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# Tannase Production from *Bacillus amyloliquefaciens* in Submerged Fermentation Through Response Surface Methodology

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**Abstract.** Tannase (Tannin acyl hydrolase) is an intracellular/extracellular enzyme produced by micro-organisms. Tannase has high market demand due to its important role in different industries. The optimization of different parameters for each micro-organism is necessary for obtaining maximum tannase yield. The aim of present study was to optimize the medium components and their concentration employing applications of response surface methodology (RSM). Ten bacterial strains isolated from fish gut content were screened for tannase producing potential. Among these, four strains, *Klebsiella oxytoca*, *Roultella ornithinolytica*, *Bacillus amyloliquefaciens* and *Enterobacter aerogenes* expressed greenish zones around their colonies. *B. amyloliquefaciens* showed highest tannase production (1.27 IU/mL) under un-optimized conditions and was selected for further work. During one factor at a time optimization of physical parameters, incubation temperature 37 °C, pH 5, inoculum size 1% and incubation period of 24 h yielded maximum tannase. To screen the significant medium components, 12 experimental runs of Plackett-Burman design for six variables (tannic acid, $K_2HPO_4$, $CaCl_2$, $MgSO_4$, $NH_4NO_3$ and yeast extract) were carried out. From these experimental runs, the enzyme assay results were analyzed using multiple regression. Three variables *i.e.,* tannic acid, $CaCl_2$ and yeast extract showed significant impact on tannase production. Concentrations of these variables were optimized using Box–Behnken design (BBD). Results of 15 experimental runs of BBD showed maximum tannase production corresponding to 0.5% tannic acid, 0.1% $CaCl_2$ and 0.275% yeast extract. The highest tannase activity was recorded at pH 7, 0.5% substrate concentration and 40 °C.

**Keywords:** tannase, *Bacillus* sp., submerged fermentation, response surface methodology

## Introduction

Tannins (tannic acid) are the polyphenolic compounds of different molecular mass, found in a variety of plants as secondary metabolic products. These compounds have capability to combine with different digestive enzymes such as β-galactosidase, protease, lipase, amylase, cellulose, some how with macromolecules like cellulose, starch, pectin and different minerals. Because of this, tannic acid is considered as anti-nutritional factor for plant consuming animals (Aguilar-zãrate *et al*., 2014; Sharma and John, 2011; Aguilar *et al*., 2001; Bhat *et al*.,1998; Lekha and Lonsane, 1997).

Tannin acyl hydrolase (EC 3.1.1.20) also called as tannase is an intracellular/extracellular, adaptive and inducible enzyme (Banerjee and Mahapatra, 2012; Aguilar *et al*., 2007) which is produced by variety of micro-organism (Selvaraj *et al*., 2019; Aguilar *et al*., 2001). This enzyme actually reduces the concentration of hydrolysable tannins (tannic acid) by hydrolyzing their esters and depside bonds to yield gallic acid, galloyl esters and glucose (Jana *et al*., 2014; Mehta *et al*., 2013). Tannase is a precious enzyme which is extensively used in multiple fields. Ability to remove tannin and producing gallic acid make this enzyme very important from the industrial point of view having applications in paints, ink, photography,brewing, beverage, health care, pharmaceutical and food industries including instantaneous tea, soft drinks having coffee flavour, wine and beer production, and decolouration of fruit juices (Selwal *et al*., 2011; Aguilar *et al*., 2007; Mohapatra *et al*., 2007; Mukherjee and Banerjee, 2004). Tannase is also used to improve animal feed as this enzyme hydrolyzes the anti nutritional compounds (Graminha *et al*., 2008).

Optimization to enhance the production of this valuable enzyme depends on the components of medium such as nitrogen, carbon, agitation, incubation time, pH and temperature. To analyze the interactive effects of the

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different factors, optimization of the medium by one factor at a time (OFAT) is known to be a potential approach (Khusro, 2016). But there is a major drawback of this technical approach that it does not consider interactive effects among the variables, consequently OFAT is not able to depict complete effects of different parameters on the process under consideration (Baåÿ and Boyacäi, 2007). Answer of the problem is in response surface methodology (RSM) which could be utilized to discover interactive effects of different variables simultaneously (Wang *et al*., 2008). RSM is statistically and mathematically approach, used for model designing, formulation, estimation of effects of multiple parameters as well as for determination of optimal culture conditions to make the responses better and minimizing the number of experiments required (De-coninck *et al*., 2000). Therefore, for optimization of enhanced enzyme production by micro-organisms, RSM is definitely a preferable technical strategy to adopt (Wang *et al*., 2008; Balusu *et al*., 2005).

