

Corn (*Zea Mays*) Leaves as Cost-Effective Substrate for Enhanced Tannase Production Under Solid State Fermentation: Optimization and Characterization

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Abstract

The current study reports the utilization of agricultural residues of corn (*Zea mays*) as lower-cost substrate for the production of tannase under solid state fermentation (SSF). Tannase producing bacterial strains were isolated from gut content of freshwater fish, *Ctenopharyngodon idella*, and highest tannase producer was identified as *Bacillus cereus* using 16S rDNA sequencing. For enhanced tannase production, *B. cereus* was investigated using one variable at a time (OVAT) followed by central composite design (CCD) of response surface methodology (RSM). Under OVAT, optimal fermentation conditions and medium composition were achieved with 70% substrate moisture, distilled water (1 ml) as enzyme extraction medium, 30°C incubation temperature, 3.0 pH, 1% inoculum size, 24 h incubation time, 150 rpm agitation, large-sized substrate particles (4 mm), non-centrifugation condition, NaCl salt, 1.5% tannic acid substrate and malt extract as organic nitrogen source for maximal enzyme synthesis. The highest tannase was produced (155.74 ± 1.67 U/ml) with 1.75% tannic acid, 0.75% NaCl and 1.25% malt extract with the application of CCD of RSM. Higher coefficient of regression ($R^2=0.9665$) indicated that second-order polynomial regression model assessed the data excellently. Further, tannase characterization depicted its maximum activity at 5.0 pH, 50°C temperature, 45 min incubation and 0.35% tannic acid.

1 Introduction

Tannase (tannin acyl hydrolase EC 3.1.1.20) is an enzyme involved in the hydrolysis of the tannic acid and yield of gallic acid and glucose (Kumar et al. 2007; Beena et al. 2011). This inducible extracellular enzyme is mainly produced by bacteria, yeast and fungi in the presence of tannic acid (Böer et al. 2009; Sharma and John 2011; Lekshmi et al. 2020). Tannase is considered an industrially important enzyme with several applications specially in pharmaceutical and food industries for gallic acid synthesis, fruit juices clarification, wine production, coffee-flavored drinks and instantaneous tea formation (Lekha and Lonsane 1997; Aguilar et al. 2007; Mohapatra et al. 2007; Madeira et al. 2011; Chávez-González et al. 2012). Furthermore, tannase is also used in animal feed preparation to enhance the nutrients bioavailability by catalyzing anti-nutritional phenolic factors (Graminha et al. 2008).

From microbial source, tannase can be produced by several methods including liquid surface, submerged and solid state fermentation but the major advantages of solid state fermentation (SSF) are its simplicity, low wastewater, less production costs and enhanced enzyme yield (Aguilar et al. 2001; Aguilar et al. 2002; Barrios-González 2012). Recently, the interest towards the utilization of agricultural residues for the production of tannase has been remarkably increased due to their lower costs and vast applications. Agricultural wastes such as olive mill waste (Aissam et al. 2005), palm kernel cake, tamarind seed powder (Sabu et al. 2005), coffee husk (Battestin and Macedo 2007), tea stalks (Xiao et al. 2015), rice straw, sugarcane bagasse (Paranthaman et al. 2010), cashew bagasse (Liu et al. 2016), different fruit seeds (Arshad et al. 2019), rice bran, coconut residues (Mansor et al. 2019), wheat bran, pomegranate peel, eucalyptus leaves, banana peel, guava (Ahmed and Abou-Taleb 2020) almond and mango leaves (Ire and Nwanguma 2020) have been utilized previously for enhanced tannase synthesis in literature. In

contrast, the use of tannic acid in pure form act as an expensive and unaffordable substrate and inducer for tannase synthesis at industrial scale (Mansor et al. 2019).

Optimizing the tannase production for maximal yield is a significant endeavor. To develop an economically feasible process, optimization of various fermentation conditions is a critical concern (Kar et al. 2009) and can be performed applying several statistical approaches for the enhancement of enzyme synthesis (Vijayaraghavan and Vincent 2015). One variable at a time (OVAT) is a traditional optimization method which involves the variation of one variable at one time by keeping all other factors constant (Singh et al. 2011). On the other hand, the response surface methodology (RSM) is being used extensively now a days for formulating experimental designs. This pragmatic modeling technique is applied to evaluate the relations present within a group of selected factors of experiment (Vinothini et al. 2019) which involves the interaction of independent variable with all other variables and its effect on the response (Baş and Boyacı 2007). In this context, the following study was designed to investigate the application of low cost agricultural wastes of corn (*Zea mays*) leaves for the production and characterization of tannase from bacterial source through the solid state fermentation using OVAT and RSM approaches for optimization.

2 Materials And Methods

2.1 Isolation and screening of tanninolytic bacteria

For isolation of tannase producing bacteria with tanninolytic potential, the gut content of *Ctenopharyngodon idella* fish was serially diluted in 0.9% sterile saline and plated on sterilized (autoclaved at 121°C and 15 psi) nutrient agar medium (2.8%) supplemented with 0.5% tannic acid. After 24 h incubation at 37°C, colonies of tanninolytic bacteria appeared on this selective medium. The initial screening was carried out following Osawa and Walsh (1993) method on the basis of zone of hydrolysis appearing around the isolates able to break down the tannic acid in medium by producing extracellular tannase. After initial screening, bacterial isolates were stored in the form of slants and subjected to tannase assay for final screening. The highest tannase producing bacterial strain was finalized to be used further for parameter optimizations.

2.2 Tannase production

One percent (1%) of each tanninolytic bacterial strains was inoculated in their respective 250 mL Erlenmeyer flask with production medium (100 mL) containing 0.5% tannic acid, 0.1% CaCl₂ and 0.275% yeast extract. Each flask was incubated for 24 h at 37°C followed by the centrifugation of medium at 8000 rpm for 15 min at 4°C. The supernatant was used as crude tannase during the enzyme assay for each bacteria.

2.3 Tannase assay

In tannase assay (Miller 1959), tannic acid was taken as substrate and glucose as standard. Solution of crude enzyme (0.5 ml) was incubated (37°C) with 0.5 ml of 0.5% tannic acid in 0.1 M acetate buffer (pH

5.0) for 2 h. After incubation, 3.0 ml di-nitro-salicylic acid was added to terminate the reaction and solution was boiled for 15 min in water bath followed by the dilution with distilled water (10 ml). Absorbance was observed at wavelength of 540 nm by spectrophotometer against blank and standard curve was formed. One tannase unit was determined by the amount of tannase enzyme that hydrolyzes 1 mM of substrate (tannic acid) during standard conditions of assay.

2.4 Identification of bacterial strain

The bacterial strain with maximal tannase synthesis potential was identified by 16S rDNA sequencing. Briefly, DNA was isolated from pure culture (overnight grown) of strain followed by the amplification of 16S rDNA using universal primers, 27 forward (AGAGTTTGATCMTGGCTCAG) and 1492 reverse (TACGG[Y]TACCTTGTTACGACTT) following the method described by Shakir (2013). The amplified DNA products were examined by agarose (1%) gel electrophoresis and further purified by GenJET™ purification kit. After purification, 16S rDNA amplicons were sequenced commercially and nucleotides were aligned using NABI BLAST. Identification of bacterial strain was performed based on sequence similarities (%) with of bacterial sequences already submitted to the database.

2.5 Solid substrate

Agricultural residues i.e., corn (*Zea mays*) leaves were obtained from Chakwal Pakistan, chopped into small pieces and sun dried properly. Dried corn leaves were grinded to fine powder to be used as solid substrate in experiments.

2.6 Optimization of fermentation conditions via OVAT

Various fermentation conditions i.e., substrate moisture content (50–90%), medium for tannase extraction (distilled water, tap water, 1% NaCl, acetate buffer of pH 4 and 5, phosphate buffer of pH 6 and 7, tris-HCl buffer of pH 8 and 9 and glycine NaOH buffer of pH 10 and 11) and volume of optimum extraction medium (1–6 ml), incubation temperature (37, 40 and 45°C), initial pH level (3–11), inoculum size (1–3%), incubation time (24, 48, 72 and 96 h), agitation effect (shaking at 150 rpm and static condition), substrate particles size (2.8, 3.4, 4.0 mm) and centrifugation (8000 rpm at 4°C for 15 min) effect were evaluated to determine their optimal value for maximum tannase production in the SSF by opting OVAT. Each optimized condition was incorporated in the next parameter optimizing experiment.

2.7 Optimization of medium composition

To assess the role of salt, carbon and nitrogen sources, fermentation medium was incorporated with several salts (NaCl, KCl, KH_2PO_4 , K_2HPO_4 , MgSO_4 , CaCl_2), tannic acid concentrations (1.5, 2.0, 2.5..., 4.0%) and organic nitrogen sources (yeast extract, malt extract and peptone) to optimize the tannase synthesis in SSF using OVAT method. The components yielding the maximum tannase was selected optimum as the result of tannase assay. Each optimized medium component was added in further optimizing experiments.

2.8 Optimization of concentrations of medium components by Central composite design

Central composite design (CCD) of RSM was applied to determine the concentrations of optimal medium components for enhanced enzyme production under SSF. For this, five-level face-centered cube design (-2, -1, 0, +1, +2) with three components requiring 17 experiments was used (Tables. 1 & 2). Medium components, tannic acid (%), NaCl (%) and malt extract (%) were independent variables while tannase production (U/ml) was the response.

ANOVA was applied to assess the significance of model and coefficients of regression. Regression predicted the tannase production as response using second order polynomial equation (Eq. 1).

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

Where Y was the response, $X_i X_j$ were independent variables while K was the variables applied in RSM. β_0 , β_{ij} and β_{ii} were intercept, interaction and quadrate coefficients respectively.

2.9 Tannase characterization

2.9.1 pH

The pH impact on tannase activity was determined by applying pH varying from 4 to 11 using OVAT. The pH value with maximum enzyme activity was considered as optimum. Acetate buffer with pH 4 and 5, phosphate buffer with pH 6 and 7, tris-HCl buffer pH 8 and 9 and 0.1 M glycine-NaOH buffer pH 10 and 11 were used for maintaining different pH values.

