Valorization of Unripe Banana Peel (Robusta) Powder for the Production of Tannase Using *Bacillus xiamenensis* in Submerged Fermentation

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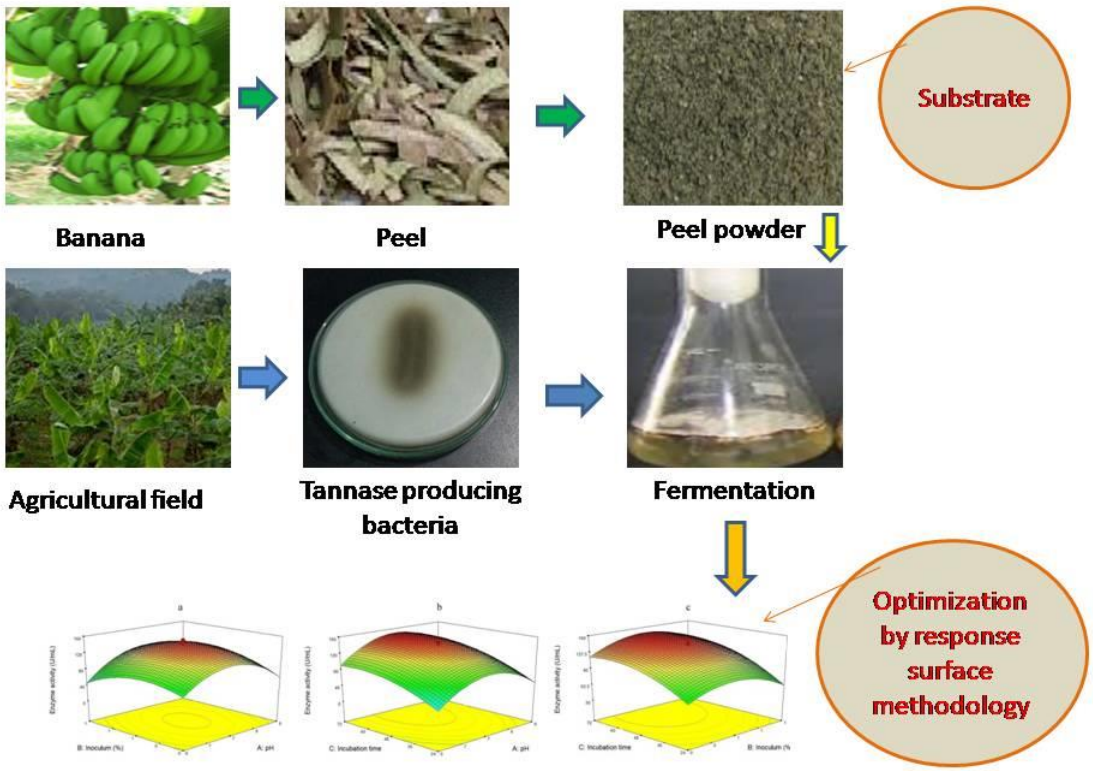
GRAPHICAL ABSTRACT



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Ahmed Alfarhan,[a] Rajakrishnan Rajagopal,[b] and Vijayaraghavan Ponnuswamy [c,*]

Tannases are industrial enzymes used in cosmetic, pharmaceutical, food, and environmental management. In the present study, 11 tannase-producing *Bacillus* spp. were isolated from agricultural soil, banana root soil, vegetable garbage, and fruit garbage. These isolated bacteria were screened using tannic acid agar plates. The zone of hydrolysis varied from 9 mm to 21 mm, and the strain *Bacillus xiamenensis* BR1 exhibited the highest activity. The dried unripe banana peel (Robusta) was powdered, and particles between 1 and 1.5 mm were used as substrate. The banana peel consists of 7.84 ± 0.15% hydrolysable tannin, which induces the production of tannase. The production medium was prepared at 10% (w/v) unripe banana peel powder. The screening experiments revealed that fermentation period, pH, inoculums, and tannic acid improved tannase production. A two-level full factorial design revealed the influence of pH, inoculums, and incubation time on tannase production (F-value=8.99; p-value=<0.0001). The optimum concentration was analyzed using a central composite design, and the model was significant (F-value = 17.03; p-value = 0.0001). Under optimal bioprocess conditions, tannase yield was 2.4-fold higher than in an unoptimized medium. The unripe banana peel can be used as a substrate for the production of tannase by *Bacillus* sp.

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Keywords: Agro-residues; Banana peel; Bacillus xiamenensis; Tannase; Response surface methodology

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INTRODUCTION

Tannase (tannin acyl hydrolase EC 3.1.1.20) is an extracellular and inducible enzyme that is involved in the hydrolysis or cleavage of the depside and ester bonds of tannins (hydrolysable), yielding gallic acid and glucose (Aguilar *et al*. 2007). Tannase is used for the preparation of gallic acid, which is the major hydrolytic product of tannic acid and is used in cosmetics, food, adhesives, and in the biosynthesis of propyl gallate and antioxidants (Aithal and Belur 2013). In the food industry, tannase is used as a clarifying agent to reduce the bitterness and haze in the case of fruit juices and beer. In wastewater treatment, tannase is used to remove tannin contaminated with polyphenic compounds (Lekha and Lonsane 1994). In addition, tannase is also applied in the manufacture of pharmaceutically important trimethoprim (Mohapatra *et al*. 2006). Living organisms such as animals, plants, and microorganisms produce tannase; however, industrial production of tannase is achieved by using microbial sources. Tannase-producing fungi and bacteria have been reported. These include fungi from the genera *Penicillium* and *Aspergillus*. Bacteria

such as *L. pentosus*, *L. paraplantarum*, and *Lactobacillus plantarum* produce tannase (Osawa *et al*. 2000; Nishitani and Osawa 2006). The fungus strains show potential activity for the degradation of tannins. Moreover, a major problem is associated with genetic complexity and a slow growth rate. But, in the case of bacteria, the genetic material can be easily manipulated, and the growth rate of bacteria is higher than that of fungi. The bacterial strains are able to survive under extreme pH, temperature, and ionic strength; hence, they may prove to be a good choice of tannase (Beniwal *et al*. 2014). In addition, tannase from bacterial sources has the potential to hydrolyze and degrade tannic acid and natural tannin effectively (Deschamps *et al*. 1983). The literature on bacterial tannase production is limited compared to fungal tannases. Microbial tannase production can be performed by either solid-state, submerged, or liquid-surface cultures (Lekshmi *et al*. 2020). The major advantages of solid-state fermentation are its high enzyme yield, lower production cost, simplicity, and less water generation (Biji *et al*. 2016; Kalaiyarasi *et al*. 2020; Arokiyaraj *et al*. 2024). The main advantage of submerged fermentation is the easy bioprocess control and product recovery.Considering these points, submerged fermentation was used in this study for the production of tannase using bacteria.

