Research Article

Tannase Production by Fungal Isolates from Tannery Effluent

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Abstract

Tannase (tannin acyl hydrolase, E.C.3.1.1.20) is an inducible enzyme that has wide applications in different food and feed industries including production of beverages, cosmetic chemicals and in clarification of beer and fruit juice. It has also been used for bioremediation of tannins from effluents of leather industries. Attempt was made for isolation of tannase producing microbes from tannery effluents using tannic acid supplemented media. On the basis of qualitative (zone of hydrolysis) assay, four fungal isolates designated as AV1, AV2, AV3 and AV4 was screened and the maximum zone was shown by AV3 isolate i.e. 2.3 cm. Quantitative tannase production by the selected four isolates (AV1, AV2, AV3 and AV4) for seven consecutive days revealed AV3 to be the maximum tannase producer (25.33 U/mL) on 5th day of incubation. Four different agrowastes i.e. spent tea powder, coconut coir, corn husk and fruit baggase were used as substrates for tannase production using selected isolate AV3 and the maximum tannase production (35.62 U/mL) was obtained using spent tea powder as substrate at pH 5.5 and temperature 40 ^{0}C. Optimization of process parameters for maximum tannase activity with central composite design using response surface methodology reveals maximum tannase activity (93.33µMmin^{-1}) at a temperature of 60^{0}C, pH 8.0 and 5% substrate concentration. On partial purification, by ammonium sulphate precipitation followed by dialysis using membrane-70, activity of the tannase increased to 153.33µMmin^{-1} and the yield of the enzyme was 83.33%. Partially purified enzyme was used for detannification of pomegranate juice and was found to reduce tannin content from 2.76 mg/mL to 1.03 mg/mL but with slight decolorization of juice

Keywords: Tannase, fungi, Response surface methodology, agrowaste, spent tea, juice detannification

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Introduction

Tannase, an important hydrolase enzyme with huge biotechnological potential has been reported from different plant parts, different animals and micro-organisms [1]. Diverse group of micro-organisms have been reported to produce tannase with varying catalytic efficiency. Tannin Acyl Hydrolase (TAH) (E.C.3.1.1.20) known as tannase, catalyzes the hydrolysis of ester bond (galloyl ester of a alcohol moiety) and the depside bond (galloyl ester of gallic acid) of hydrolysable tannins. It was accidentally found by Tieghem (1867), [2] when an investigation on formation of hydrolyl esters of tannic acid by tannase brought about the liberation of glucose, gallic acid and different galloyl esters of glucose [3]. It has a pH steadiness extending 3.5-8.0, and optima of 5.5-6.0, temperature strength stuck in the vicinity of 30 and 60^{0}C, temperature optima with reference to 30– 40^{0}C, an isoelectric purpose of 4.0– 4.5 and an atomic weight in the vicinity of 186 and 300 kDa.

Tannase has commercial application in drinks and food industries, chemical-pharmaceutics, production of animal feed and brewing. It has numerous important applications in food, feed, beverage, brewing chemical and pharmaceutical industries [4, 5]. In brewing as well as wine industries, the enzyme is used for removal of chill haze formation of beer and wines [6]. It has been used for reducing the hydrolysable tannin levels in poultry feed, in order to improve feed effectiveness [7]. Tanneries effluents contain tannins in huge amounts, mainly polyphenols which are toxic, here the usage of tannase is an inexpensive and efficient treatment for the degradation of these compounds.

Microbial tannase production at industrial level using tannic acid as a substrate is fairly expensive. Therefore, it was trusted that agricultural wastes containing tannin could be considered as a alternative source of tannic acid for producing this enzyme. Majority of enzyme production is done by fungi and bacteria [8]. Filamentous fungi of the *Aspergillus* and *Penicillium* have been broadly used for tannase production. Among bacterial isoltes *Lactobacillus apodemi, Klebisella pneumonia, Enterobacter cloacae, Pseudomonas aeruginosa* and *Bacillus licheniformis* are tannase producers [9].