2.9.2 Temperature

To determine the effect of temperature, activity of tannase was observed by incubating it at different temperatures (20–90°C) with optimized pH under OVAT. Incubation temperature associated with maximum enzyme activity was recorded as optima.

2.9.3 Incubation time

At optimal pH and temperature, enzyme (crude) was incubated for different times i.e., 15, 30, 45..., 75 min using OVAT. Incubation time showing the highest tannase activity was taken as optimum.

2.9.4 Substrate concentration

Different substrate (tannic acid) concentrations (0.25, 0.30, 0.35..., 0.60%) were used under previously optimized conditions using OVAT to record the activity. Concentration corresponding to maximal enzyme activity was recorded as optimum substrate concentration.

2.10 Data analysis

Experiments were performed in triplicates. Student's t-test and one way ANOVA followed by Tukey's test was applied on data to determine the significant differences ($P < 0.05$) by using IBM SPSS statistics 20 software and Microsoft Excel 2019. Results were presented in as mean \pm standard deviation. STATISTICA software (99th edition) was used for RSM statistics.

3 Results

3.1 Screening and identification of tannase producing bacteria

During initial screening, colonies of three tanninolytic bacterial strains were appeared with zone of clearance on tannic acid incorporated medium. These strains were coded as A1, A2 and A3 and subjected to the tannase assay where A3 depicted the highest production of tannase enzyme with value of 2.21 ± 0.05 U/ml (Fig. 1).

The strain A3 was identified as *Bacillus cereus* with the application of 16S rDNA nucleotide sequence. Neighbour-joining tree showing phylogenetic relationships with closely related bacterial strains has been displayed in Fig. 2.

3.2 Optimization of fermentation parameters

3.2.1 Initial substrate moisture

Moisture of substrate plays a vital role in SSF during enzyme productivity. The effect of substrate moisture content on the tannase production by *B. cereus* depicted that the enzyme synthesis increased with increase in moisture (distilled water) contents and the maximal tannase synthesis (50.94 ± 1.02 U/ml) was attained with 70% initial substrate moisture. However, further increase in moisture contents caused the decline in tannase production (Fig. 3).

3.2.2 Tannase extraction medium and its volume

Experiments with different extraction mediums were performed in SSF and results in Fig. 4 revealed that optimum tannase yield (53.33 ± 0.87 U/ml) was achieved with distilled water while lowest value (25.39 ± 3.7 U/ml) was obtained using acetate buffer of pH 4. The volume of optimal extraction medium (distilled water) was optimized by applying its different volumes from 1 to 6 ml. Results (Fig. 5) depicted 1 ml as optimum volume for maximal enzyme synthesis (75.24 ± 0.64 U/ml) which tend to decrease with further increase volume with lowest enzyme production (16.90 ± 0.82 U/ml) on 6 ml.

3.2.3 Incubation temperature

Temperature is a vital component during a fermentation process that influence the product significantly. Experiments revealed the optimum temperature for tannase synthesis from *B. cereus* to be 30°C with

enzyme value 77.01 ± 0.28 U/ml. The further increase of temperature resulted in the decline of enzyme production (Fig. 6).

3.2.4 Effect of pH

The effect of pH was evaluated by experiments on different pH ranging from 3 to 11. The maximum tannase production (82.79 ± 1.07 U/ml) was observed at pH 3.0 which declined sharply with the increase of pH as shown in Fig. 6.

3.2.5 Inoculum size

Inoculum size is very significant for metabolite synthesis in SSF (Pandey, 1994). Different inoculum sizes (1–3%) of *B. cereus* were applied during fermentation process for substrate consumption and enzyme production. The highest tannase production (87.29 ± 0.79 U/ml) was obtained with 1% and further increase in size decreased the enzyme value (Fig. 6). The lowest enzyme synthesis (10.58 ± 0.86 U/ml) was observed with 3% inoculum size.

3.2.6 Effect of incubation time

Results in Fig. 6 displayed that the optimum incubation time 24 h with enzyme production 90.20 ± 0.46 U/ml. While the enzyme value decreased with further increase in incubation time and no enzyme synthesis was recorded at 94 h.

3.2.7 Agitation

This study supported that agitation (150 rpm) had positive effect on enzyme production (106.62 ± 1.87 U/ml) by *B. cereus* compared to static state (85.48 ± 2.20 U/ml) as shown in Fig. 6.

3.2.8 Substrate particle size

Substrate with different particle size was used in experiments of *B. cereus* SSF for tannase production. The results demonstrated that the highest tannase synthesis (108.60 ± 2.08 U/ml) was achieved with large sized particles (4.0 mm) whereas minimum enzyme (71.30 ± 2.19 U/ml) was obtained with medium sized particles (3.4 mm) of substrate (Fig. 7).

3.2.9 Centrifugation

Experiments were performed to evaluate the effect of centrifugation on tannase synthesis during enzyme extraction. Results depicted that tannase productivity was significantly higher (108.60 ± 2.08 U/ml) without centrifugation (Fig. 8).

3.3 Optimization of medium composition

3.3.1 Salts

Among different salts applied in the medium, NaCl enhanced tannase production from *B. cereus* the most (109.78 ± 1.18 U/ml). While in the presence of MgSO_4 , the lowest enzyme productivity (87.24 ± 1.47 U/ml)

was recorded (Fig. 9).

Figure 9. Effect of different salts on tannase production from *B. cereus*. Bars with different not sharing common alphabets are significantly different.

3.3.2 Concentrations of tannic acid

The effect of different concentrations of tannic acid (carbon source) was examined on tannase production using corn SSF. The results depicted that the maximum tannase production (112.18 ± 0.75 U/ml) was achieved with 1.5% of tannic acid. The production was recorded to be decreased with further increase of concentration where minimum enzyme was obtained with highest concentration of 4% with the value of 31.56 ± 0.80 U/ml (Fig. 10).

3.3.3 Organic nitrogen sources

Effect of supplement of three organic nitrogen sources i.e., yeast extract, malt extract and peptone on tannase productivity has been shown in Fig. 11. Among applied nitrogen sources, malt extract was the most suitable for enhanced enzyme production (128.82 ± 2.25 U/ml) while yeast extract (114.31 ± 1.77 U/ml) had the least effect.

3.4 Optimization of concentrations of medium components by Central composite design

In the study, concentrations of optimal medium components were optimized by central composite design (CCD) of RSM under SSF. Tannase production values ranging from 94.86 ± 1.74 U/ml to 155.74 ± 1.67 U/ml was observed in 17 runs with highest tannase value (155.74 ± 1.67 U/ml) corresponding to 3rd run with 1.75% tannic acid, 0.75% NaCl and 1.25% malt extract in medium composition (Table 2). The response for CCD was predicted by second order polynomial regression Eq. (2).

Table 1
Range (5 levels) of three independent variables used in CCD.

Independent variables	Codes	Levels				
		-2	-1	0	+1	+2
Tannic acid (%)	A	1.00	1.25	1.5	1.75	2.00
NaCl (%)	B	0.10	0.25	0.50	0.75	1.00
Malt extract (%)	C	0.50	0.75	1.00	1.25	1.50

Table 2
CCD design and responses for tannase synthesis by *B. cereus* employing different concentrations of A: tannic acid (%), B: NaCl (%) and C: malt extract (%) in SSF.

Run	A	B	C	Tannase production (U/ml)		Residue value
				Observed	Predicted	
1	1.50	0.10	1.00	125.5289	123.7173	1.81157
2	1.50	0.50	0.50	127.9556	127.8549	0.10064
3	1.75	0.75	1.25	155.7415	150.7769	4.96460
4	1.50	1.00	1.00	143.9823	146.9973	-3.01495
5	2.00	0.50	1.00	132.3138	135.8763	-3.56250
6	1.75	0.25	1.25	145.4743	144.9202	0.55408
7	1.50	0.50	1.00	140.5966	140.2814	0.31524
8	1.00	0.50	1.00	116.5049	114.7979	1.70696
9	1.25	0.75	0.75	145.2328	143.9314	1.30146
10	1.25	0.25	0.75	117.7036	120.8127	-3.10906
11	1.50	0.50	1.50	135.7735	137.7297	-1.95618
12	1.75	0.75	0.75	144.9038	143.1460	1.75777
13	1.25	0.75	1.25	122.1929	122.0414	0.15145
14	1.25	0.25	1.25	123.1543	123.0565	0.09777
15	1.75	0.25	0.75	114.8596	113.1556	1.70409
16	1.50	0.50	1.00	139.2895	140.2814	-0.99191
17	1.50	0.50	1.00	138.4503	140.2814	-1.83104

$$Y = 61.5478 + 68.5826A + 115.3669B - 59.0701C - 59.7771A^2 - 31.0872B^2 - 29.9564C^2 + 27.4871AB + 118.0834AC - 96.5351BC \quad (2)$$

Here Y denoted the response (tannase production) while A, B and C were tannic acid (%), NaCl (%) and malt extract (%) respectively during CCD design.

ANOVA results (Table 3) for CCD depicted the model statistically significant with F-value of 22.43577 and $P < 0.01$. Among three independent variables, B (NaCl (%)) was found significant ($P < 0.05$). The value of coefficient of determination (R^2) was obtained as 0.9665 which explained 96.65% of total variations in sample while just 3.35% variance was not described by the model. Regression model with $R^2 > 0.95$ show high correlation. High similarities between observed and predicted values indicated the model statistically

strong (Table 2, Fig. 14). Contour plots and desirability charts (Figs. 13 & 14) further explained the interaction of tannic acid (%), NaCl (%) and malt extract (%).

Table 3
Results for ANOVA displaying regression model obtained from CCD used for medium optimization, A: tannic acid (%), B: NaCl (%) and C: malt extract (%).