Second order approach such as Box-Behnken, Central composite, Doehlert and Plackett-Burman designs are extensively used in RSM as they can deal with a large variety of functional forms and this plasticity allows them to more closely approximate the true response surface (Xiao *et al*., 2007). The following investigation was planned for the screening of potential tannase producing bacteria isolated from gastrointestinal tract of fish with selection of medium components and their optimization to harvest higher production of tannase using response surface methodology (RSM). One factor at a time (OFAT) approach was employed for the optimization of physical parameters as well as enzyme activity.

## Material and Methods

**Screening of tannase producing bacteria.** Ten bacterial strains *Bacillus pumilus, Bacillus amyloliquefaciens, Enterobacter aerogenes, Bacillus flexus, Roultella ornithinolytica, Aeromonas hydrophila, A. bestiarum, Klebsiella oxytoca, A. allosaccharophila, A. media*, previously isolated from the gut of freshwater fish, *Catla catla* (thaila) were obtained to evaluate their tannase producing potential. Nutrient agar medium plates containing 0.5% tannic acid and pH 5 were used for qualitative estimation of tannase producing potential of different strains as described by Osawa and Walsh (1993). These plates were then incubated at temperature of 37 °C for 96 h after shifting the inoculum (10 µL) on the plate in the form of a dot. A greenish zone was formed around the colonies of tannase producing bacteria showing positive results for tannase production. The quantitative estimation (enzyme assay) was carried out on corresponding bacteria which showed positive results.

**Tannase production.** Tannase production was carried out by using production media of Kumar *et al*. (2015) with minor modification. The fermentation media comprised of tannic acid (0.5%), $MgSO_4$ (0.2%), $CaCl_2$ (0.1%), $K_2HPO_4$ (0.05%) and yeast extract (0.5%) was taken in 250 mL Erlenmeyer flask, pH was adjusted to 5 and then medium was sterilized by autoclaving at 121 °C and 15 Psi pressure for 15-20 min. Then each strain was inoculated with 1% inoculum under aseptic conditions in each flask and incubated for 24 h at 37 °C. After incubation, culture was centrifuged at 8000 rpm at 4 °C for 15 min. and then supernatant was secured as crude enzyme.

**Tannase enzyme assay.** Miller methods (Miller, 1959) was adopted for tannase enzyme assay. Crude enzyme (1m L) was added in 1mL of substrate (0.5% tannic acid in acetate buffer), followed by incubation at 37 °C for 30 min and then kept in the boiling water bath for 15 min for the de-activation of enzyme substrate activity. One milliliter solution from this enzyme substrate system was taken in a test tube and then 3 mL of DNS (dinitro salicylic acid) was added in it. The test tube was placed in water bath for 10 min. Solution in the test tube was then diluted with distilled water up to 10 mL volume. Optical density of the solution was recorded by the spectrophotometer at 540 nm against blank (having distilled water instead of crude enzyme, rest components are same). All tests were carried out in triplicates. Among four bacterial isolates, the strain (*Bacillus amyloliquefaciens*) showed highest enzyme was selected for further studies.

**Optimization of cultural parameters through one factor at a time approach.** Different cultural parameters such as incubation temperature (30 °C, 37 °C and 45 °C), initial medium pH (3, 5, 7 and 9), inoculum size (1%, 2% and 3%) and incubation time periods (24 h, 48 h, 72 h and 96 h) were optimized for maximal tannase production from *B. amyloliquefaciens* under submerged fermentation.

**Experimental design and statistical analysis.** To select the components of medium, Plackett-Burman design (PBD) was used. Six (6) medium ingredients with their minimum (negative) and maximum (positive) values

were selected as displayed in Table 1. Twelve (12) experiments were conducted to select the suitable medium ingredients having significant impact on higher tannase production. After PBD experiments, the ingredients having significant impact on tannase production was further optimized using Box-Bhenken Design (BBD). The three levels *i.e.*, +, 0 and – of the selected variables were mentioned in Table 2. The fifteen experiments of BBD were carried out for optimization of concentration of medium components. All the statistical modeling was done as described by Khalid *et al*. (2017).