Materials and Methods

Screening of the tannase producing micro-organisms

Sample collection Effluent samples were collected from Leo tanneries located at Leather Complex, Jalandhar and Common Effluent Treatment Plant (CETP), Focal point, phase V, Ludhiana. The collected samples were diluted and plated on the agar medium plates supplemented with tannic acid @1 %. Tannase producing micro-organisms were selected quantitatively by zone of hydrolysis formed by the isolates [10]. Quantitative estimation of the tannase producers was done following the method of Mondal *et al* (2001) [11]. Standard graph of tannic acid was prepared with various concentrations of 1% tannic acid ranging from 1mg/mL to 10mg/mL, from every concentrations of tannic acid. The reaction mixture consisting of substrate tannic acid 0.3 mL and 0.3 mL enzyme was incubated at 30^{o}C for a incubation period of 30 mins. The enzymatic reaction was stopped by the addition of 3 mL BSA solution, which precipitated the remaining tannic acid. The tubes were centrifuged (5000g x 5 min). The supernatant was discarded and the resultant precipitate was dissolved in 3 mL SDS–triethanolamine solution. Subsequently 1 mL of $FeCl_3$ reagent was added and absorbance was measured at 530 nm, against the blank.

Optimization of physico-chemical conditions for tannase production

Various parameters (temperature, pH and substrate concentration) was that influenced enzyme yield were optimized. The strategy followed was to optimize each parameter independent of the others and subsequently optimal parameters were employed in all experiments.

Impact of temperature

To study the effect of incubation temperature for maximum tannase production, the flasks with the production medium were inoculated and incubated at different temperatures viz., 25, 30, 35, 40, 45 and 50˚C. Tannase production was determined on the 5th day of incubation.

Impact of pH

The pH of the production medium was varied from 4.5, 5.0, 5.5, 6.0 and 6.5 by preparing minimal medium in desired buffers. The flasks were incubated with fungal bits from 48hr old culture and kept at optimized temperature for 5 days. Amount of tannse produced was estimated by method given by Mondal *et al* (2001) [11].

Impact of substrate concentration

Concentration of substate (tannic acid) in the media was varied from 0.05 to 2.50% and tannase production was studied at optimized temperature and pH by method of Mondal *et al* (2001) [11].

Tannase production using different Agrowaste

Four different substrates spent tea waste, corn husk, coconut coir and fruit baggase were used for the production of tannase. The Agro wastes Corn Husk, Fruit baggasse, Coconut coir and spent tea were powdered into 100 mesh (0.15 mm) fine powders by using laboratory grinder at 3000 rpm and were preserved in a sealed plastic bag at 4˚C to prevent any possible degradation or spoilage. The physico-chemical factors such as incubation time, temperature and pH optimized were considered for the enzyme production. The enzyme production was determined by the method of Mondal *et al* (2001) [11].

Optimization of process parameters for maximum tannase activity using response surface methodology (RSM)

For optimization, the analysis of variance (ANOVA) for the overall effect of three factor variables i.e. substrate concentration (1.0-5.0%), temperature (20-60ºC) and pH (4.0-8.0) on the response variable i.e. tannase activity according to the fitted model was done using software Statgraphics Centurian XVI.I and the least significant factor affecting the response variable was selected. The three dimensional plots and contour plots according to the fitted model were drawn using the software. Response Surface Methodology was used for regression analysis of the experiment. 16 sets of various combinations (Table 5) were obtained, under which experiments were performed for determining the tannase activity (U) as response variable.

Partial purification of the enzyme and its tannase activity

The crude enzyme was treated with ammonium sulphate at 75% concentration. Precipitated protein was collected by centrifugation at 10,000 rpm for 15 min at 4 ºC. The supernatant was discarded and the precipitates were dissolved in 0.1M citrate phosphate buffer (pH 5). The dialysis membrane-70 (HIMEDIA) was used for the dialysis process as per the protocol by Nandi and Chatterjee (2016) [12].

Application of tannase in detannification of pomegranate juice

Pomegranate juice is a rich source of tannins, so to check the efficiency of the tannase enzyme obtained was tested. 10 mL of the juice was taken in the test tube and added with 1 mL of partially purified tannase. The control test tube received 1mL of distilled water along 10 mL of the juice. The test tubes were incubated at 37^{o}C for 2 hrs with gentle shaking. The test tube was then placed in water bath at 50^{o}C for 10 min to deactivate the enzyme. 1mL of the sample was taken from each test tubes and the tannin content was measured [13].

The tannin content in the fruit juice was measured following the protein precipitation method by tannins [14].