Effect	SS	DF	MS	F-value	P-value
Model	2267.010	9	2667.010	22.43577	0.000234
A	29.7499	1	29.7499	2.7499	0.147587
A ²	274.6254	1	274.6254	24.46080	0.001664
B	105.6279	1	105.6279	9.40824	0.018137
B ²	51.6661	1	51.6661	4.60188	0.069102
C	26.2260	1	26.2260	2.33595	0.170263
C ²	68.9683	1	68.9683	6.14299	0.042303
AB	23.6107	1	23.6107	2.10300	0.190294
AC	435.7404	1	435.7404	38.81126	0.000433
BC	291.2197	1	291.2197	25.93885	0.001410
Error	78.5901	7	11.2272		

3.5 Tannase activity

3.5.1 pH

Experiment was performed to find the effect of pH on the tannase activity by applying pH ranging from 4 to 11. Results indicated the maximum activity (171.94 ± 5.95 U/ml) at pH 5 after which activity was observed to decrease at higher pH values (Fig. 15).

3.5.2 Temperature

Investigation on tannase activity at different temperatures (20–90°C) revealed that the enzyme activity increased with the elevating temperature, While the maximum activity (182.46 ± 0.57 U/ml) was observed at 50°C (Fig. 15). Thereafter, the decline in activity was seen with temperature increase.

3.5.3 Incubation time

When the crude tannase was incubated for time periods varying from 15 to 165 min, the highest tannase activity (192.86 ± 0.60 U/ml) was observed after 45 min of incubations (Fig. 15). Further incubation caused the decreased activity value.

3.5.4 Tannic acid concentration

Optimum tannic acid concentration was recorded by conducting experiments at various concentrations varying from 0.25 to 0.60%. Figure 15 indicated 0.35% tannic acid as optimum for enhanced tannase activity (201.17 ± 0.66 U/ml), thereafter the activity was observed to decrease.

4 Discussion

Generally, tannic acid is referred as toxic for bacterial species, however several bacteria have developed the potential to degrade this toxic material to utilize it as a source of carbon and energy (Aissam et al. 2005). In current study, *B. cereus* with tannase synthesis potential was isolated from the gut content of *Ctenopharyngodon Idella*. Several reports have confirmed the presence of tannase producer microbes from different ruminant and non-ruminant herbivores digestive tract (Bhat et al. 1998; Odenyo et al. 1999; Goel et al. 2005) but few studies are available on the isolation of such microbes from fish gut (Mandal and Ghosh 2013a; Talukdar et al. 2016). Ray et al. (2012), Mandal and Ghosh (2013a), Talukdar et al. (2016) reported the isolation of numerous tannase producing microbes from gut of several freshwater fishes. Tannin in plants has inhibitory effect for animals feeding (Goel et al. 2005) and also on omnivorous and herbivorous fish species (Al-Owafeir 1999; Becker and Makkar 1999). So, the occurrence of tannase producing bacteria in fish gut to degrade the tannin might be the consequence of co-evolution between compounds like tannins and such fishes (Mandal and Ghosh 2013a). Apart from fish, Mondal et al. (2001) reported the isolation of tannase producing *B. cereus* from soil sample. Other tannase producer such as *Staphylococcus lugdunensis* was isolated from human feces (Noguchi et al. 2007), *Bacillus velezensis* from soil (Lekshmi et al. 2020), *Serratia marcescens* strain IMBL5 from intestinal tract of *Onthophagus babirusa* IMBL (Thiyonila et al. 2020) and *Bacillus gottheilii* M2S2 from tannery effluent (Subbalaxmi and Murty 2016).

In this study, various conditions affecting the fermentation process of tannase production from *B. cereus* were optimized using OVAT under SSF. Moisture in substrate plays an important role for enzyme productivity in SSF and it is required for microbial expansion, substrate bulging and product generation (Pandey et al. 2000). In current study, tannase synthesis increased with moisture increase until the maximum enzyme value was obtained with 70% initial substrate moisture (distilled water), but beyond this level the enzyme production was recorded to decrease. In SSF, Aharwar and Parihar (2018) described the optimum moisture range at 40–90% for higher tannase production. Lima et al. (2014) also observed the 70% moisture as optimum for tannase production from *Penicillium montanense* URM 6286 which was observed to decrease at higher moisture level. Banerjee et al. (2007) reported that highest tannase synthesis from *Aspergillus aculeatus* DBF9 was observed with 80% moisture level in SSF. Moisture content beyond the optimal level reduced the enzyme production, as further increase in moisture level decreases the oxygen availability for microorganisms (Kumar et al. 2007). At very small or high moisture

content, low enzyme synthesis could be due to low degradation of organic matter in solid substrate (Mandal and Ghosh 2013b). In general, tannase is an extracellular enzyme which is extracted using buffer or water (Aguilar et al. 2007). Experiments with different extraction mediums indicated the best results with distilled water (1 ml) for tannase production from *B. cereus*. Treviño-Cueto et al. (2007) also used water as extraction medium for tannase. Vatter and Shetty (2003) applied 5 ml of water extract for tannase extraction. However, Sabu et al. (2006) used 50 ml of 0.05 M citrate buffer with 5.0 pH. In investigation of Rodrigues et al. (2008) 0.05 M citrate buffer with pH 5.0 was applied.

During enzyme production, temperature plays a vital role as it may denature or inhibit the enzyme and even cause the death of cells (Sabu et al. 2006). In current investigation, the optimum temperature was recorded as 30°C while enzyme production decreased at higher temperature values. In support to our obtained results, Aharwar and Parihar (2018) documented the general range for tannase synthesis as 25–35°C in SSF. Banerjee et al. (2007) also reported 30°C for *Aspergillus aculeatus* DBF9 tannase in SSF and synthesis decreased at higher temperature. Sabu et al. (2005) observed 30°C as optimum for tannase production using agricultural wastes in SSF by *Aspergillus niger* ATCC. However for *Enterobacter cloacae* strain 41 it was 50°C (Govindarajan et al. 2019). Lower enzyme production at higher temperature could be because of microbial denaturation (Muslim et al. 2015). Specific activities are decreased at higher and lower temperatures due to thermal effects on microbial growth and rate of enzyme reaction inside cell that reflects on vital enzyme creation (Brühlmann 1995). pH is also a significant factor during the metabolite production (Selwal et al. 2010). When pH ranging from 3 to 11 was applied, the maximum tannase production from *B. cereus* was recorded at pH 3 while the synthesis decreased at higher pH. In SSF, the tannase synthesis mostly depicts pH optima in acidic range (Aharwar and Parihar 2018). Mondal et al. (2001) obtained optimal tannase production at pH 4.5 for *B. cereus* which decreased with increasing pH. While Sabu et al. (2006) produced the highest tannase production from *Lactobacillus* sp. ASR-S1 at pH 4.8 in SSF. Various investigations on tannase production reported pH of 5.0 (Selvaraj and Vytal 2017). pH may affect the active sites and amino acids of enzymes (Sabu et al. 2006). As enzymes are proteins, ionic nature of carboxylic and amino group attached with surface of protein could be affected with pH change which would further influence the enzymatic catalytic activity. Very low or higher pH could significantly affect the process of fermentation because of enzyme inactivation (Jana et al. 2013).

An appropriate size of inoculum is essential for metabolite production in SSF (Rodrigues et al. 2008; Pandey et al. 1999). Results of this report indicated the highest tannase production when 1% inoculum size of *B. cereus* was used while the synthesis decreased significantly with higher sizes (2% and 3%). Similar to our investigation, Beniwal et al. (2010) documented the higher tannase production when 1% *Enterobacter cloacae* MTCC 9125 inoculum size was used in SSF and enzyme value was observed to decrease with higher size. While inoculums of 1% and 2% also have been reported for *Lactobacillus* sp. ASR-S1 tannase production at optimum (Sabu et al. 2006) and *Aureobasidium pullulans* DBS66 respectively (Banerjee et al. 2007). Inoculum size actually controls and reduces the lag phase, greater inoculum size enhances the moisture which leads in the decrease of growth and synthesis of enzyme (Sharma et al. 1995). But after a limit, the inoculum size would cause lower enzyme synthesis, that could

be due to increased biomass and exhaustion of nutrients (Ramirez-Coronel et al. 2003; Sabu et al. 2006). Among different incubation periods (24–96 h), maximum enzyme was produced after 24 h incubation while tannase synthesis decreased with further passage of incubation time. While no enzyme production was detected at 96 h incubation. Similar to our study, Ayed and Hamdi (2002) reported 24 h as optimum incubation time for tannase synthesis from *Lactobacillus plantarum* in SSF which declined at higher periods. Sabu et al. (2006) also reported similar results for tannase synthesis. The highest tannase production from *Klebsiella pneumoniae* MTCC 7162 was reported between 20–30 h by Sivashanmugam and Jayaraman (2011). Lower production after applying prolonged incubation could result due to the nutrients exhaustion in the medium with passage of time, leading to decline in enzyme synthesis. Decreased enzyme synthesis may also be because of enzyme inhibition and denaturation with prolonged incubation (Gautam et al. 2002). However, Sabu et al. (2005) reported optimal tannase synthesis after 120 h.

Results supported that agitation at 150 rpm had positive effect on enzyme production compared to static state. Optimum agitation speed for tannase production generally lies between 100–200 rpm (Jana et al. 2014). Belur et al. (2010a, b) reported tannase synthesis from *Serratia ficaria* with maximum tannase at 150 rpm agitation. Govindarajan et al. (2019) found the higher tannase yield on 100 rpm from *Enterobacter cloacae* strain 41 while Kumar et al. (2015) found it to be 103.34 rpm. Natarajan and Rajendran (2009) observed optimum tannase at 120 rpm agitation from *Lactobacillus plantarum*. Selwal et al. (2010) observed maximum tannase yield from *Pseudomonas aeruginosa* at 200 rpm agitation. Agitation is a significant factor for mass transfer, proper medium mixing and dissolving of oxygen and heat (Darah et al. 2011). The agitation requirement also depends on the nature of cultured microbe (Jana et al. 2014). In contrast to our finding, for *Serratia marcesans* static condition was more suitable for tannase production (Sheela et al. 2016). The highest tannase productivity was achieved with large sized particles whereas minimum enzyme was obtained with medium sized particles of substrate. Yee et al. (2011) also reported the highest tannase synthesis with larger particle size of substrate in SSF. However, Madeira Jr et al. (2015) reported the optimal tannase synthesis with small sized particles. Although smaller particles have lower surface area for more microbial interaction leading to bioconversion (Kar et al. 1999) but very small particles may cause agglomeration of substrate and result in the decrease of surface for reaction which could reduce the enzyme synthesis (Krishna 2005). Larger particles have better aeration and respiration capacity with better reaction (John et al. 2006). In this study, the enhanced tannase synthesis was obtained when crude tannase was separated by filtration as compared to the centrifugation. Sabu et al. (2006) separated the *Lactobacillus* sp. ASR-S1 crude tannase by filtering using Whatman No.1 filter paper under SSF. While in many reports tannase produced during microbial source is separated through centrifugation during SSF (Madeira et al. 2015; Subbalaxmi and Murty 2016).