**Effect of pH on tannase enzyme activity.** For optimization of pH for tannase, the crude enzyme was incubated with 0.5% substrate of various pH ranging from 4 to11 in appropriate buffer. Tannase activity was recorded using standard enzyme assay procedure as mentioned above.

**Effect of substrate concentration on tannase enzyme activity.** Optimization of substrate concentration was determined by incubating crude enzyme with various substrate concentrations (0.35%, 0.4%, 0.45%, ……., 0.65% tannic acid in buffer of optimized pH) and enzyme assay was performed as stated above.

**Effect of temperature on tannase enzyme activity** The crude enzyme with optimized substrate concentration and pH was incubated at different temperature (30 °C, 40 °C, 50 °C and 60 °C). Enzyme activity was then measured by standard tannase assay procedure as cited above.

**Statistical analysis.** All obtained data were analyzed statistically through Minitab v. 17.0, while statistica v. 99 was used for response surface modeling. All experiments were performed in triplicates and readings were displayed in the form of mean ± standard deviation.

## Results and Discussion

**Selection of bacteria.** Four strains out of ten which were isolated from the gut of fish captured from river Ravi, Pakistan showed greenish zones around their colonies and gave positive results for tannase production (Table 3). The strains having positive test for tannase production was further subjected to quantitative estimation and results revealed that *Bacillus amyloliquefaciens* showed highest enzyme production (1.269 U/mL) followed by *Enterobacter aerogenes, Roultella ornithinolytica* and *Klebsiella oxytoca* (Table 4) after 24 h of incubation at 37 °C. For the tannase production, first strain *Lactobacillus plantarum* was isolated from the waste of olive mill (Wilson *et al*., 2009; Ayed and Hamdi, 2002). Bacterial strain capable of degrading tannin was isolated from goat and sheep feces (Mosleh *et al*., 2014). Mohapatra *et al*. (2009) recorded the capacity of tannase enzyme production from *Bacillus licheniformis*as 0.356 U/mL. Beniwal *et al*. (2010) reported that *Enterobacter cloacae* was capable to produce 0.6 U/mL tannase on MSM medium. *Serratia ficaria* was reported to have tannase enzyme activity as 0.4 U/mL in MSM medium under submerged fermentation (Belur *et al*., 2010). Sivashanmugam and

**Table 1.** Nutrients used for PB design.

| Nutrient code | Nutrient | Low (-1) (g/100 mL) | High (+1) (g/100 mL) |
|---|---|---|---|
| $X_1$ | Tannic acid | 0.05 | 0.5 |
| $X_2$ | $K_2HPO_4$ | 0.05 | 0.1 |
| $X_3$ | $CaCl_2$ | 0.02 | 0.1 |
| $X_4$ | $MgSO_4$ | 0.02 | 0.1 |
| $X_5$ | $NH_4NO_3$ | 0.05 | 0.1 |
| $X_6$ | Yeast extract | 0.05 | 0.5 |

**Table 2.** Ranges of the independent variables (significant factors) used in BBD

| Variable | Levels | | |
|---|---|---|---|
| | -1 | 0 | + 1 |
| Tannic acid (%) | 0.05 | 0.275 | 0.5 |
| $CaCl_2$ (%) | 0.02 | 0.06 | 0.1 |
| Yeast extract (%) | 0.05 | 0.275 | 0.5 |

**Table 3.** Bacterial isolates results for plate test

| Strains | Codes | Results |
|---|---|---|
| *Bacillus pumilus* | 100 | _ |
| *Bacillus amyloliquefaciens* | 52 | + |
| *Enterobacter aerogenes* | 33 | + |
| *Bacillus flexus* | 74 | _ |
| *Roultella ornithinolytica* | 11 | + |
| *Aeromonas hydrophila* | 9 | _ |
| *Aeromonas bestiarum* | 32 | _ |
| *Klebsiellaoxytoca* | 20 | + |
| *Aeromonas allosaccharophila* | 19 | _ |
| *Aeromonas media* | 35 | _ |

+ = Zone producted; - = No zone produced

Jayaraman (2011) found to have 3.9U/mL tannase activity at 37 ºC after incubation of 28 h by *Klebsiella pneumoniae*.