Results and Discussion

Screening of the tannase producing micro-organisms

Different micro-organisms which were able to grow on tannic acid supplemented media which included 5 fungi, 3 bacteria, 2 actinomycetes and 1 yeast. Fungal isolates were screened on PDA medium containing 1% tannic acid for their capacity to produce tannase by utilizing the tannic acid in plate assay method. From all the selected isolates (AV1, AV2, AV3 and AV4), AV3 produced a maximum zone of hydrolysis i.e. 2.3 cm (**Table 1**). The tannase production by four fungal isolates (AV1, AV2, AV3 and AV4) was studied continuously for seven days (**Table 2**). Maximum tannase production was observed on fifth day of incubation (25.33 U/mL) by AV3.

Table 1 Qualitative tannase assay of selected fungal isolates

S. No.	Isolate	Diameter of zone (cm)	Diameter of colony (cm)	Zone of hydrolysis (cm)* = Diameter of zone - Diameter of colony
1	AV1	1.2	0.5	0.7±0.03
2	AV2	2.4	1.2	1.2±0.01
3	AV3	4.5	2.2	2.3±0.06
4	AV4	3.9	2.0	1.9±0.17
CD@5%				0.3

*Readings expressed as the average of three values.
Medium used: Potato dextrose agar supplemented with 1% tannic acid
pH of medium: 5.5
Incubation time: 72 hrs
Incubation temperature: 28±2^{o}C

Table 2 Quantitative of tannase assay of selected fungal isolates

S. No.	Incubation period (days)	Tannase units (U/mL)			
		AV1	AV2	AV3	AV4
1.	3rd day	3.33±0.17	5.00±0.11	1.60±0.15	1.43±0.05
2.	4th day	6.02±0.39	5.66±0.36	5.33±0.01	6.00±0.23
3.	5th day	12.70±0.05	9.30±0.23	25.33±0.96	21.00±0.23
4.	6th day	5.66±0.01	9.00±0.28	5.60±0.28	10.00±0.69
5.	7th day	1.00±0.11	3.60±0.40	2.00±0.11	2.06±0.42
CD w.r.t days		0.89			
CD w.r.t fungal isolates		0.80			
CD (p≤5%) (days x fungal isolates)		1.79			

Optimization of physico-chemical conditions for tannase production

Impact of temperature upon tannase production

The temperature's impact upon enzyme production was studied by varying the incubation temperatures at 25-50^{0}C with a difference of 5^{0}C and by maintaining all other parameters stable i.e., pH 5.5 with concentration of tannic acid

@ 1% (w/v). The results are depicted in **Table 3**. With an increase in the incubation temperature, the tannase production was observed to increase at a rapid rate. The maximum tannase production with 1% tannic acid as substrate was found to be 23.33 U/mL at 40^0C on the fifth day of incubation. Calvalcanti *et al* (2017) [15] studied temperature's effect on tannase production by *A. niger* ANG 18 and *A. fumigatus* CAS21. The two species produced maximum tannase with an optimum temperature at 30^0C.

Table 3 Impact of temperature upon the tannase production

S. No.	Temperature (^{0}C)	Tannase units (U/mL)
1.	25	3.35±1.76
2.	30	8.83±0.70
3.	35	13.16±0.23
4.	40	23.33±2.36
5.	45	16.83±2.36
6.	50	11.6±1.41
	CD (p≤5%)	3.57
Incubation period -5 days Media- minimal media supplemented with tannic acid @1% pH- 5.5		

Impact of pH upon tannase production

The impact of pH on tannase production was considered by experimenting at different pH (4.0-8.0) keeping other parameters constant i.e., temperature at 40^0C and concentration of tannic acid @ 1% (w/v). The results are given in **Table 4**. The highest tannase production was found at pH 5.5. Sahora *et al* (2015) [16] studied the influence of pH on tannase production using different pH values of the growth medium. They reported that the increase in production of tannase with an increase in the pH from 3.5 to 5.0 followed by a decline. This may be due to salt formation of tannic acid at high pH, or possibility of low activity of tannase at higher pH which might have led to unavailability of tannic acid for the utilization and hence the minute tannase production was observed.