Tannase enzyme require metallic ions for catalytic activity expression properly (Selwal et al. 2010). In this study, among all applied salts in the medium, NaCl enhanced the tannase production most. Various investigations have established that different salts affect differently tannase from various microbial sources. Similar results were obtained by Sabu et al. (2006) for *Lactobacillus* sp. ASR-S1. Bhoite and

Murthy (2015) reported inorganic salts NaCl and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ to act as enhancer for tannase synthesis in SSF. For tannase synthesis from *Bacillus gottheilii* M2S2, KH_2PO_4 , MgSO_4 , NaCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ had positive effect in an investigation by Selvaraj and Vytla (2017). Tannic acid is an additional source of carbon (Jana et al. 2014). Our results found the 1.5% tannic acid optimum for tannase production from *B. cereus* in SSF which tend to decrease which increasing tannic acid concentration. In literature its concentration value ranges from 1–12% (Seth and Chand 2000; Banerjee et al. 2007; Rodrigues et al. 2008; Madeira Jr. et al. 2011). For *Lactobacillus* sp. ASR-S1, 0.6% tannic acid with substrate yielded the highest tannase (Sabu et al. 2006). Zou et al. (2015) and Rodrigues et al. (2008) reported 2% and 2.5% tannic acid respectively as optimum for tannase productivity in SSF. While Selvaraj and Vytla (2017) obtained tannic acid (4%) as optimum for the synthesis of tannase. As tannase is referred as inducible enzyme that require tannin/tannic acid for its synthesis from microorganisms (Mansor et al. 2019). But at higher concentration, the decreased tannase synthesis could be due to the toxicity of tannic acid for microorganism and negative effect on their growth (Jana et al. 2012). Apart from carbon, source of nitrogen is also significant in the medium for microbial growth and enzyme production (Chandrasekaran 1991). Among nitrogen sources used, the results indicated the malt extract as optimal for enhanced enzyme production while yeast extract had the least impact on enzyme synthesis. Similarly, Lata and Rani (2016) reported established the malt extract as most suitable organic nitrogen source for microbial growth and tannase production. Lal and Gardner (2012) reported the peptone with maximum tannase productivity among all used organic nitrogen sources. Kumar et al. (2007) reported the decrease in tannase production by use of complex nitrogen source, yeast extract. Yeast extract as organic nitrogen source is rarely chosen in traditional SSF due to its very slight effect on the synthesis of enzyme as reported by Wu et al. (2018).

Under CCD of RSM, optimal tannase production from *B. subtilis* using corn leaves was achieved with 1.75% tannic acid, 0.75% NaCl and 1.25% malt extract in medium composition. Lekshmi et al. (2020) with pomegranate peel as substrate, obtained the highest tannase by *B. velezensis* TA3 using tannic acid (0.68%) in medium component optimized by CCD under SSF. Selvaraj and Vytla (2018) determined 1% tannic acid as medium component using CCD for optimal tannase synthesis by *B. gottheilii* M2S2. RSM optimized components of 7.49% tannin, 8.11% glucose, 2.25% yeast extract and 9.26% $(\text{NH}_4)_2\text{SO}_4$ were obtained by Wu et al. (2018) for highest tannase productivity from *Aspergillus tubingensis* CICC 2651 using tea stalk under SSF. So, the requirement of medium composition and their concentrations could vary depending on microorganism or substrate used.

Tannase was characterized by assessing the impact of physical factors such as pH, temperature, incubation period and concentration of substrate on enzyme activity. pH influences the enzyme reaction by affecting the ionization state of enzyme amino acids (Jana et al. 2014). To determine the optimal pH, different pH values (4–11) were applied where optimal enzyme activity was obtained with pH 5 and activity was observed to reduce with increasing pH. Tannase is considered as acidic protein which usually has enhanced activity in acidic range (Jana et al. 2014). Similarly, Rodríguez et al. (2008) reported optimum pH as 5 for tannase activity produced by *Lactobacillus plantarum* CECT 748^T. whereas

tannase activity pH optima for *Bacillus cereus* KBR9 and *Bacillus licheniformis* KBR 6 was 4.5 and pH 5.75 respectively (Mondal and Pati 2000; Rodríguez et al. 2008). Several other investigators have reported the optimal pH from 4 to 5 (Gayen and Ghosh 2013; Bagga et al. 2015; Kumar et al. 2015; Farag et al. 2018). However, some reports also indicated the activity in alkaline range (Iwamoto et al. 2008). Microbial tannases generally show the optimal activity within the temperature range of 20–60°C (Yao et al. 2014). In this investigation, tannase activity at different temperatures (20–90°C) indicated that as temperature elevated, the activity was observed to increase which became maximum at 50°C. However, beyond this level the activity decreased at higher temperature. In agreement to our investigation, Lima et al. (2014) recorded the highest tannase activity at 50°C for *Penicillium montanens*. Jana et al. (2013) observed *Bacillus subtilis* tannase activity optimum at 40°C while Gayen and Ghosh (2013) reported optima at 35–40°C. However, Madeira Jr. et al. (2015) reported 70°C for optimal tannase activity in SSF. The enzyme kinetic energy increases with the increase of temperature which aids the enzymatic reaction. However, after a point the potential energy becomes very high and breaks the weak bonds in 3D structure which as the result causes the inactivation and denaturation of enzyme or structure of the substrate (Mukherjee and Banerjee 2006). Maximum tannase activity was shown after 45 min of incubations and further rise in time duration caused the decreased enzyme activity. The decreased activity with prolonged period could be the result of denaturation of enzyme with time (Gautam et al. 2002). Optimum tannic acid concentration (%) was recorded at 0.35% while enzyme activity decreased with increasing concentration that could be due to saturation of all active sites by substrate.

5 Conclusion

Utilization of tannin at industrial level is limited due to its higher production costs. During recent past, several investigations have focused on different approaches to minimize the production costs as well as screening of potential tannase producer microbial strains. The current study reported the low-cost production and statistical optimization of tannase enzyme through the biodegradation of agricultural residues, corn (*Zea mays*) leaves by *B. cereus*. The obtained results indicated that *Zea mays* leaves held the potential to be used as substrate for tannase production in environmental and economical friendly manner. However, levels of tannin vary in different plants according to agro-climatic conditions, therefore, more agricultural wastes from various plants are required to evaluate for their commercial utilization.

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

All authors agreed for publication of this article

Availability of data and materials

The sequence of the strain is already available on NCBI

Competing interests

No competing interest exist

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Authors' contributions

HAS, JIQ supervised the project, IJ, MAY conducted experiments and wrote first draft, SA, MK, CA helped in literature review and data interpretation, MI analyze the data and critically reviewed the final draft.

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Figures

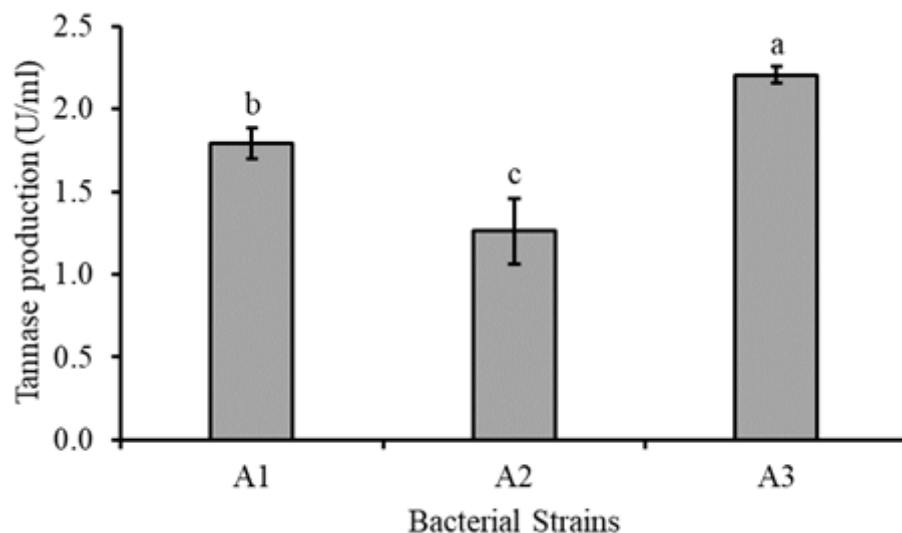


Figure 1

Tannase production of bacterial strains as the result of tannase assay. Different alphabets on bars show them significantly different.