**Optimization of temperature on tannase production.** In present study, after incubation of 24 h, the optimal enzyme synthesis (9.82±0.165 U/mL) was observed at 37 °C followed by 2.614±0.269 and 2.407±0.227 U/mL at 45 °C and 30 °C, respectively (Fig.1). For *Bacillus sphaericus* maximum enzyme (3.12 U/mL) was produced at 37 °C (Raghuwanshi *et al*., 2011). Similarly, the highest tannase enzyme synthesis (15.69 U/mL/min) for *Bacillus megaterium* was reported at 37 °C (Tripathi *et al*., 2016). Aftab *et al*., (2016), however reported the maximum production at 41 °C for tannase enzyme being produced by *Bacillus subtilis* in submerged fermentation.

**Optimization of pH on tannase production.** *B. amyloliquefaciens* showed the best enzyme production at pH 5 and enzyme units reached up to 9.828±0.016 U/mL (Fig. 1).Minimum value of tannase enzyme was recorded as 0.192±0.038 U/mL at pH of 7, while at pH 3 and pH 9, 0.423±0.182 and 0.453±0.041 enzyme units/mL were produced respectively. Our results were comparable with Aftab *et al.* (2016) as he also reported the highest tannase production at pH 5.0 using *Bacillus subtilis* in submerged fermentation. For *Bacillus sphaericus* maximum enzyme (3.12 IU/mL) was produced at pH 6 (Raghuwanshi *et al*., 2011). *Bacillus megaterium* was reported to have maximum tannase enzyme production (13.06 U/mL) at pH 6. This showed that slight acidic pH favoured enzyme production.

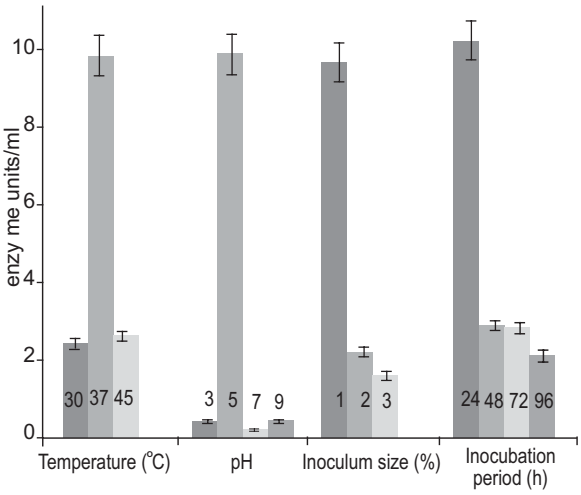


**Fig. 1.** Effect of temperature, pH, inoculum size and incubation period on tannase production.

**Optimization of inoculum size on tannase production.** Enhanced tanase production 9.639±0.282 U/mL was observed corresponding to 1% of inoculum size (Fig. 1). During present studies, best results were obtained with 1% inoculum (9.635 U/mL), while the tannase production was shown to be reduced with the increasing concentration of inoculum. Minimum enzyme production (1.609±0.183 U/mL) was recorded with highest inoculum size *i.e*., 3%, while for 2% inoculum size 2.221±0.340U/mL were produced. Increase in inoculum size (number of cells) results in the rapid biomass proliferation leading to higher enzyme synthesis. But beyond a limit, enzyme synthesis could decline due to increase in nutrient uptake competition in micro-organism and exhaustion of nutrients due to increased biomass (Kashyap *et al*., 2002), while in contrast, for *Bacillus megaterium* higher tannase activity was observed at 10% inoculum (Tripathi *et al*., 2016).

**Optimization of incubation time on tannase production.** In this study, *B. amyloliquefaciens* showed highest enzyme production (10.204 U/mL) when the incubation time was 24 h. Raghuwanshi *et al*. (2011) reported 36 h as optimum incubation time for *Bacillus sphaericus.* Optimum incubation time for *Klebsiella pneumonia* MTCC 7162 was reported to be 20-30 h (Sivashanmugam and Jayaraman, 2011). Decline in tannase activity might be due to decreased tannic acid leading to increase in glucose concentration produced by hydrolysis of tannic acid (Tripathi *et al*., 2016).