Agricultural activities produce waste, which affects the environment and ecosystem. Lignocellulosic material is one of the major plant residues;it containscellulose, hemicelluloses, and lignin (Mamma *et al*. 2008). Agro-industrial wastes such as sugar cane bagasse, coffee by-products, and pomegranate peels are major sources of micro- and macronutrients that improve the production of enzymes and microbial growth. Agro-residues were used for the production of tannase in solid-state fermentation (SSF). Some bacteria have been utilized for the production of tannase. Bacteria such as *Staphylococcus lugdunensis* (Chaitanyakumar and Anbalagan 2016), *Staphylococcus lugdunensis* (Jiménez *et al*. 2014), and bacteria of the genera *Lactobacillus* (Ueda *et al*. 2014) produce extracellular tannase.

Banana (*Musa* spp.) is a major edible crop in subtropical and tropical regions. In 2019, about 116 million metric tons of banana fruits were produced. The fruit comprises 75% water and 25% dry matter. The unripe banana is generated in large quantities and is considered waste (Ishak *et al*. 2020). Therefore, the utilization of unripe banana peel is useful to reduce environmental pollution and minimize the production cost of tannase. Bacterial strains from the natural environment could have better adaptation strategies and improved genetic variability for product formation than conventional species (El-Sheikh *et al*. 2020). Hence, it would be useful to analyze new bacterial strains from the natural environment for tannase production.

Traditional methods have been used for the optimization of bioprocess conditions by changing one factor at a time and keeping the other factors constant. This method is costly and laborious, and the one-variable-at-a-time approach fails to provide any new information about the interaction between the selected variables. To overcome these limitations, the response surface methodology (RSM) is recommended to analyze the interactions between the selected variables. Response surface methodology is used to determine the optimum bioprocess conditions and interaction between various selected nutritional and physiological factors (Marraiki *et al*. 2020). In this study, unripe banana peel was used as a substrate for the production of tannase in solid-state fermentation by RSM.

EXPERIMENTAL

Materials

The chemicals used in this study were purchased from Sigma-Aldrich, USA. The culture media were procured from Himedia Laboratories, Mumbai, India. The unripe banana peel was collected locally.

Determination of Total Carbohydrate, Lignin, and Ash

The unripe banana peel was steam-cooked for 30 min, and the total carbohydrate, lignin, and ash content (%) were determined. To determine total carbohydrate content, 0.2 mL of sample was combined with 5 mL of the anthrone reagent and mixed by vortexing. It was incubated at 90 °C for 15 min. The reaction mixture was cooled, and the absorbance of the sample was read at 629 nm against a blank (Mirpoor *et al*. 2020). The unripe banana peel was dried for two days at 60 °C, and 0.1 g sample was extracted with acetone. Lignin content of the sample was estimated using the acetyl bromide method. Briefly, 0.1 mL extract was dried and redissolved in 5 mL 20% (v/v) AcBr-acetic acid reagent and heated for 2 h at 50 °C under constant stacking. It was immediately stored at −20 °C for 20 min and was thawed. The sample was transferred into a volumetric flask containing NaOH (2 M, 5 mL) and 12 mL acetic acid (100%). The absorbance of the sample was read at 280 nm using a UV-visible spectrophotometer at 280 nm. The ash content of the unripe banana peel was determined by heating 50 g of unripe banana peel for 20 h at 950 °C.

Banana Peel and Proximate Analysis

The unripe banana peel was sundried for ten days, ground mechanically, and passed through a standard sieve to obtain particle sizes between 1 and 1.5 mm. Alkaline treatment was performed using sodium hydroxide at 10%, 20%, and 30%. The banana peel slurry concentration was maintained at 10% (w/v). The pretreatment was performed at 50 ºC for 60 min. Lignocellulosic materials are a good feedstock to produce tannase. The pretreatment method is focused on improving hemicelluloses, and cellulose accessibility. The pretreatment method converted highly complex lignocellulosic structures into simple hemicelluloses, cellulose and lignin. The lignin and cellulose content was determined as described previously (Liu *et al*. 2020).

Isolation of Tannase-Producing Bacteria by Enrichment Method

An enrichment technique was used to improve the growth of tannase-producing bacteria. A total of eight samples (n = 8), including agricultural soil, banana root soil, vegetable garbage, and fruit garbage, were collected in sterile containers and stored at 4 °C. About 1 g of sample was mixed in an Erlenmeyer flask containing 50 mL of minimal medium containing KH_2PO_4: 0.5 g/L, K_2HPO_4: 0.5 g/ L, $MgSO_4$: 2.0 g/ L, NH_4Cl: 3.0 g/ L, and $CaCl_2$: 1.0 g/ L and supplemented with tannic acid (w/v. 1%). The pH of the culture medium was set to 6.5. The Erlenmeyer flasks were incubated in an orbital shaker incubator at 37 °C for 24 h. The culture was transferred (5 mL) to the newly prepared culture medium, and we repeated this experiment one more time. Then it was serially diluted, plated on nutrient agar medium, and incubated for 24 h at 37 °C. The isolated bacterial colonies were subcultured in nutrient agar slants and stored at 4 °C in the refrigerator (Wang *et al*. 2020).

Tannase Screening

The isolated bacterial strains were screened for tannase production using tannic acid agar plates. Briefly, nutrient agar medium was sterilized, and filter sterilized (1%, w/v) tannic acid was supplemented. The isolated bacterial strains were inoculated on these plates and incubated for 2 to 3 days at 37 °C. The isolates showing dark brown coloration around the colonies were considered tannase-producing bacteria (Lekshmi *et al*. 2020).