Table 4 Impact of pH upon the tannase production

S. No.	pH	Tannase units (U/mL)
1.	4.0	2.22±0.82
2.	4.5	4.50±2.12
3.	5.0	18.31±0.43
4.	5.5	21.37±1.82
5.	6.0	11.37±1.47
6.	6.5	5.38±2.89
7.	7.0	3.65±0.77
8.	7.5	2.80±0.63
9.	8.0	2.50±0.70
	CD (p≤5%)	3.43755
Incubation period- 5days Media- Minimal Media supplemented with tannic acid @1% Temperature- 40^oC		

Impact of substrate concentration upon tannase production

Different concentrations of tannic acid were used (0.05, 0.45, 0.85, 1.25, 1.65, 2.05 and 2.50 %) maintaining other parameters constant at 40^oC and pH 5.5. **Table 5** depicts the result that as the tannic acid production increases with an increase in its concentration. The tannase production was highest (37.67 U/mL) for concentration of tannic acid @1.65% after an incubation period of 5 days. Sharma *et al* (2017) [17] reported highest tannase production (20 U/mL) by *Aspergillus* sp. at 1% (w/v) tannic acid concentration when incubated at 32^0C for 96 hr.

Tannase production using different agro wastes

Tannase production was studied by using different substrates viz. fruit baggase, corn husk, coconut coir and spent tea.

The flasks containing Minimal Media supplemented with different substrates @16.5 mg/mL were incubated at 40^0C, pH at 5.5 for 5 days. The maximum tannase production (35.62 U/mL) was observed on spent tea as substrate (**Table 6**). Kuppuswamy *et al* (2012) [18] used the powdered agro wastes of green-gram husk, black-gram husk, red-gram husk, tea dust, tamarind seed powder, groundnut shell and rice bran for the tannase production. The substrates tamarind seed powder and red-gram husk had a maximum enzyme production of 67.59 U/mL and 76.49 U/mL after an incubation period of 96 hr.

Table 5 Impact of substrate concentration upon the tannase production

S. No.	Concentration of tannic acid (%)	Tannase units (U/mL)
1.	0.05	0.31±0.12
2.	0.45	0.84±0.16
3.	0.85	4.77±0.72
4.	1.25	8.15±1.20
5.	1.65	37.67±1.16
6.	2.05	18.05±1.44
7.	2.50	4.84±0.21
	CD (p≤5%)	2.09
Incubation period- 5days Incubation temperature-40^{o}C Incubation pH-5.5		

Table 6 Tannase production on different agro waste at optimised conditions

S. No.	Substrates	Tannase units (U/mL)
1.	Fruit baggase	5.95±0.49
2.	Corn husk	16.85±0.42
3.	Coconut coir	9.56±0.94
4.	Spent tea	35.62±0.35
	CD(p≤5%)	1.342

Optimization of conditions for tannase activity with response surface methodology (RSM)

Statgraphics Centurian XVI.I software was used for the optimization of different conditions for maximum tannase activity by AV3 isolate using spent tea as a substrate. Fermentation was carried out under stationary conditions using 100 mL of minimal media supplemented with spent tea as a substrate for each of 16 runs according to RSM plan and the response was studied in terms of tannase activity (μMmin^{-1}) (**Table 7**).

Table 7 Experimental response profile for optimization of process parameters for tannase activity using AV3 isolate

Run	Temperature (^{0}C)	pH	Substrate concentration (%)	Observed tannase Activity (μM min^{-1})	Observed Desirability	Predicted Desirability
1	20.0	4.0	1.0	40.66	0.47	0.51
2	60.0	4.0	1.0	20.08	0.26	0.27
3	20.0	8.0	1.0	3.21	0.10	0.0
	60.0	8.0	1.0	21.22	0.27	0.34
5	20.0	4.0	5.0	28.97	0.35	0.37
6	60.0	4.0	5.0	9.77	0.16	0.13
7	20.0	8.0	5.0	35.72	0.42	0.49
8	60.0	8.0	5.0	93.33	1.0	0.83
9	6.36	6.0	3.0	30.41	0.37	0.34
10	73.63	6.0	3.0	30.28	0.37	0.43
11	40.0	2.64	3.0	42.3	0.49	0.45
12	40.0	9.36	3.0	47.5	0.54	0.61
13	40.0	6.0	0.1	1.11	0.078	0.058
14	40.0	6.0	6.36	18.33	0.25	0.30
15	40.0	6.0	3.0	16.66	0.23	0.25
16	40.0	6.0	3.0	20	0.26	0.25