**Optimization of medium components for tannase production using response surface methodology.** In contrast to single variable optimization approach, RSM is a more precise technique for the determination of parameters under optimized conditions. To find out which variable has a significant impact, several components of medium were evaluated by Plackett-Burman design (PBD). PBD is a fraction two level factorial design which enables to investigate the 'n-1' variables during at least 'n' experiments. In twelve (12) experimental runs, six variables were screened *i.e.,* tannic acid,$CaCl_2$, $K_2HPO_4$ ,$NH_4NO_3$ ,$MgSO_4$ and Yeast extract. The response obtained from these experiments was examined by multiple regression (Table 5). Results supported three variables *i.e.* tannic acid, $CaCl_2$ and yeast extract to be significant for tannase production.

**Optimization by Box-Bhenken design.** A model consisting of fifteen trial experiments were designed using Box-Behnken Design (BBD) with three levels

were applied and results shown in Table 6. The obtained response was calculated using second-order polynomial regression equation (eq. 1).Results disclosed that the highest tannase synthesis was obtained at 0.5% tannic acid, 0.1% $CaCl_2$ and 0.275% yeast extract. The observed values of enzyme production were almost near to the predicted tannase enzyme production depicting the accuracy of the model (Fig. 2).

**Table 4.** Isolated bacterial strains and their enzyme production

| Strains | Tannase activity(U/mL) |
|---|---|
| *Roultella ornithinolytica* | 0.929b±0.010 |
| *Klebsiellaoxytoca* | 0.838b±0.101 |
| *Enterobacter aerogenes* | 1.020b±0.121 |
| *Bacillus amyloliquefaciens* | 1.269a±0.00 |

**Table 5.** Plackett–Burman design for screening of importantvariables for tannase production

| $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | $X_6$ | Tannase production (U/mL) |
|---|---|---|---|---|---|---|
| 0.05 | 0.05 | 0.1 | 0.1 | 0.1 | 0.05 | 27.99 |
| 0.05 | 0.05 | 0.02 | 0.1 | 0.1 | 0.5 | 22.98 |
| 0.5 | 0.05 | 0.1 | 0.02 | 0.05 | 0.05 | 27.61 |
| 0.5 | 0.1 | 0.02 | 0.1 | 0.1 | 0.05 | 27.73 |
| 0.5 | 0.05 | 0.02 | 0.02 | 0.1 | 0.5 | 23.87 |
| 0.05 | 0.05 | 0.02 | 0.02 | 0.05 | 0.05 | 24.75 |
| 0.05 | 0.1 | 0.02 | 0.02 | 0.05 | 0.5 | 25.16 |
| 0.5 | 0.1 | 0.1 | 0.02 | 0.1 | 0.5 | 26.58 |
| 0.5 | 0.1 | 0.02 | 0.1 | 0.05 | 0.05 | 25.77 |
| 0.5 | 0.05 | 0.1 | 0.1 | 0.05 | 0.5 | 26.95 |
| 0.05 | 0.1 | 0.1 | 0.02 | 0.1 | 0.05 | 26.92 |
| 0.05 | 0.1 | 0.1 | 0.1 | 0.05 | 0.5 | 24.71 |

**Table 6.** Observed and predicted values of tannase produced through Box-Bhenken design.

| $X_1$ | $X_3$ | $X_6$ | Tannase activity (IU/mL) | | |
|---|---|---|---|---|---|
| | | | Observed | Predicted | Residual |
| 0.5 | 0.06 | 0.05 | 11.990 | 11.970 | 0.020 |
| 0.05 | 0.06 | 0.05 | 8.780 | 8.780 | 0.000 |
| 0.275 | 0.1 | 0.5 | 11.400 | 11.435 | -0.035 |
| 0.5 | 0.02 | 0.275 | 11.600 | 11.655 | -0.055 |
| 0.5 | 0.06 | 0.5 | 10.300 | 10.300 | 0.000 |
| 0.275 | 0.06 | 0.275 | 12.600 | 12.533 | 0.066 |
| 0.5 | 0.1 | 0.275 | 13.400 | 13.365 | 0.035 |
| 0.275 | 0.02 | 0.5 | 12.400 | 12.345 | 0.055 |
| 0.275 | 0.1 | 0.05 | 12.600 | 12.655 | -0.055 |
| 0.275 | 0.06 | 0.275 | 12.500 | 12.533 | -0.033 |
| 0.275 | 0.02 | 0.05 | 9.790 | 9.755 | 0.035 |
| 0.05 | 0.06 | 0.5 | 11.800 | 11.820 | -0.020 |
| 0.05 | 0.1 | 0.275 | 11.870 | 11.815 | 0.055 |
| 0.05 | 0.02 | 0.275 | 11.500 | 11.535 | -0.035 |
| 0.275 | 0.06 | 0.275 | 12.500 | 12.533 | -0.033 |