Production of Tannase

The isolated bacteria were cultured in a 250-mL Erlenmeyer flask containing 50 mL of mineral salt medium with 0.1% tannic acid (pH 6.5). It was autoclaved, and the isolated bacteria were inoculated (0.1 mL) (1×10^8 colony-forming units/mL) individually, which were previously cultivated in nutrient broth medium for 18 h at 37 °C. The culture was incubated for 3 days at 37 °C, and the culture was centrifuged at 5000×g for 10 min. The cell-free extract was used for the determination of tannase activity.

Tannase Assay

Tannic acid was prepared in 0.1 M phosphate buffer (pH 6.5) and used as a substrate. To determine the tannase assay, 0.25 mL of tannic acid (1.0%) was mixed with 0.1 mL of crude tannase and incubated for 30 min at 37 °C. After 30 min of incubation, 2 mL of bovine serum albumin (0.1%) precipitated the remaining tannic acid from the medium. In the control tube, inactive enzymes were included. The reaction mixture was centrifuged at 5000×g for 10 min, and the final precipitates were suspended in SDS-triethanolamine (2.5 mL, 1%). Then, 1 mL of 0.13 M $FeCl_3$ was added with the reaction mixture and incubated for 15 min to stabilize the color. After 15 min, the absorbance of the sample was read at 530 nm against the reagent blank. The absorbance value was directly proportional to the amount of gallic acid in the reaction mixture. The standard curve was performed with gallic acid (10 to 100 µmol). One unit of tannase was defined as the amount of enzyme that released 1 µmol of gallic acid per min under standard assay conditions (Mondal *et al*. 2001).

Characterization of a Tannase-Producing Strain

The maximum tannase-producing bacterial strain was characterized by biochemical, morphological, and molecular methods. The strain BR1 was cultured in nutrient broth medium and incubated for 24 h at 37 °C. Genomic DNA was extracted from the culture using a DNA purification kit (Qiagen, Germany), and the purity was tested using agarose gel electrophoresis. The 16S rDNA was amplified using forward (27F) and reverse (1492R) primers. The amplified 16S rDNA sequences were sequenced using Applied Biosystems, and the sequences were analyzed using the BLAST alignment search tool (Al-Dhabi *et al*. 2020).

Analysis of Hydrolysable Tannins

Approximately 0.5 g of unripe banana peel was mixed with 20 mL of double-distilled water and boiled for 15 min. Then, 100 mL of acetate buffer (0.1 M, pH 5.5) was added, and the round bottom flasks were placed on the orbital shaker for 30 min at 150 rpm. The sample was centrifuged at 5000×g for 10 min, and the residue was removed. This sample was further transferred to a test tube, 0.25 mL of 0.1% of $FeCl_3$ solution was added, and incubated for 10 min. The absorbance was read at 550 nm against a blank, and the development of blue-black or brown colour was observed (Romani *et al*. 2006).

Optimization of Tannase Production by the Traditional Method

The effect of the incubation period on tannase production (1, 2, 3, and 4 days) was analyzed at 37 °C. The effect of pH on tannase production was performed between pH values of 6.0 and 8.0 for 3 days. To determine the inoculum level on tannase production, the selected strain was cultured in nutrient broth medium and inoculated at a 0.2 to 1% level (1×10^6 CFU/mL). In order to determine the optimum tannic acid content for tannase production, tannic acid was supplemented at 0.1 to 0.5%. The control experiment was run for all sets of experiments.

Screening of Variables by a Two-level full Factorial Design

Response-surface methodology (RSM) using a two-level full factorial design was used to screen the optimum variables for improved tannase production. The variables and their levels were agitation period (50 to 150 rpm), pH (6 to 8), inoculum (0.2 to 1%), incubation period (24 to 72 h), and tannic acid (0.1 to 0.5%).These values were optimized by a statistical approach. The pH of the culture medium was adjusted using 0.1 N NaOH or 1 N HCl. Tannic acid was filter sterilized, and it was added at an appropriate concentration. The culture medium was prepared, sterilized, and inoculated with various concentrations of inoculum. A total of 16 experiments were performed to satisfy the polynomial model, which is established on a two-level full factorial design. A design expert (version 8.0.1) was applied to design the experiment. An analysis of variance (ANOVA) was used to test the significance, and a p-value of <0.01 was considered statistically significant (Al Farraj *et al*. 2020).

Central Composite Design

The bioprocess factors selected for the central composite design were based on two-level full factorial design experiments. The three significant variables were selected to analyze the optimum bioprocess conditions for tannase production. The variables were analyzed at five different levels (-1.414, -1, 0, +1, and +1.414). The central composite design experiment comprises 20 experiments, and the experiments were designed according to the experimental matrix design of Design Expert software. All experiments were carried out in triplicate, and an average value was used for analyses (Vijayaraghavan and Vincent 2014).

Central Composite Design

One-way analysis of variance (ANOVA) was performed and the p-value <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Composition of Banana Peel and Hydrolysable Tannin

The composition of unripe banana peel was determined. Total carbohydrate content was 78.9+5.4% and lignin content was 21.01+1.1%. The ash content was 0.09+0.001% (Fig. 1). The hydrolysable tannin content of the unripe banana peel was 7.84 ± 0.13%.

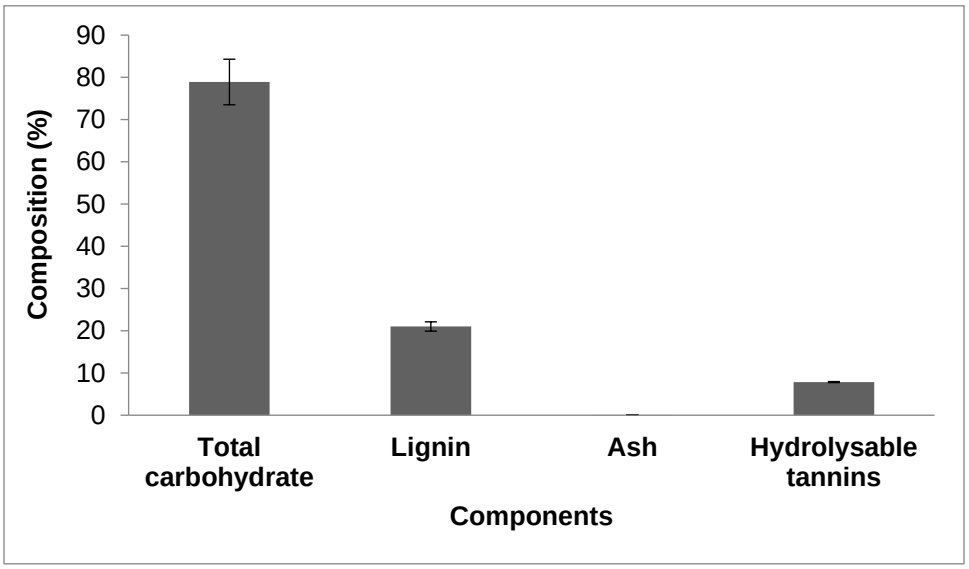

Fig. 1. Composition of unripe banana peel. The unripe banana peel was collected, dried and steam-cooked, and % composition was determined.