Results from Table 5 showed that in minimal media broth inoculated with AV3 isolate had maximum tannase activity (93.33µMmin^{-1}) was found at 5% substrate concentration, 40^{0}C temperature and pH 8.0 after an incubation of 5 days. The regression equation of the fitted model for tannase activity is:

$$\text{Tannase activity} = 247.85 - 3.03\ X_1 - 54.38 * X_2 - 14.61 * X_3 + 0.012 * X_1^2 + 0.36 * X_1 * X_2 + 2.53 * X_2^2 + 3.96 * X_2 * X_3 - 0.79 * X_3^2$$

Where X_1=temperature; X_2 = pH; X_3 = substrate concentration

Effect of combined cultural conditions on enzyme activity by AV3. Contour plots of estimated response surface showing the effect of different parameters on tannase activity were studied. Results (**Figure 1**) revealed that the maximum tannase activity falls in the range of 90-150 µMmin^{-1}at varying substrate concentration (1-5%), temperature (20-60^{o}C) and pH (4.0-8.0).

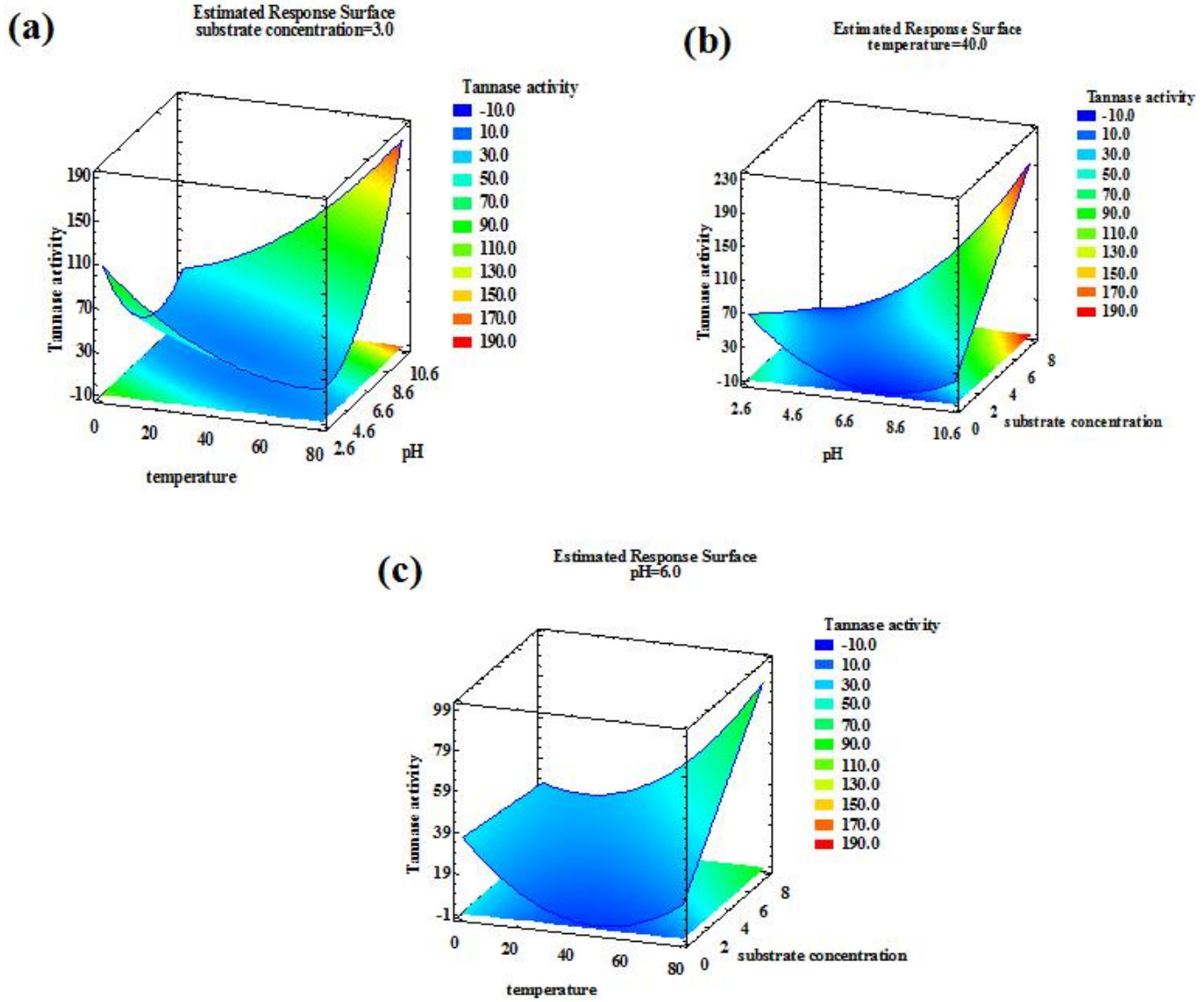

Figure 1 3D Response surface plots representing interaction between significant process parameters on tannase activity. (a) Effect of temperature and pH at constant substrate concentration, (b) Effect of pH and substrate concentration at constant temperature, (c) Effect of temperature and substrate concentration at constant pH

Molkabadi *et al* (2015) [19] optimized production of tannase by mutant strain, *Penicillium* sp. EZ-ZH390 in submerged fermentation by RSM using central composite design (CCD). The results showed that a temperature of 30^{0}C, rotation rate of 85 rpm and incubation period of 24 hr led to maximum tannase production. At these conditions, tannase activity reached to 21.77 U/mL/hr and tannase productivity increased at least about 3.55 times (0.26 U/mL/hr).

Partial purification of the enzyme and its tannase activity

Assay conditions were optimized in the previous section 4.5 and the enzyme activity was recorded at optimized condition i.e. 5% substrate concentration, 60^0C temperature and pH 8.0. The activity of the partially purified enzyme was (153.33µMmin^{-1}) high as compared to the activity given by the crude enzyme (93.33µMmin^{-1}). The comparison of the tannase activity of the crude enzyme and the partially purified enzyme is depicted in **Table 8**. Abou-Bakr *et al* (2013) [20] studied the tannase activity from the promising fungus isolated from tannery soil sample, identified as *Aspergillus niger* and had a tannase activity of 305U/mL after the purification of the enzyme. It was comparatively high as compared to other isolates used in his study.