Tannase activity (U/mL) = $5.734 + 12.772\ X_1 + 16.024\ X_3 + 27.093\ X_6 - 12.551X_1^2 + 121.615\ X_3^2 - 23.317\ X_6^2 + 39.722\ X_1.X_3 - 23.259X_1.X_6 - 105.833\ X_3.X_6$ ..... eq1

Analysis of variance (ANOVA) evaluated the significant components which were optimized by BDD. The values of F and P express the independent variables' individual as well as interactive effects. This analysis technique was used for the assessment of effects of variables as well as probably existing interactions. The model was found very significant as well as sufficient also to exhibit the actual relationship between the response and significant variables as indicated by the small P-value (<0.001). Regression equation gained from ANOVA represented $R^2$ (multiple correlation coefficient) to be 0.999 (Table 7). This value estimates the fraction of overall variation in the analyzed data exhibiting that this model is proficient to explain 99.88% of the variation in response ($R^2$ value should be near to 1.0 for a good statistical model).

**Contour Plots for tannase production by *B. amyloliquefaciens.*** Figure 3 (a,b,c,) adepicts the impact of tannic acid ($X_1$) and $CaCl_2$($X_3$) on tannase enzyme synthesis by *B. amyloliquefaciens* in submerged fermentation while yeast extract ($X_6$) was kept constant.

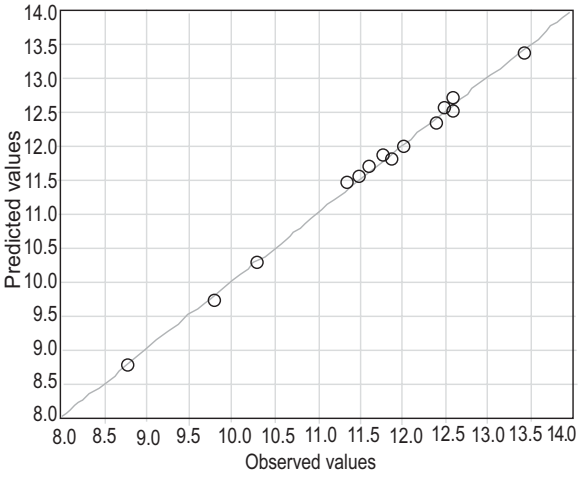


**Fig. 2.** Residual plots of experimental vs. predicted values for alkaline tannase production.

**Table 7.** Analysis of variance for tannase production through Box-bhenken design.

| Effect | SS* | DF** | MS*** | F-value | P-value |
|---|---|---|---|---|---|
| Intercept | 7.415 | 1 | 7.415 | 1515.344 | 0.000 |
| $X_1$ | 3.082 | 1 | 3.082 | 629.867 | 0.000 |
| $X_{12}$ | 1.490 | 1 | 1.490 | 304.656 | 0.000 |
| $X_3$ | 0.124 | 1 | 0.124 | 25.370 | 0.003 |
| $X_{32}$ | 0.139 | 1 | 0.139 | 28.570 | 0.003 |
| $X_6$ | 13.869 | 1 | 13.869 | 2834.337 | 0.000 |
| $X_{62}$ | 5.144 | 1 | 5.144 | 1051.390 | 0.000 |
| $X_1$* $X_3$ | 0.511 | 1 | 0.511 | 10.474 | 0.000 |
| $X_1$* $X_6$ | 5.546 | 1 | 5.546 | 1133.384 | 0.000 |
| $X_3$* $X_6$ | 3.629 | 1 | 3.629 | 741.626 | 0.000 |
| Error | 0.024 | 5 | 0.004 | | |
| R=0.999; $R_2$ = 0.998; Adj. $R_2$ = 0.996 | | | | | |

*sum of square; **degree of freedom; ***means of square.