Alkaline Treatment

The unripe banana peel (Robusta type) was treated with various concentrations of sodium hydroxide (10 to 30%), and the optimum concentration was detected. At 10% sodium hydroxide treatment, cellulose and lignin contents were 75±3.5%, and 6.9±0.2%, respectively. The present study revealed that 20% sodium chloride was optimum for the maximum cellulose (75 ± 3.5%) and lignocellulose yield (11.6 ± 0.2%). At 30%, cellulose (81 ± 3.5%) and lignin content (9.3 ± 0.54%) decreased. This might be caused by the degradation of lignin and cellulose at higher alkaline concentrations (Fig. 2).

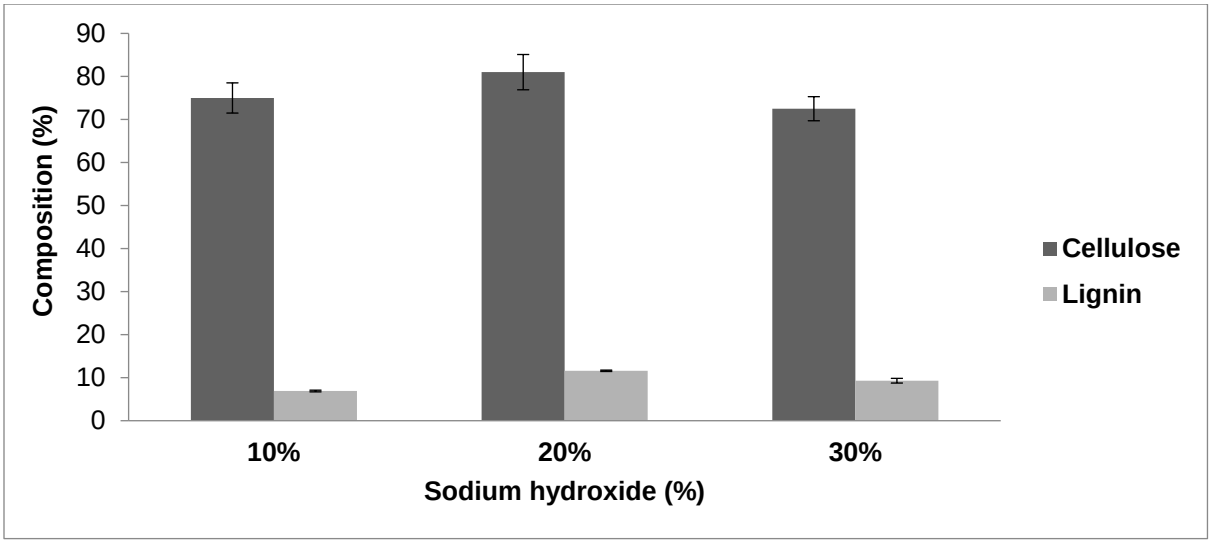

Fig. 2. Cellulose and lignin content (%) of unripe banana peel treated with various concentrations (10 to 30%) of sodium hydroxide at 50 °C for 60 min

Screening of Bacteria for Tannase Production

Tannin acyl hydrolase is one of the important industrial enzymes used in food processing industries and tannin waste management. In view of its economic importance, the present study was carried out to isolate tannin-degrading enzyme-producing *Bacillus*

spp. from the natural environment. In our study, we used an enriched medium containing tannic acid for the isolation of tannin-degrading bacteria. A total of 43 morphologically different bacteria were obtained from the different samples. In order to select only *Bacillus* strains, the bacteria showing Gram-positive, endospore-forming, rod-shaped, and catalase-positive strains were selected. Eleven isolates exhibited these properties and were used for screening experiments. A dark color zone formed on a minimal agar medium containing tannic acid. The zone of hydrolysis ranged from 9 mm to 21 mm (Fig. 3a). Among the strains, the isolate BR1 showed a greater zone of clearance (21 mm) than other isolates (Fig. 3b). Tannic acid substrate agar plates were used for the screening of bacteria for tannase production. Raghuwanshi *et al*. (2011) isolated tannase-producing bacteria using a minimal medium agar plate containing 1% tannic acid. The screened bacteria showed a clear zone of hydrolysis and formed black precipitates around the positive culture. Enriched medium was used for the isolation of target bacteria; this method is widely used (Wilson *et al*. 2009).