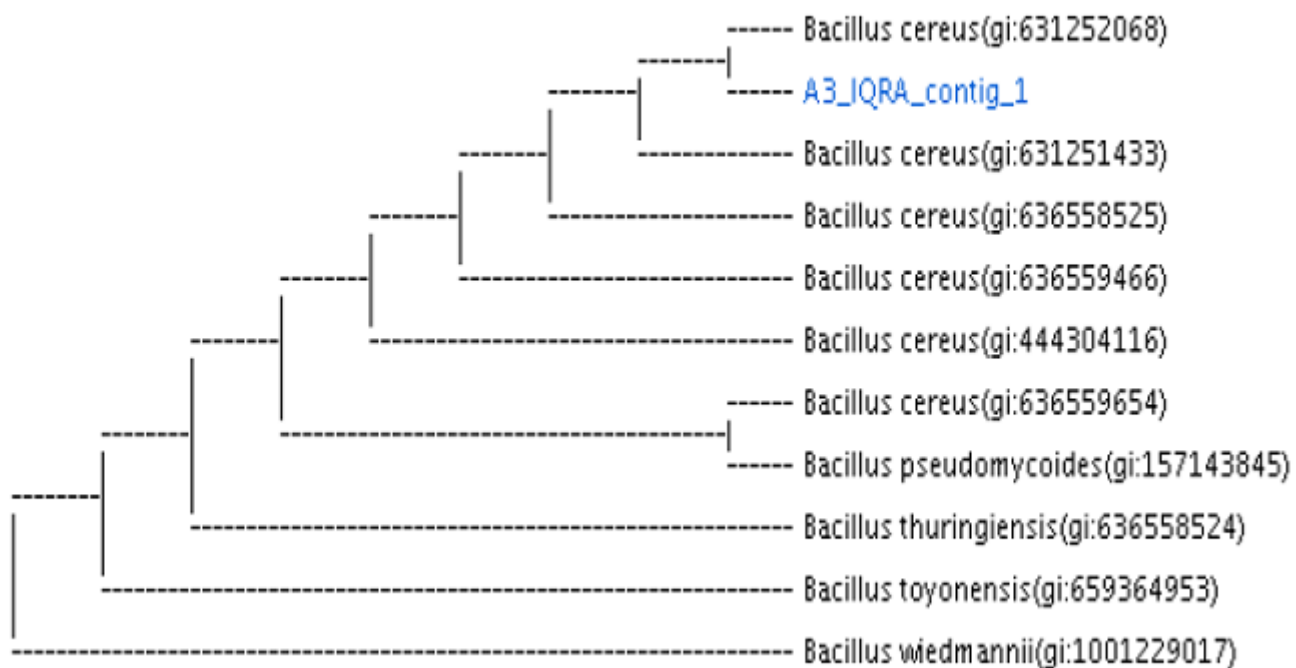


Figure 2

Phylogenetic (Neighbour-joining tree) analysis based on 16S rDNA nucleotide sequence indicating the A3 strain as *Bacillus cereus*.

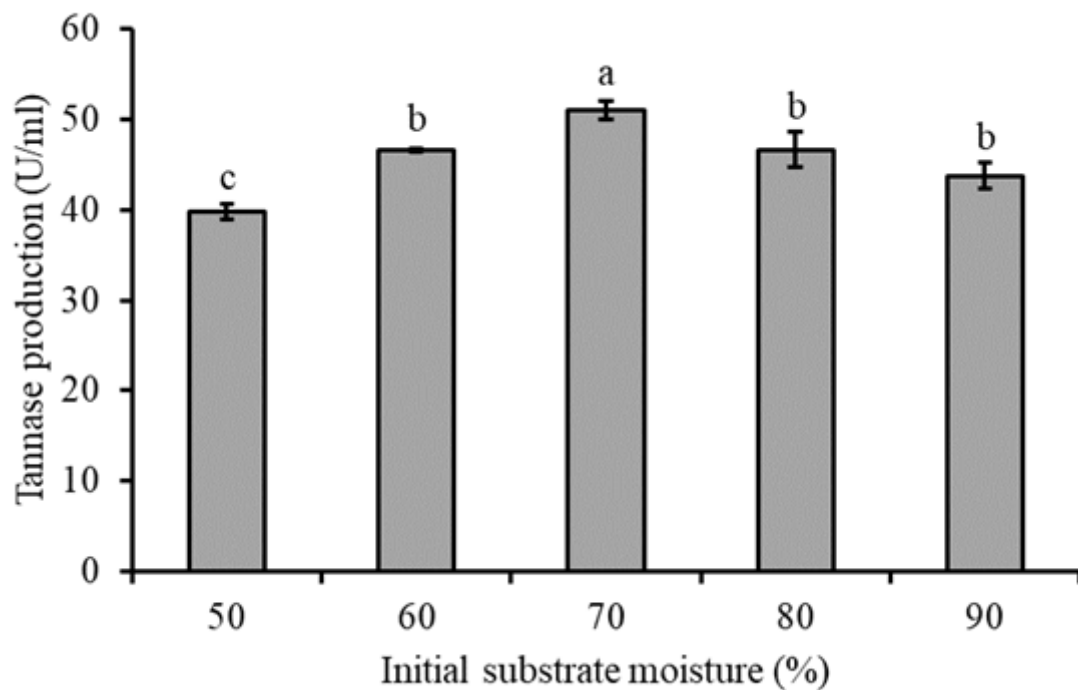


Figure 3

Effect of initial substrate moisture (%) on tannase production from *B. cereus*. Different alphabets on bars show them significantly different.

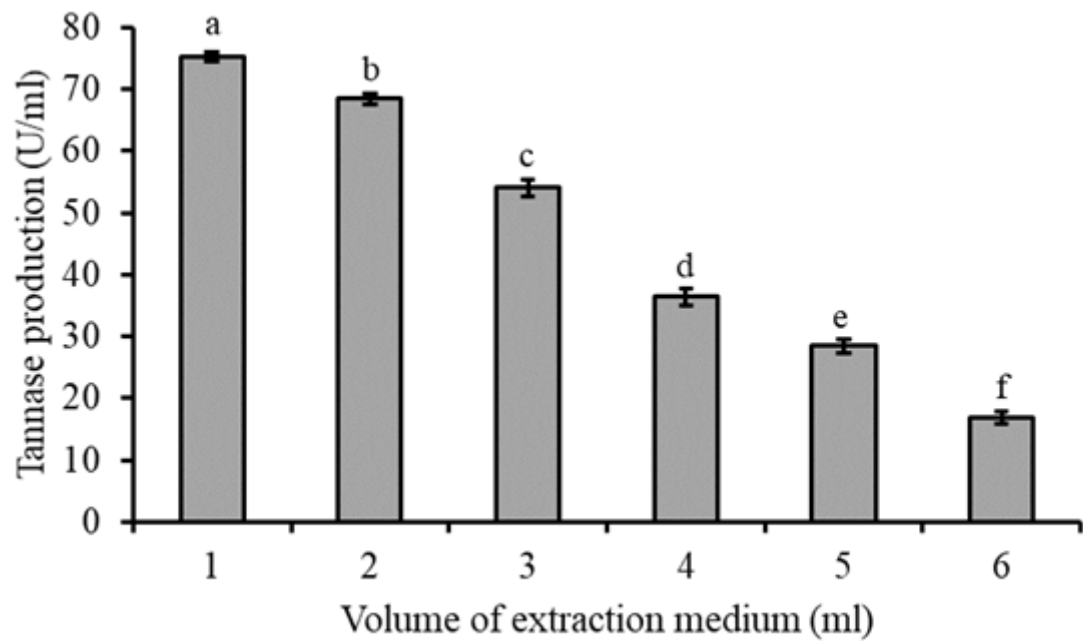


Figure 5

Effect of volume of optimum extraction medium on tannase production from *B. cereus*. Different alphabets on bars show them significantly different.

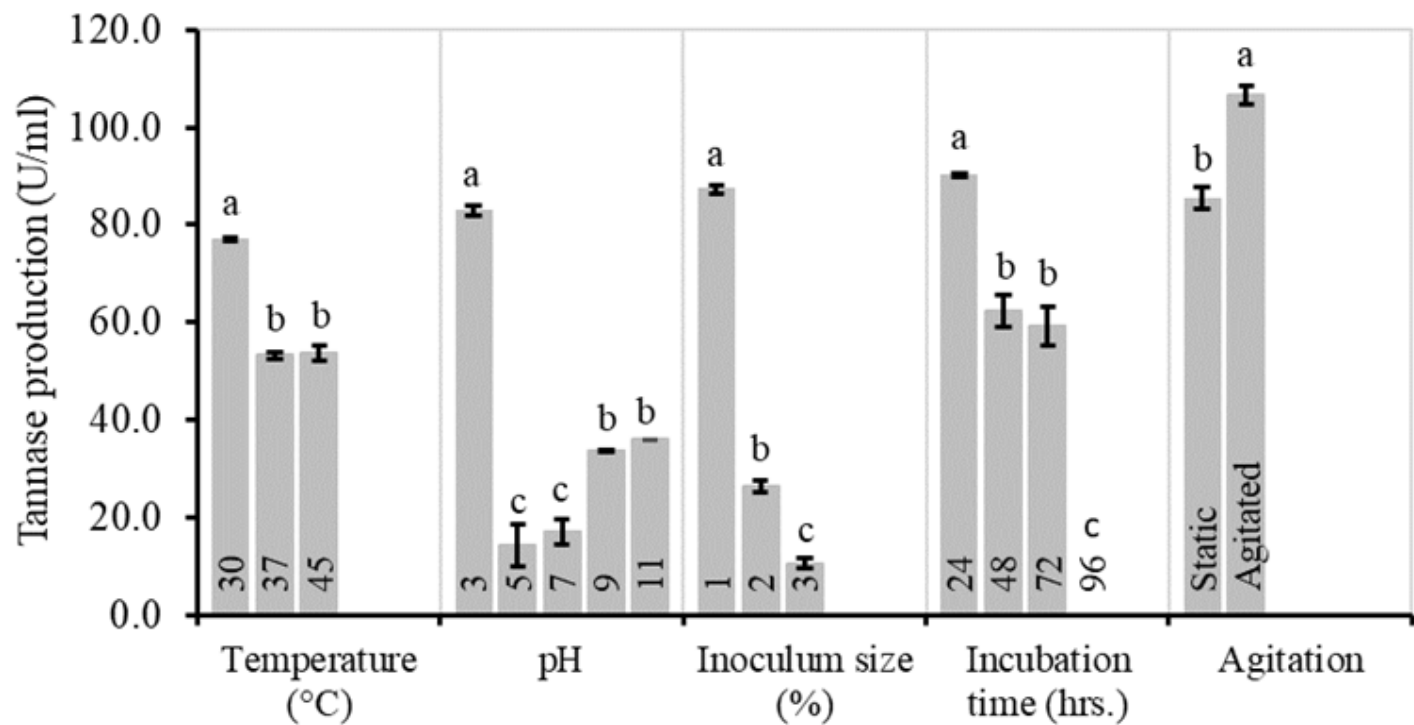


Figure 6

Effect of different parameter on tannase production from *B. cereus*. Bars not sharing same alphabet are different significantly.