Figure 3b depicts the correlation of tannic acid ($X_1$) and yeast extract ($X_6$) on microbial tannase synthesis by *B. amyloliquefaciens* in submerged fermentation while $CaCl_2$ was kept constant. Figure 3c illustrates the effect of $CaCl_2$($X_3$) and yeast extract ($X_6$) on microbial tannase production by *B. amyloliquefaciens* in submerged fermentation when tannic acid was kept constant. These graphs indicated that all these three components play significant role in tannase production.

Figure 4 depicts the desirability charts for tannase production. This chart illustrated that using concentration of $X_1$ 0.275, $X_3$ 0.06 and $X_6$ 0.275, the maximum tannase production would be 14.098 U/mL, while minimum value would be 9.238 U/mL and medial value would be 11.699 U/ml obtained.

**Effect of pH on enzyme activity.** For the study of the effect of pH on tannase activity, the reaction mixture was incubated at several pH and result showed that 7.0 was the optimal pH for *B. amyloliquefaciens* tannase (Fig. 5). In many researches, pH ranging from 3.0 to 6.0 has been recorded to be optimum for the tannase activity (Sabu *et al*., 2005; Ramirez-Coronel *et al*., 2004). For *Bacillus subtilis* (Aftab *et al*., 2016) and *B. subtilis* PAB2 (Kumar *et al*., 2015), the optimum pH for tannase activity was reported as 5.

**Effect of temperature on enzyme activity.** For the discovery of optimal temperature for tannase, the reaction mixture was incubated at various temperatures and it was found that the enzyme was most reactive at 40 °C. Further increasing temperature describe in (Fig. 6) from