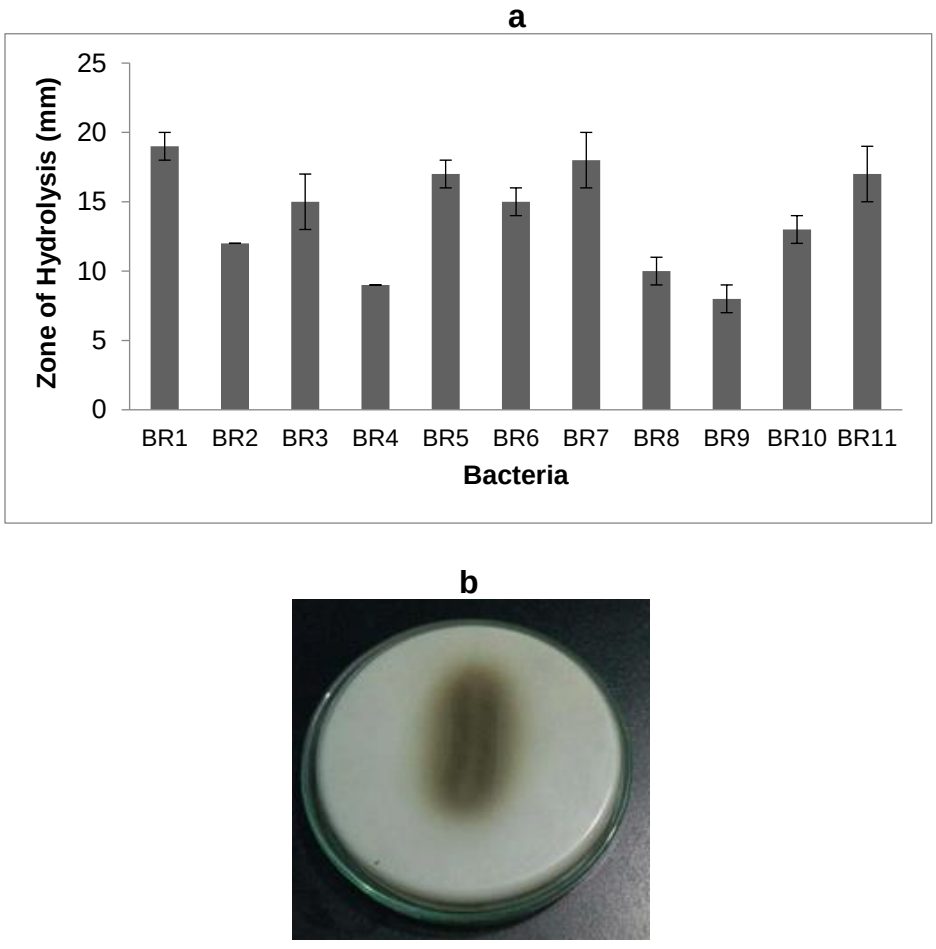

Fig. 3. Screening of *Bacillus* spp. for the production of extracellular tannase on tannic acid agar plates after 74 h of incubation. (a) zone of clearance of *Bacillus* sp. (mm) on tannic acid agar plates; (b) extracellular tannase production by *Bacillus* sp. BR1

Extracellular Tannase Production

The morphologically different bacterial strains were differentiated and sub-cultured. Only *Bacillus* spp. was selected for extracellular tannase screening based on their ability to degrade tannic acid in agar plates. The enzyme activity of all 11 selected *Bacillus*

sp. was analyzed from the crude enzyme fraction after 48 h in a broth medium containing 0.5% tannic acid. The result was depicted in Fig. 4 and showed maximum enzyme production by the strain BR1 (21 ± 1.1 U/mL) after 72 h ($p<0.001$). Tannase activity was detected in the cell-free extract of all cultures, and enzyme production varied widely. Among the strains, seven bacterial isolates showed >10 U/mL enzyme activity in the cell-free extract. There are several tannase-producing *Bacillus* species that have been reported. The productivity of tannase varied based on the strains used for cultivation and the culture medium. *Bacillus* species such as *B. licheniformis* (Mondal *et al*. 2000), *B. polymyxa* (Deschamps *et al*. 1983), *B. sphaericus*(Raghuwanshi *et al*. 2011), *B. cereus* (Mondal *et al*. 2001), *B. gottheilii* (Subbalaxmi and Murty 2016), and *B. massiliensis* (Belur *et al*. 2012).

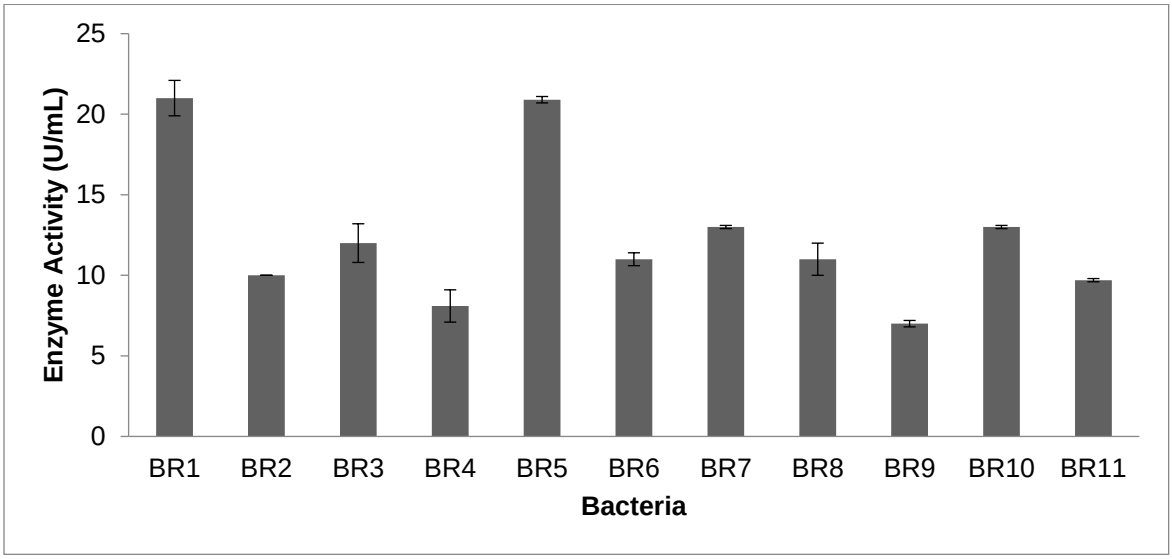

Fig. 4. Production of tannase by the *Bacillus* sp. isolated from the natural environment. The selected 11 bacteria were cultured for 72 h and tannase production was estimated from the supernatant.

Characteristics of Isolated *Bacillus xiamenensis*

The isolated strain BR1 was observed to be endospore-forming, gram-positive strains, motile organisms, and subpolar flagella. It formed dull white, round, non-luminescent, and non-pigmented colonies with irregular margins. The developed colonies were 1- to 2-mm in diameter after 24 h of incubation at 37 °C. The selected bacterial strains grew well between 0 to 5% sodium chloride concentrations, and optimum growth was observed at 2.5% sodium chloride concentrations. The isolated bacterial strains produced tryptophan deaminase, beta-galactosidase, catalase, and gelatinase. The characterized isolate utilized citrate and was positive for catalase activity. The selected strain showed sensitivity to tetracycline, cephalothin, chloramphenicol, streptomycin, and enthromycin. The strain BR1 was identified as *B. xiamenensis* based on 16S rDNA sequence analysis and showed 98.76% similarity to *B. xiamenensis*.