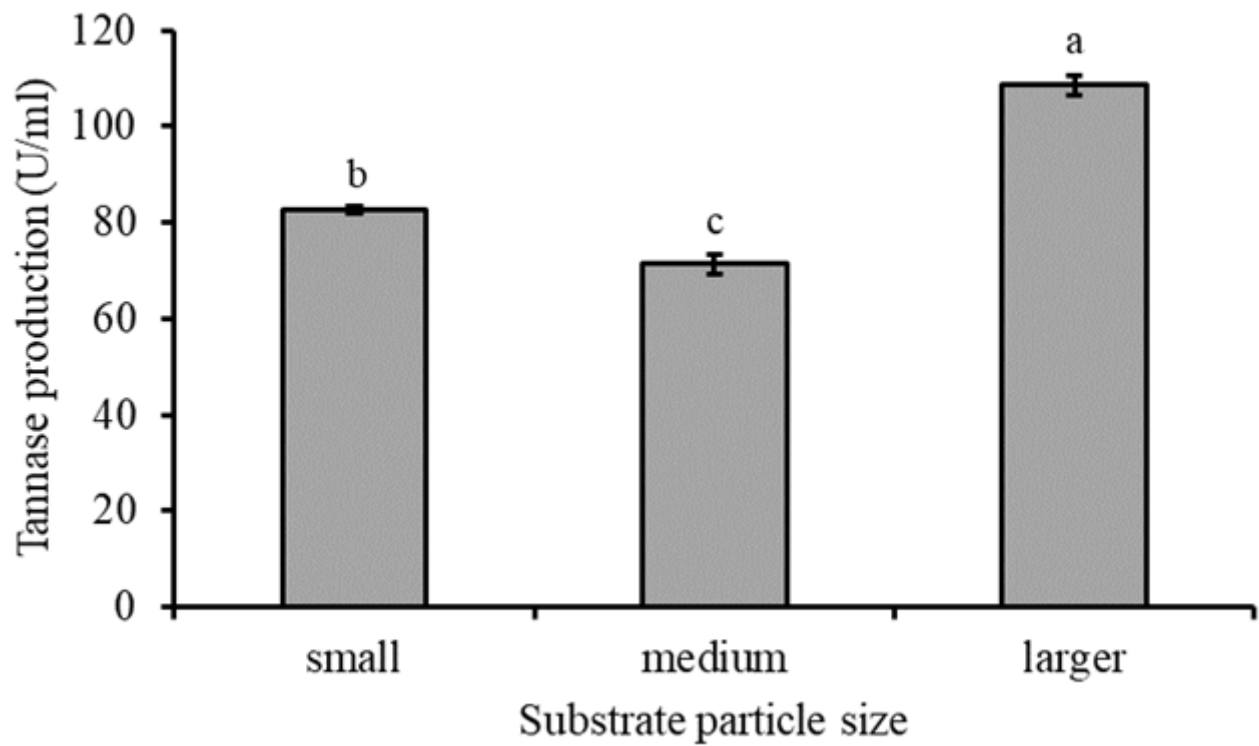


Figure 7

Effect of substrate particle size on tannase production from *B. cereus*. Different alphabets on bars show them significantly different.

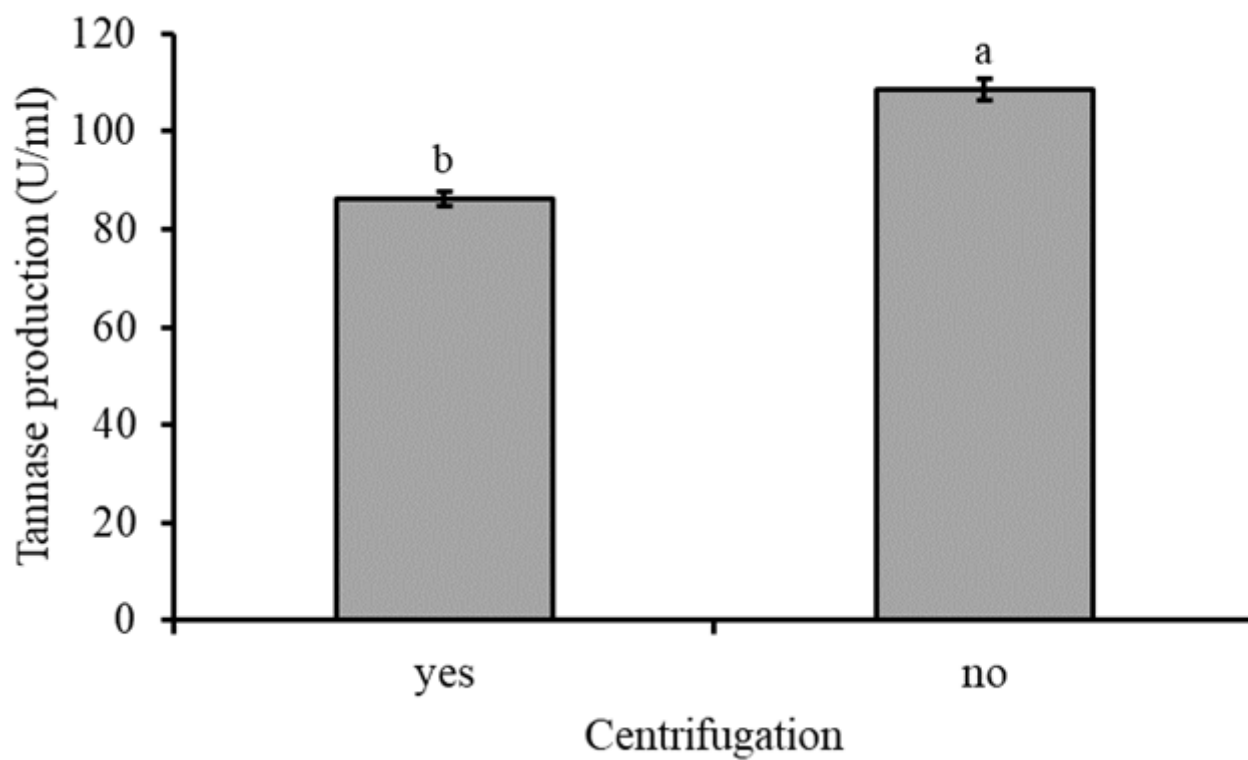


Figure 8

Effect of centrifugation on tannase production from *B. cereus*. Different alphabets on bars show them significantly different.

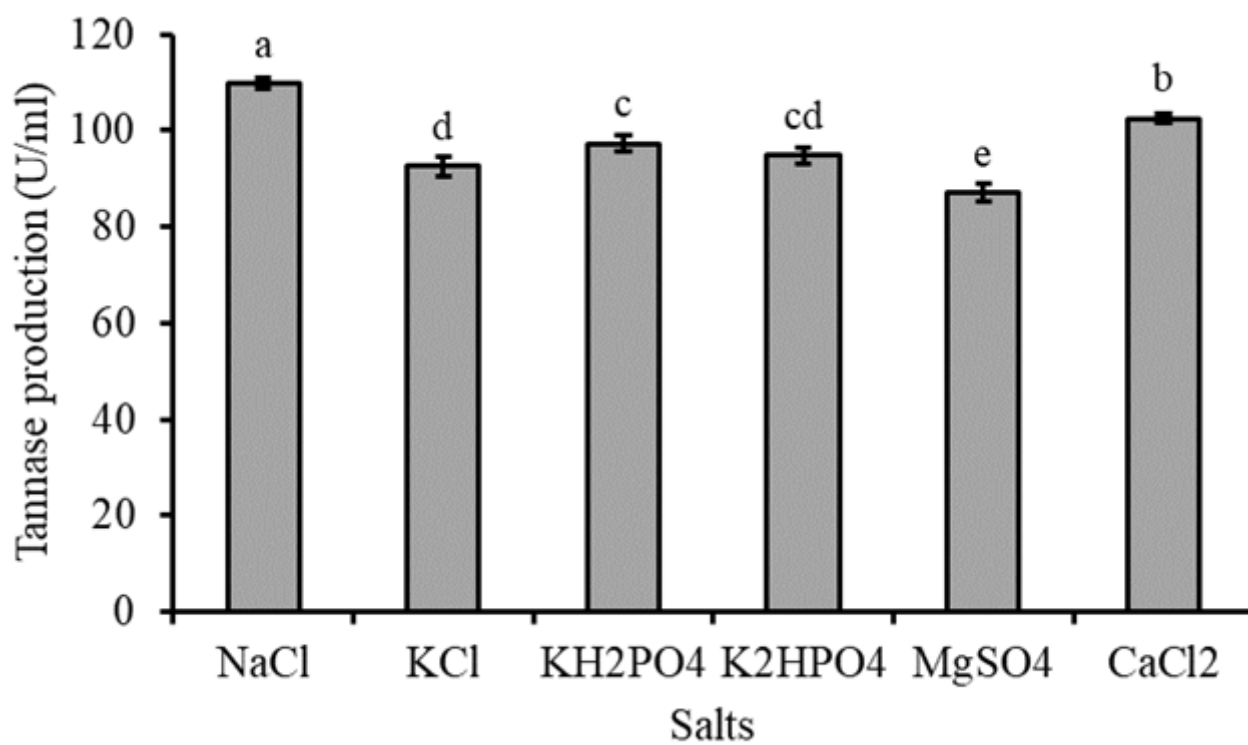


Figure 9

Effect of different salts on tannase production from *B. cereus*. Bars with different not sharing common alphabets are significantly different.