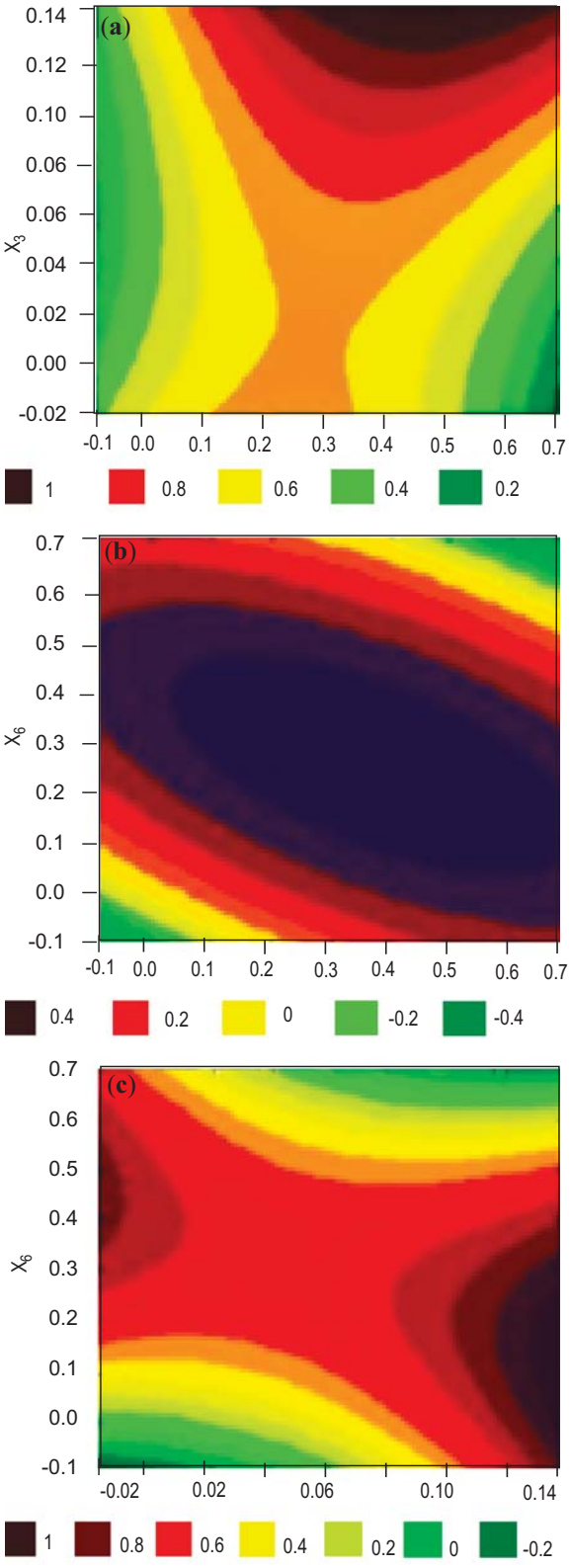


**Fig. 3.** Contour plots for the effects of yeast extract, tannic acid and $CaCl_2$ on tannase production i-e **a,b,c.**

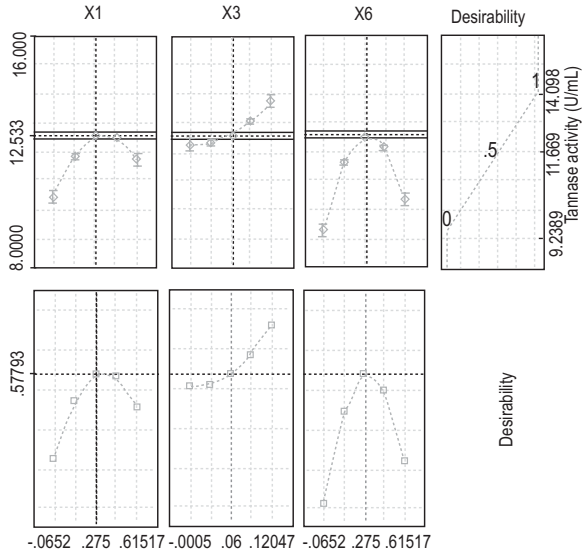


**Fig. 4.** Desirability chart for tannase production.

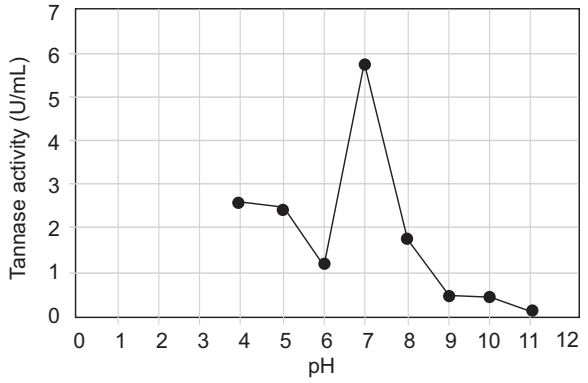


**Fig. 5.** Effect of pH on tannase activity.

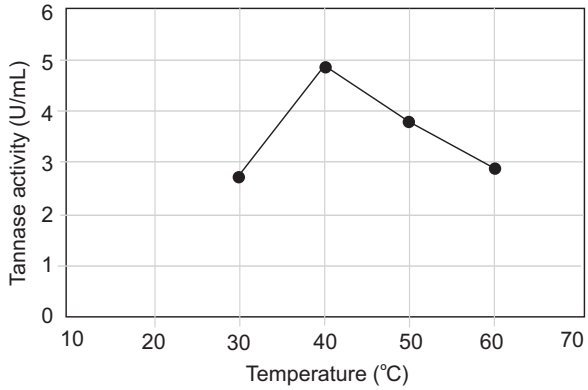


**Fig. 6.** Effect of temperature on tannase activity.

40 °C to onward resulted decline in enzyme activity. Kumar *et al*. (2015) reported the optimum temperature for tannase activity synthesized from the bacterial strain *B. subtilis* PAB2 as 40 °C, that is in accordance to our present results. Optimum temperature for tannase extracted from *Bacillus subtilis* was reported to be 45 °C by Aftab *et al*. (2016). Tannase produced from *Rhodococcus* NCIM 2891 exhibited optimum activity at 30 °C (Nadafand Ghosh, 2011).

## Conclusion

Results of this study showed that optimization of process parameters are pre-requiste for the enhancement of enzyme production in submerged fermentation process. Response surface methodology effectively improved enzyme production by optimizing various cultural and nutritional parameters. The findings of present investigation could be employed for further scale up in industrial exploration.

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