Optimization of Tannase by Traditional Method

The maximum production of tannase, 29.3 ± 1.9 U/mL, was observed after 72 h incubation, and pH 7.5. Enzyme production was depleted at acidic pHs. The results showed that optimal inoculum was obtained with 0.6% inoculum and enzyme activity depleted at higher inoculum concentration. The results showed that optimal enzyme production was

obtained after 72 h incubation. Tannase production was induced by the lower concentration of tannic acid (0.1 to 0.3%) in the culture medium. However, at higher concentrations (>0.3%) tannase activity was depleted. Because of its industrial application, enzyme production was initially optimized by the one-variable-at-a-time approach method. The impact of various bioprocess factors, such as pH, fermentation period, inoculums, and tannic acid content, was optimized.

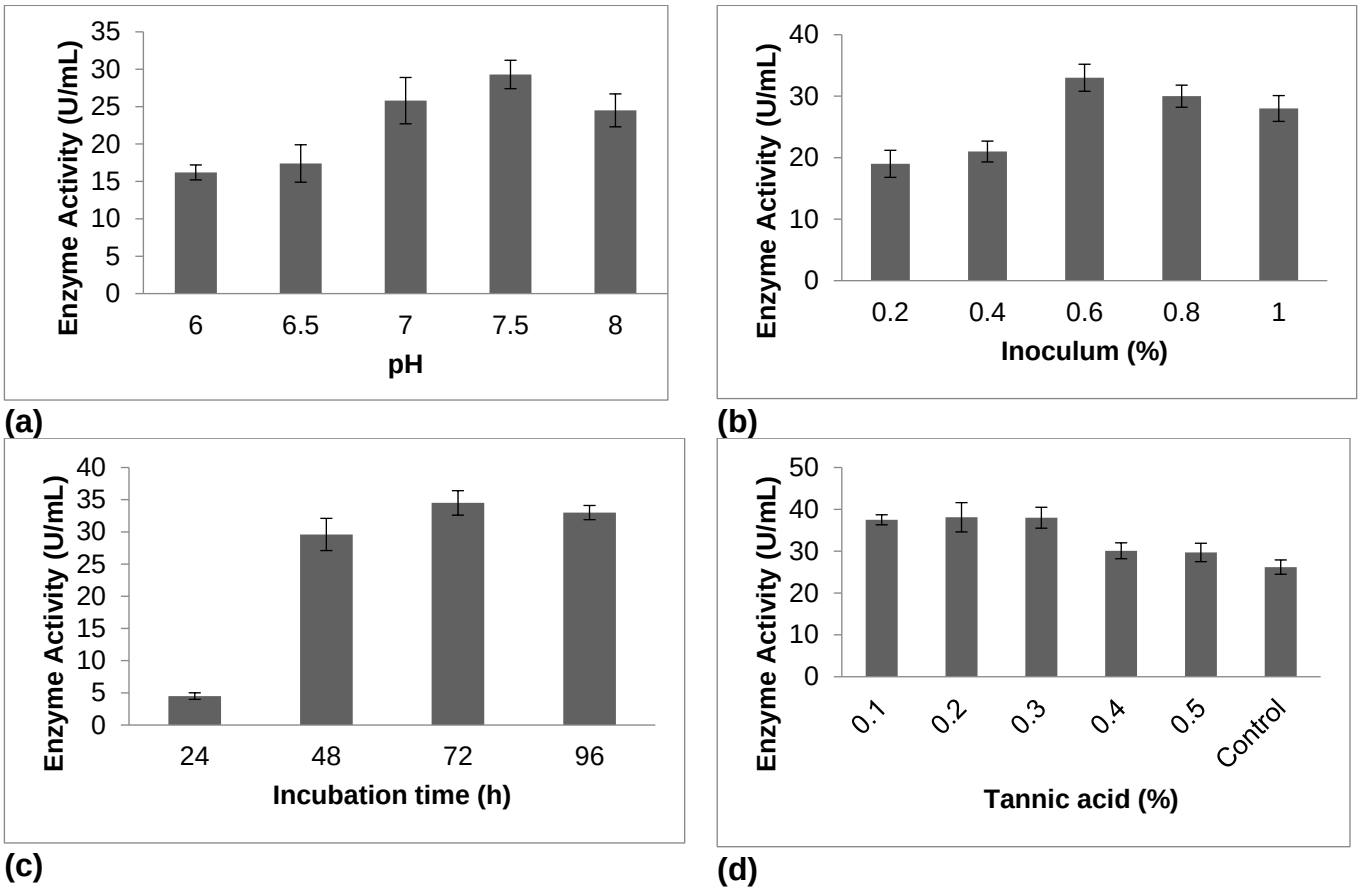

Fig. 5. Effect of pH (a), inoculums (b), incubation time (c), and tannic acid (d) on tannase production. The unripe banana peel (10%) was used as the substrate for the production of tannase, and the result was expressed in U/mL.

Screening of Variables for Tannase Production in Unripe Banana Peel Medium

The unripe banana peel was used as a low-cost medium for the production of tannase in a submerged fermentation system. The unripe banana peel is rich in cellulose and tannin. Tannase is an inducible enzyme; hence, a tannin rich substrate is preferred to meet the requirement of tannin in the medium. In earlier studies, cheap agro-industrial wastes such as cashew testa (Viswanath*et al*. 2015), castor bean residues (Madeira *et al*. 2011), tea stalks (Xiao *et al*. 2015), and coffee husks (Battestin and Macedo 2007) were used as substrates to produce microbial tannase. The earlier studies optimized bioprocess conditions on the basis of a one-variable-at-a-time approach, including enzyme activity. In the traditional one-variable-at-a-time approach, pH, inoculums, incubation time, and tannic acid were found to influence tannase production. Hence, these four variables were selected to optimize tannase production in unripe banana peel medium. A 32-run, two-level full factorial design was performed to determine the most significant factors influencing

tannase yield. As described in Table 1, tannase production varied widely, which revealed the significance of optimizing bioprocess conditions to achieve maximum enzyme production. The results were fitted onto the following equation:

$$\text{Enzyme activity (Y)} = +29.34+3.96A+10.14B+6.59C+8.21D$$
$$+3.72AC+3.57AD+3.29AE+ 3.73BE+4.33CD+8.52BCD$$
$$+3.17BDE-2.98ABDE \quad (1)$$

where A represents agitation speed, B represents pH, C represents inoculums, and D represents incubation period. E represents tannic acid. The effect estimate was positive for agitation speed, pH, incubation period, and inoculums.