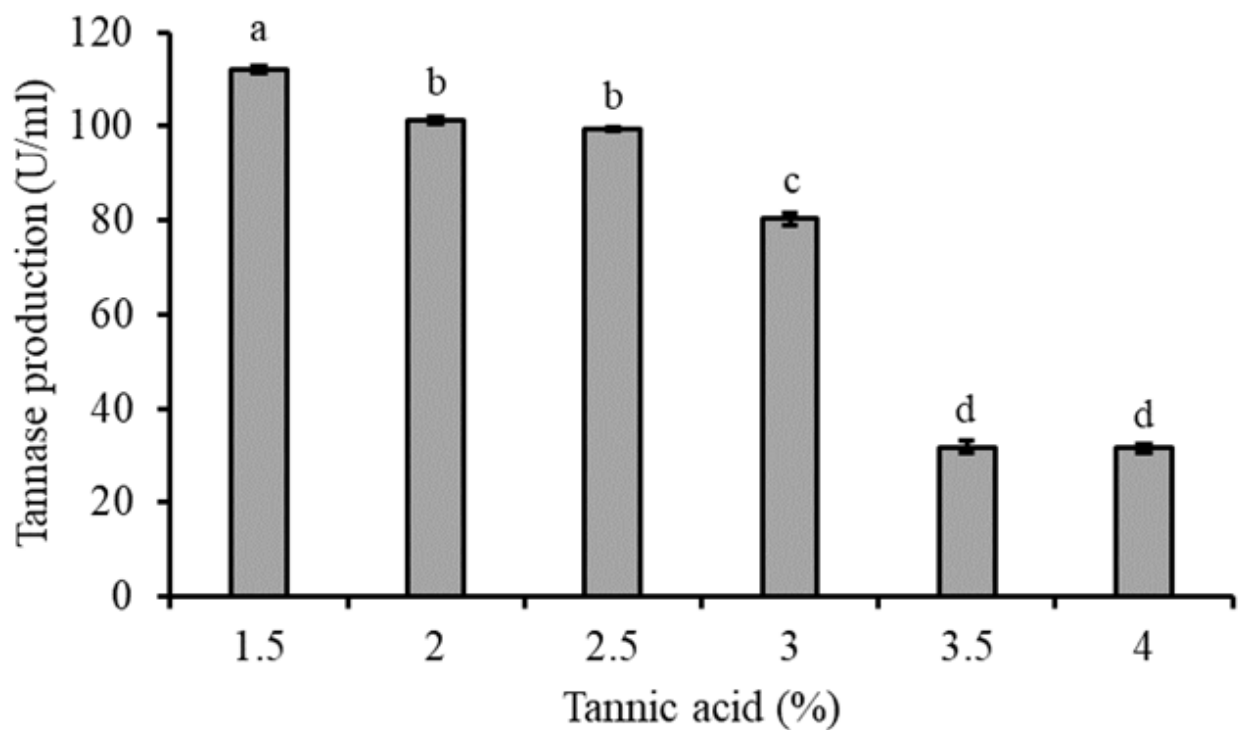


Figure 10

Effect of tannic acid concentrations (%) on tannase production from *B. cereus*. Different alphabets on bars show them significantly different.

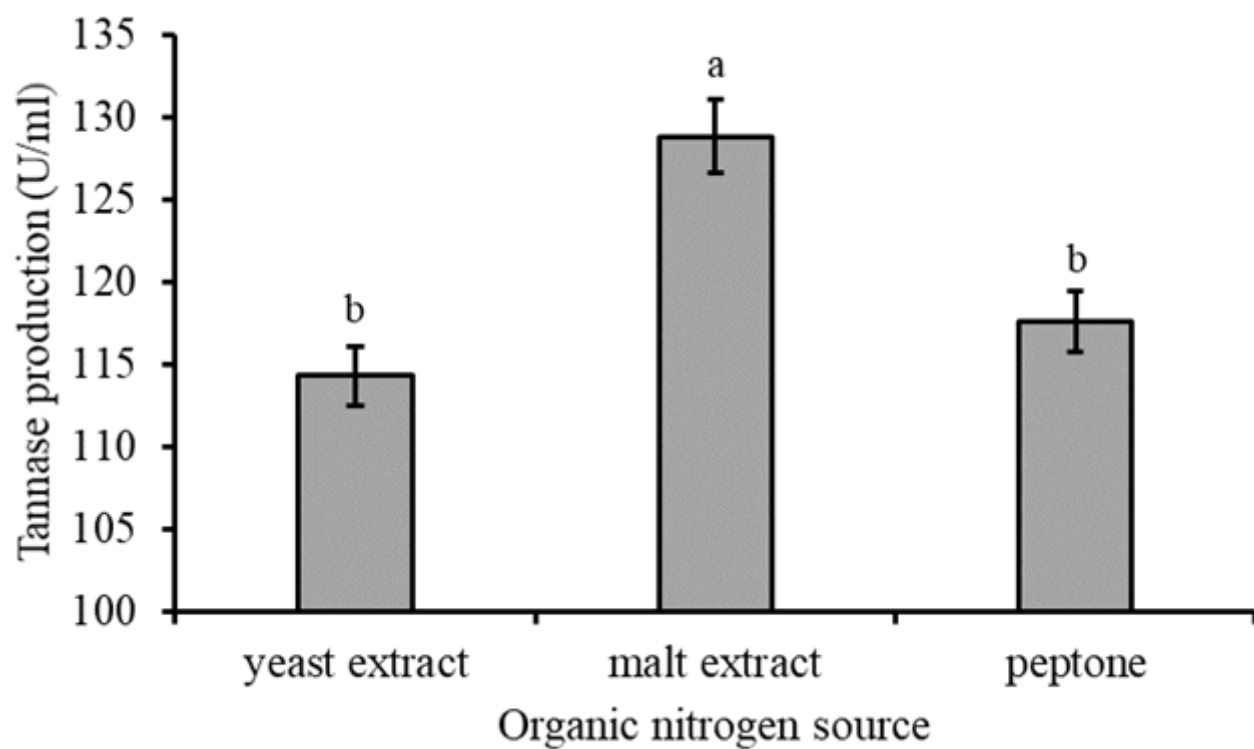


Figure 11

Effect of organic nitrogen sources on tannase production from *B. cereus* Different alphabets on bars show them significantly different.

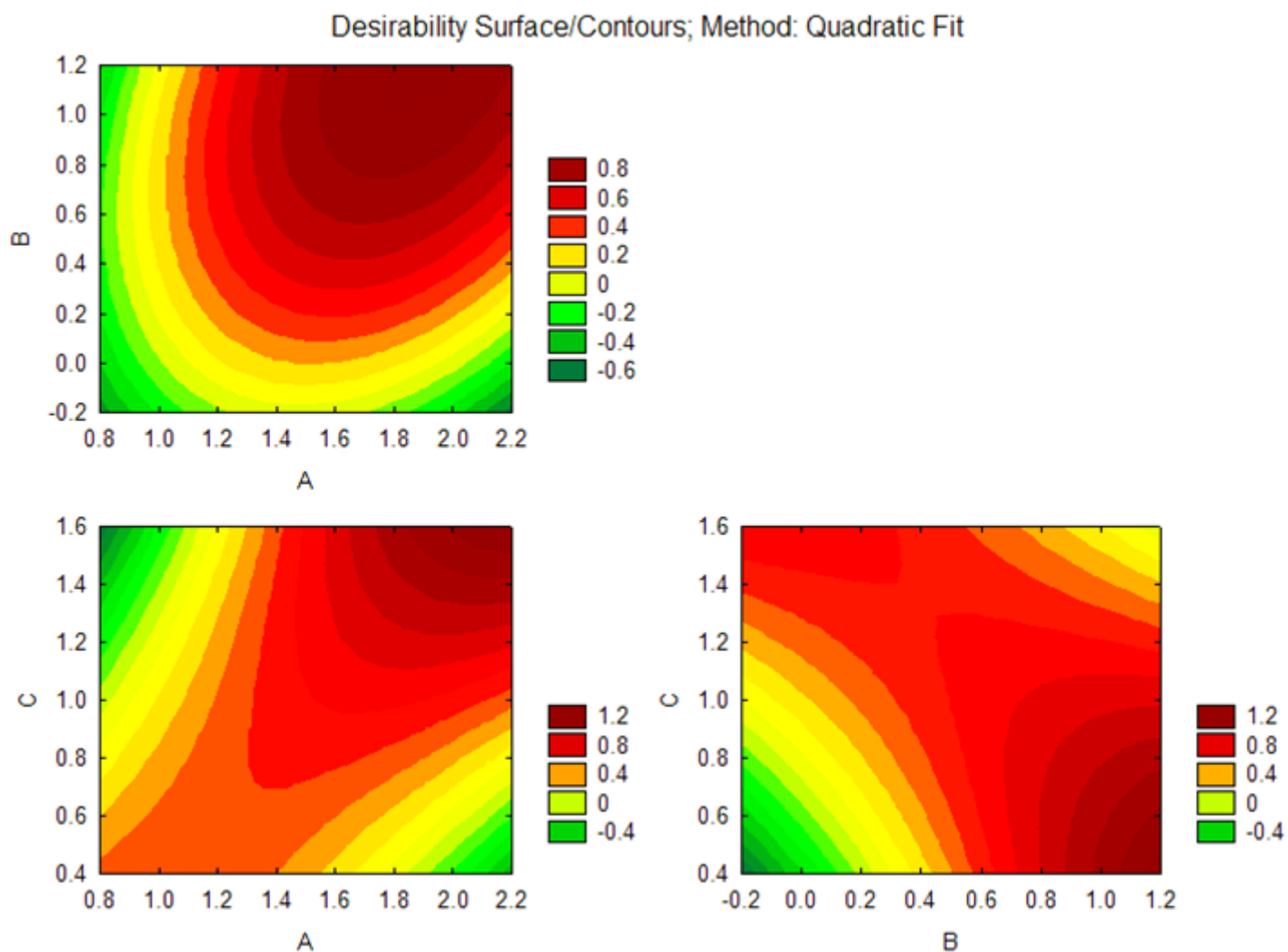


Figure 12

Contour plots displaying interaction of medium components, A: tannic acid (%), B: NaCl (%) and C: malt extract (%) during CCD for optimized tannase synthesis under SSF.

