi:10.1016/j.procbio.2005.09.013
67. Krishna C. Solid-state fermentation systems—an overview. Crit Rev Biotechnol. 2005;25(1-2):1-30. doi:10.1080/07388550590925383
68. Madeira Jr JV, Ferreira LR, Macedo JA, Macedo GA. Efficient tannase production using Brazilian citrus residues and potential application for orange juice valorization. Biocatal Agric Biotechnol. 2015;4(1):91-7. doi:10.1016/j.bcab.2014.11.005
69. Subbalaxmi S, Murty VR. Process optimization for tannase production by *Bacillus gottheilii* M2S2 on inert polyurethane foam support. Biocatal Agric Biotechnol. 2016;7:48-55. doi:10.1016/j.bcab.2016.05.004
70. Ni H, Chen F, Jiang ZD, Cai MY, Yang YF, Xiao AF, et al. Biotransformation of tea catechins using *Aspergillus niger* tannase prepared by solid state fermentation on tea byproduct. LWT-Food Sci Technol. 2015;60(2):1206-13. doi:10.1016/j.lwt.2014.09.010
71. Mansor A, Ramli M, Rashid NA, Samat N, Lani M, Sharifudin S, et al. Evaluation of selected agri-industrial residues as potential substrates for enhanced tannase production via solid-state fermentation. Biocatal Agric Biotechnol. 2019;20:101216. doi:10.1016/j.bcab.2019.101216
72. Seth M, Chand S. Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*—optimisation of process parameters. Process Biochem. 2000;36(1-2):39-44. doi:10.1016/S0032-9592(00)00179-5
73. Madeira Jr JV, Macedo JA, Macedo GA. Detoxification of castor bean residues and the simultaneous production of tannase and phytase by solid-state fermentation using *Paecilomyces variotii*. Bioresour Technol. 2011;102(15):7343-8. doi:10.1016/j.biortech.2011.04.099
74. Chandrasekaran M. Combined effect of environmental factors on spoilage bacteria. Fish Technol. 1991;28:146-53.
75. Wu C, Zhang F, Li L, Jiang Z, Ni H, Xiao A. Novel optimization strategy for tannase production through a modified solid-state fermentation system. Biotechnol Biofuels. 2018;11(1):92. doi:10.1186/s13068-018-1093-0
76. Lima JSd, Cruz R, Fonseca JC, Medeiros EVd, Maciel MdHC, Moreira KA, et al. Production, characterization of tannase from *Penicillium montanense* URM 6286 under SSF using agroindustrial wastes, and application in the

clarification of grape juice (Vitis vinifera L.). Sci World J. 2014;2014:182025. doi:10.1155/2014/182025
77. Lekshmi R, Nisha SA, Kaleeswaran B, Alfarhan A. Pomegranate peel is a low-cost substrate for the production of tannase by *Bacillus velezensis* TA3 under solid state fermentation. J. King Saud Univ. Sci. 2020;32 (3):1831-7. doi:10.1016/j.jksus.2020.01.022
78. Iwamoto K, Tsuruta H, Nishitaini Y, Osawa R. Identification and cloning of a gene encoding tannase (tannin acylhydrolase) from *Lactobacillus plantarum* ATCC 14917T. Syst Appl Microbiol. 2008;31(4):269-77. doi:10.1016/j.syapm.2008.05.004
79. Rodriguez H, de las Rivas B, Gymez-Cordovīs C, Mucoz R. Characterization of tannase activity in cell-free extracts of *Lactobacillus plantarum* CECT 748T. Int J Food Microbiol. 2008;121(1):92-8. doi:10.1016/j.ijfoodmicro. 2007.11.002
80. Mahmoud AE, Fathy SA, Rashad MM, Ezz MK, Mohammed AT. Purification and characterization of a novel tannase produced by *Kluyveromyces marxianus* using olive pomace as solid support, and its promising role in gallic acid production. Int J Biol Macromol. 2018;107:2342-50. doi:10.1016/j.ijbiomac.2017.10.117
81. Mukherjee G, Banerjee R. Effects of temperature, pH and additives on the activity of tannase produced by a co-culture of *Rhizopus oryzae* and *Aspergillus foetidus*. World J Microbiol Biotechnol. 2006;22(3):207-12. doi:10.1007/s11274-005-9022-3
82. El-Fouly M, El-Awamry Z, Shahin AA, El-Bialy HA, Naeem E, El-Saeed GE. Biosynthesis and characterization of *Aspergillus niger* AUMC 4301 tannase. J Am Sci. 2010;6(12):709-21.
83. Iqbal H, Kapoor A. Tannin degradation efficiency of tannase produced by *Trichoderma harzianum* MTCC 10841 and its biochemical properties. Int J LifeSc Bt & Pharm Res. 2012;1(4):106-17.
84. Rana NK, Bhat TK. Effect of fermentation system on the production and properties of tannase of *Aspergillus niger* van Tieghem MTCC 2425. J Gen Appl Microbiol. 2005;51(4):203-12. doi:10.2323/jgam.51.203