Table 1. Production of Tannase Using Unripe Banana Peel Medium in Two-Level Full Factorial Design

Run	A	B	C	D	E	Tannase(U/m)
1	50	6	0.2	24	0.5	5.01
2	150	8	1	24	0.1	20.09
3	50	6	0.2	72	0.1	40.2
4	50	8	1	24	0.5	20.3
5	150	8	1	72	0.1	95
6	150	6	1	72	0.5	37.6
7	50	8	1	72	0.1	35.09
8	50	8	0.2	72	0.1	25.09
9	150	8	1	72	0.5	90.3
10	50	6	1	72	0.5	7.42
11	150	8	0.2	72	0.5	52.09
12	150	6	1	24	0.1	25.4
13	50	6	0.2	72	0.5	5.2
14	50	6	1	72	0.1	31.5
15	150	8	1	24	0.5	34.92
16	50	6	1	24	0.5	20.3
17	50	8	0.2	72	0.5	30.52
18	50	6	0.2	24	0.1	5.42
19	150	8	0.2	24	0.1	34.9
20	50	8	1	24	0.1	20.42
21	50	8	0.2	24	0.5	29.52
22	150	6	0.2	72	0.1	20.1
23	150	6	1	24	0.5	19.8
24	150	6	0.2	24	0.5	4.9
25	150	6	0.2	72	0.5	30.2
26	150	6	0.2	24	0.1	2.57
27	150	6	1	72	0.1	25.7
28	50	8	0.2	24	0.1	39.1
29	50	6	1	24	0.1	25.9
30	150	8	0.2	72	0.1	9.63
31	50	8	1	72	0.5	65.14
32	150	8	0.2	24	0.5	29.52

A: agitation speed; B: pH; C: inoculums; D: incubation period; and E: tannic acid

The adequacy of the two-level full factorial design was analyzed, and the factors that were analyzed to have a significant effect were tested using Fisher's test for analysis of variance (ANOVA). ANOVA revealed that the designed model was statistically significant. The F-value of the model was 8.99, and the p-value was <0.001 (Table 2). There was only a 0.01% chance that a "model F-value" this large could occur due to noise. The values of "Prob>F" <0.05 indicate the selected 2-level full factorial model terms are statistically significant. In our study, B, C, D, CD, and BCD are statistically significant model terms. The R-squared value was 0.8503, and the adjusted R-squared value was 0.7557. The "pred R-squared" value was in reasonable agreement with the "adj R-squared" value of 0.7557. The screened variables showed positive regression coefficients, and only significant variables (pH, inoculum, and incubation period) were selected for further optimization using CCD and RSM.

Table 2. Analysis of Variance of Two-Level Full Factoria Design for Tannase Production

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob> F
Model	12501.8	12	1041.8	8.9	< 0.0001
A-Agitation speed	500.7	1	500.7	4.3	0.0514
B-pH	3288.8	1	3288.8	28.3	< 0.0001
C-Inoculum	1390.0	1	1390.0	11.9	0.0026
D-Incubation period	2156.7	1	2156.7	18.6	0.0004

Optimization of Variables by Central Composite Design

Based on the findings of a two-level full factorial design, the interactions between the selected variables (pH, inoculum, and incubation period) were tested and optimized using a CCD. A total of 20 experiments were analyzed in triplicate; their corresponding enzyme activities are illustrated in Table 3. This study determined optimal physical and nutritional sources that improved tannase productivity in submerged fermentation. In run 14 (pH 7.0, 0.7% inoculums, and 48-h fermentation), the enzyme activity reached its maximum. The value obtained after the optimization process was significantly higher than the traditional one-variable approach to optimization. These results revealed that optimizing bioprocess factors on the basis of a one-variable-at-a-time approach has several limitations. This is due to the fact that the optimization of agro-residues still requires a suitable mathematical model for optimization. The tannase production by *B. xiamenensis* was improved by the statistical method *via* a two-level full factorial design and a central composite design. The statistical method optimization revealed that an increase in fermentation period causes an increase in significant activity. The amount of tannase activity varied widely. In addition, the second-order polynomial was used to explain tannase production and interaction among the factors as below:

$$\text{Enzyme activity } (Y) = +140.89 + 6.27A - 0.92B + 27.38C + 11.00AB + 2.08AC + 4.79BC - 49.85A^2 - 26.88B^2 - 19.26C^2 \quad (2)$$

In Eq. 2, Y is the tannase activity, whereas A, B, and C are pH, inoculum, and incubation period, respectively. The ANOVA of the designed central composite design model was significant (p<0.0001), and the F-value of the model was 17.03. When analyzing the F-and

p-values, pH and inoculum were shown as having non-significant effects; however incubation time showed significant p-value (<0.0004) (Table 4). There was only a 0.01% chance that a "model F-value" so large could occur due to noise. In this case, C (p-value = 0.0004), A^2(0.0001), B^2 (0.0004), and C^2 (0.0035) were significant model terms. The R^2 value for the CCD model was 0.98, and the adjusted R^2 value was 0.97, which showed that 98% of the observed variation in the enzyme yield could be clearly explained by the model. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. In this model, the ratio of 11.113 indicates an adequate signal. This model can be used to navigate the design space.