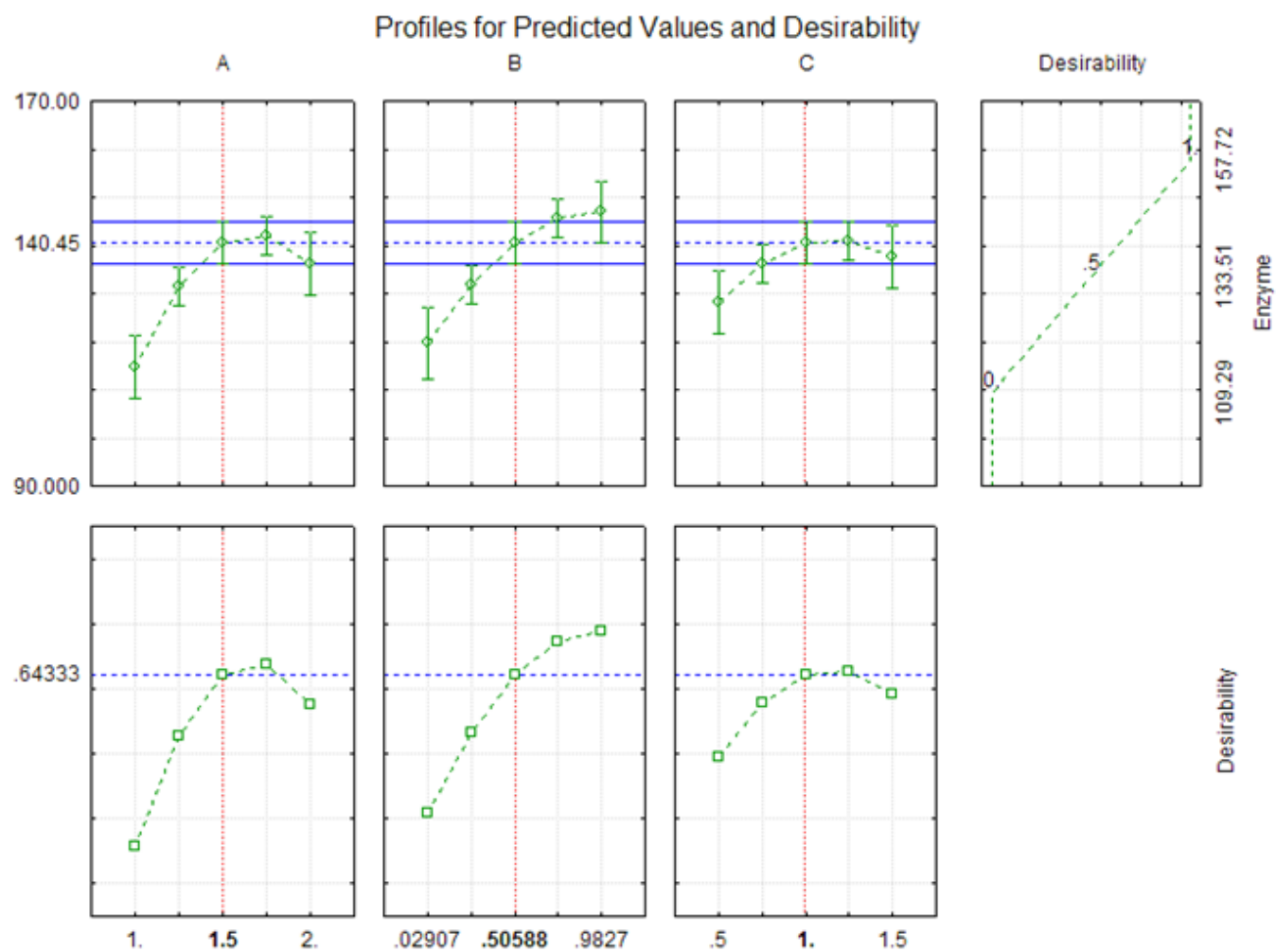


Figure 13

Desirability chart for medium components, A: tannic acid (%), B: NaCl (%) and C: malt extract (%) during CCD for optimized tannase synthesis under SSF.

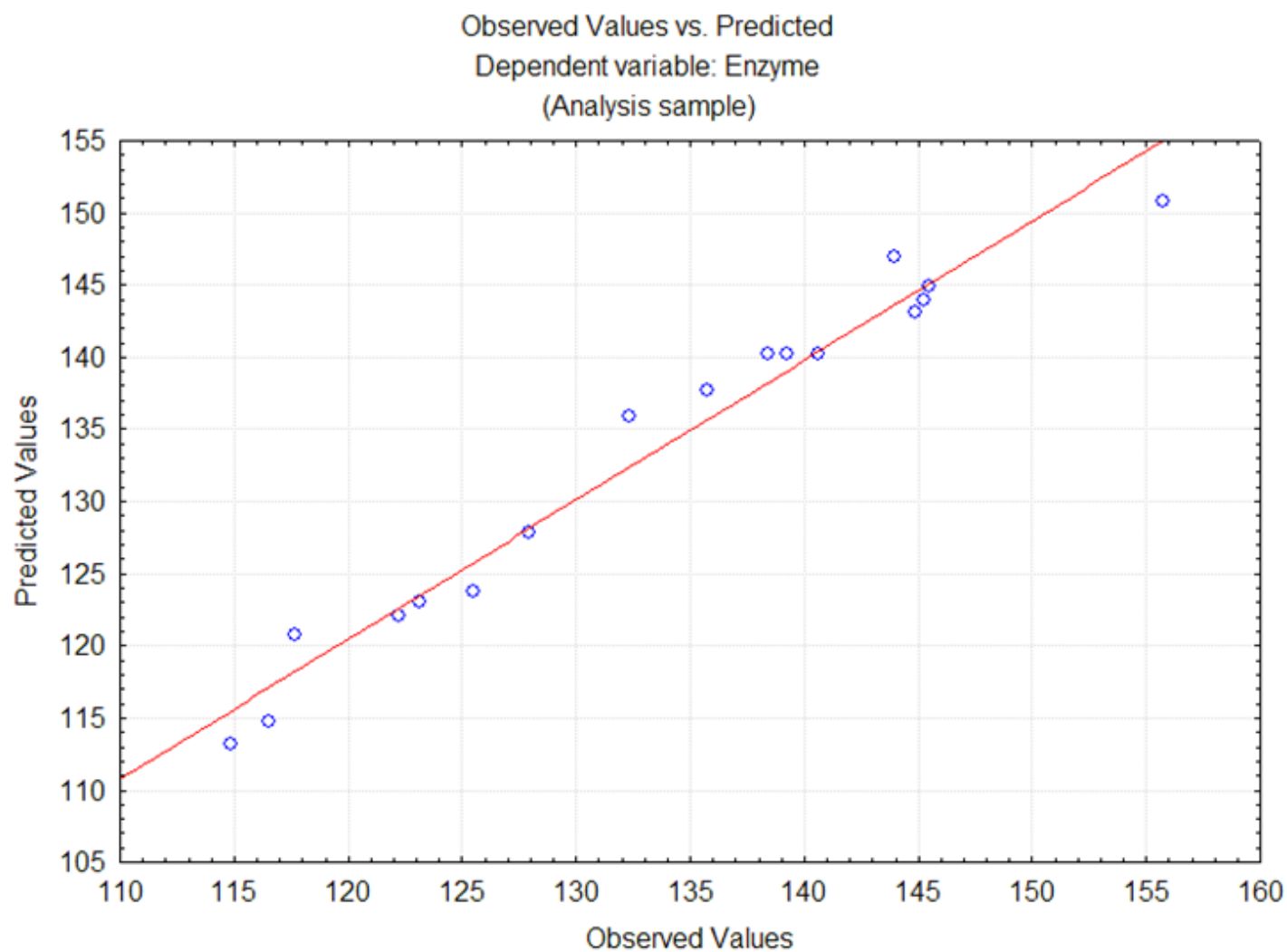


Figure 14

Residual plot representing the relationship between observed and predicted values obtained from CCD for medium optimization.

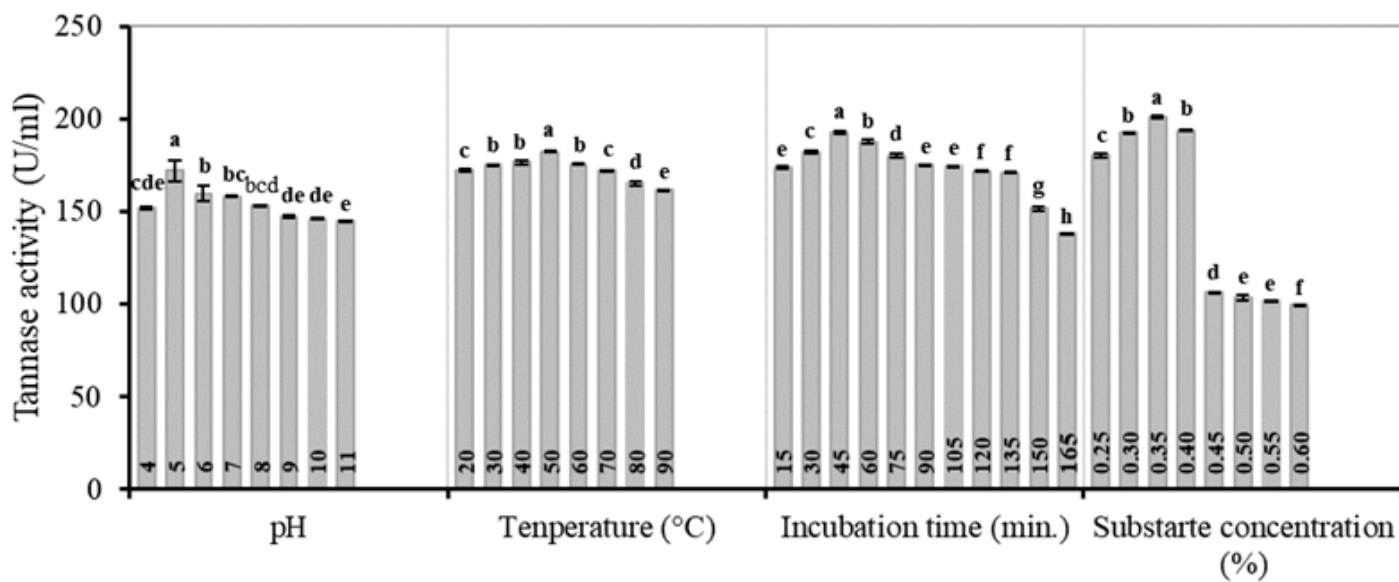


Figure 15

Effect of several parameters on the activity of tannase synthesized by *B. cereus*. Bars not sharing same alphabet are different significantly.