Table 8 Comparison of the tannase activity before and after purification

S. No.	Treatment	Tannase activity (µM min^{-1})	%increase in tannase units	Yield (%)
1	Crude enzyme	93.33	1	100
2	75% ammonium sulphate fractionation and dialysis	153.33	64.28	83.33

Application of tannase in detannification of pomegranate juice

The tannase was used for the detannification of the pomegranate juice by the method discussed in section 3.8. The enzyme activity was observed to be 112.61µMmin^{-1} in the juice sample. Partially purified enzyme was used for detannification of pomegranate juice and was found to reduce tannin content from 2.76 mg/mL to 1.03 mg/mL thus leading to 62% decrease in the tannin contents. Nandi and Chatterjee (2016) [12] obtained the enzyme from *Aspergillus niger* MTCC 2525 by using rice husk as the substrate for the solid state fermentation. The enzyme was applied in the detannification of fruit juice, 56% decrease in the tannin content was observed with an activity of 173U/mg of the purified enzyme.

Conclusions

On the basis of formation of zone of formation and the maximum tannase production, AV3 isolate was selected for further studies. From the different experimental set the optimum temperature was considered to be 40^0C, pH 5.5 and substrate concentration 1.65% by using AV3 isolate. At the optimized conditions, four different agricultural waste (fruit baggase, coconut coir, corn husk and spent tea powder) were used to replace tannic acid from media. Maximum tannase production (35.62 µMmin^{-1}) was obtained by using spent tea as a substrate at the optimized physico- chemical conditions. Among all the 16 sets obtained using RSM, set 8 was selected as it had maximum tannase activity i.e. 93.33µMmin^{-1}. The enzyme activity after the partial purification increased from 93.33 to 153.33 µMmin^{-1} showing 64.28% increase in the tannase activity over the crude enzyme. The partially purified enzyme was used for the detannification of the pomegranate juice. The enzyme activity lead to 62% decrease in the tannin contents in the juice but with slight decolorization of juice.

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Publication History
Received 06th Oct 2017
Revised 20th Oct 2017
Accepted 20th Oct 2017
Online 30th Oct 2017