Table 3. Central Composite Design for Tannase Production Using Unripe Banana Peel Substrate

Runs	A	B	C	Enzyme Activity (U/mL)
1	6	0.2	72	73
2	6	0.2	24	8.57
3	7	0.7	48	122
4	6	1.2	72	34.97
5	7	-0.1409	48	78.4
6	7	0.7	7.636972	32.39
7	8	1.2	24	21.91
8	7	0.7	48	133.9
9	7	0.7	88.36303	144.9
10	5.318207	0.7	48	1.3
11	7	1.540896	48	55.8
12	8	0.2	72	43.9
13	7	0.7	48	143.8
14	7	0.7	48	152.5
15	7	0.7	48	149.2
16	8	1.2	72	113.8
17	8	0.2	24	35.1
18	7	0.7	48	143.2
19	6	1.2	24	15.32
20	8.681793	0.7	48	2.95

A:pH, B: inoculum, C: incubation period

Table 4. Central Composite Design and Analysis of Variance

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob> F
Model	57021.95	9	6335.773	17.03435	< 0.0001
A-pH	536.8464	1	536.8464	1.443364	0.2573
B-Inoculum	11.58533	1	11.58533	0.031148	0.8634
C-Incubation time	10241.55	1	10241.55	27.53542	0.0004
AB	967.78	1	967.78	2.601972	0.1378
AC	34.48651	1	34.48651	0.09272	0.7670
BC	183.457	1	183.457	0.493242	0.4985
A^2	35815.1	1	35815.1	96.29242	< 0.0001
B^2	10412.47	1	10412.47	27.99495	0.0004
C^2	5347.196	1	5347.196	14.37646	0.0035
Residual	3719.41	10	371.941		
Lack of Fit	3096.157	5	619.2314	5.1	0.0816
Pure Error	623.2533	5	124.6507		
Cor Total	60741.36	19			

The increased hydrolysable tannin content may be considered a good inducer for tannase activity. In this study, the addition of tannic acid showed improvement in tannase activity. However, a high concentrations of tannic acid did not have any impact on tannase production. This finding revealed that the selected substrate is the source of tannin and is enough to induce tannase production. The utilization of agro-industrial products into valuable biochemicals helped solve environmental pollution. The response surface plot revealed that lower incubation periods, pH, and inoculums caused a decrease in tannase activity, and it increased up to 63 h of incubation, pH 7.34, and 0.64% inoculum. After that, the tannase activity declined. pH is one of the significant factors affecting tannase production. The variation of tannase yield due to pH variation was reported previously (Rodríguez *et al*. 2008). In a similar fashion, decreased tannase biosynthesis was observed at lower and higher incubation times. Enzyme production was less at 24 h of incubation and reached its maximum after 60 h of incubation. A drop in tannase production beyond 63 h of incubation may be attributed to the reduced availability of nutrient components in the culture medium. To analyze the interaction and to visualize the effects of the selected two factors (one at the midpoint) on tannase activity, a 3D plot was obtained in Design Expert software analysis based on enzyme activity. The 2D contour plots and 3D response surface plots were presented in Figs. 6 and 7. The shape of the curve is used to predict the response. The elliptical shape of the curve indicated good interactions, and the graphs are convex in nature, which revealed that the selected factors for optimization were well defined. The shape of the contour plot is useful to analyze the extent and nature of the interactions.

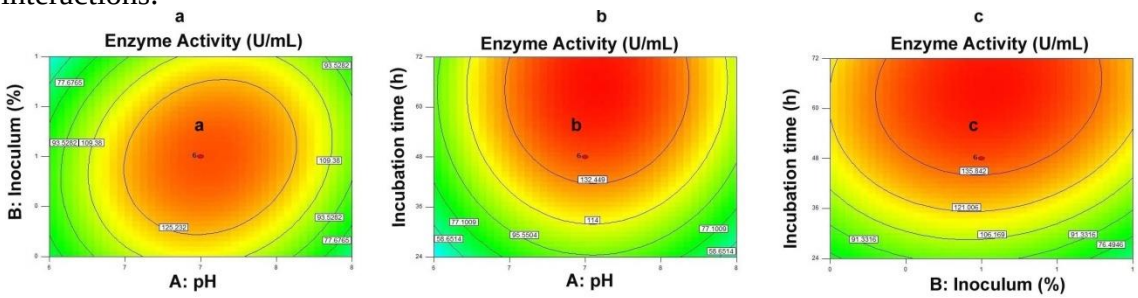

Fig. 6. The 2D contour plots for the production of tannase and the analysis of interactive effects between variables (a) pH and inoculation, (b) pH and incubation time, and (c) inoculum and incubation time (h).

Figure 6 shows the insignificant interaction between pH and inoculums. Moreover, incubation time interacted significantly with pH and inoculums and significantly influenced tannase production (Fig. 7). The 2D contour plots and 3D response surface plots revealed that all the selected process parameters showed significant interactive effects. The optimum bioprocess conditions obtained in the present study are in agreement with the previous reports (Varadharajan *et al*. 2015; Kumar *et al*. 2016).The predicted concentration of the factors obtained from the Design-Expert software was tested using experiments. To confirm the predicted experimental model, triplicate experiments were used, and the tannase activities were determined. The predicted tannase activity was very close to experimental results, which validated the designed model. Consequently, the designed model was considered to be reliable for improving tannase production from *B. xiamenensis*.

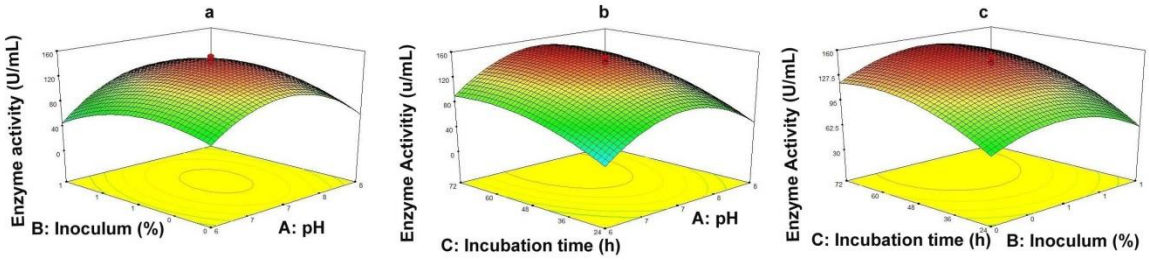

Fig. 7. 3D contour plots for the production of tannase and analysis of interactive effects between variables. (a) pH and inoculation, (b) pH and incubation time, and (c) inoculum and incubation time.

CONCLUSIONS

1. The unripe banana peel was used as the substrate for the production of tannase in submerged fermentation by *Bacillus xiamenensis*. Fermentation period, pH, inoculums, and tannic acid improved tannase production. The unripe banana peel can be used as a substrate for the production of tannase to reduce the production cost of the enzyme.
2. The unripe banana peel consists of an increased amount of hydrolysable tannin, which induces the production of tannase. Under optimal bioprocess conditions, tannase yield was 2.4-fold higher than in an unoptimized medium.

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