Chapter 4

Purification and characterization of tannase enzyme from *Aspergillus niger* CEPC
Chapter 4

Purification and characterization of tannase from *Aspergillus niger* CEPC 11

4. Introduction

The significance and utility of tannase enzymes has prompted great research from 1867 onwards resulting in many publications and many patents in this area of research (Beniwal et al. 2013). The purification process is one of the less developed aspects on tannase. Most of the published purification protocols consist on multistep procedures able to obtain a highly purified enzyme but with a low-recovery yield. The common strategy used for the purification of tannase was based on protein concentration followed by ion exchange and/or gel filtration chromatography (Beniwal et al. 2003; Bharadwaj et al. 2003 and Mahendran et al. 2005).

Tannase has been purified from a variety of fungi like *A. flavus* (Yamada et al. 1968), *A. oryzae* (Iibuchi et al. 1968), *Candida* sp.(Aoki et al. 1976), *Penicillium chrysogenum* (Rajkumar and Nandy 1983) and *A. niger* (Barthomeuf et al. 1994). Based on extracellular and intracellular nature of enzyme production, culture filtrate as such or mycelia extract after sonication (Yamada et al. 1968) were used as the crude enzyme.

As an initial step of purification, ammonium sulphate precipitation (Yamada et al. 1968; Sabu et al. 2005a; Naidu et al. 2008) or acetone precipitation
were attempted (Beverini and Metche 1990; Lekha and Lonsane 1994) which resulted in an initial concentration as well as purification. Aoki et al. (1976) and Lekha and Lonsane (1994) reported the failure of ammonium sulphate to precipitate tannase because of the very low yield. Tannase from *A. Awamori* MTCC 9299 was purified using ammonium sulfate precipitation followed by ion exchange chromatography. A purification fold of 19.5 with 13.5 % yield was obtained (Chhokar et al. 2009). Precipitation of tannase using polymers 1-90 % such as polyethylene glycol, poly vinyl alcohol and dextran have been reported (Naidu et al. 2008). Ultra filtration membranes were also used in concentrating the enzyme recently (Sharma et al. 2007; Marco et al. 2009).

In the present study, tannase enzyme produced from cashew testa using *A. niger* CEPC 11 was purified to homogeneity using a combination of steps including, Ammonium sulphate and acetone precipitation, dialysis and gel filtration chromatography. After purification, the by-product Gallic acid was confirmed by FTIR, HPLC and HPTLC. All the experiments were done at 4ºC unless otherwise specified.

4.1.1 Materials and Methods

4.1.1. Enzyme purification

4.1.1.1 Ammonium sulphate and Acetone precipitation of crude enzyme preparation

Enzyme production was carried out using solid state fermentation under optimized Conditions as described in Chapter 2 (Section 2.1.8). Extraction of the
enzyme was performed as outlined under section 2.1.6.1. Partial purification of enzyme was done by Acetone and Ammonium sulphate Precipitation. The tannase in the culture supernatant (crude extract) was precipitated by adding pre-chilled acetone to the supernatant in the ratio 2:1 (by volume) and it was kept at 4°C overnight. This resulted in increased enzyme precipitation. The precipitated enzyme was separated from the solution by centrifugation process (8000 rpm at 4°C for 20 minutes). The supernatant was discarded carefully without dislodging the protein pellet. Acetone was allowed to evaporate from the uncapped tube at room temperature for 30 min. Care was taken not to over dry the pellet and the enzyme pellets was resuspended in minimal amount of 0.2M acetate buffer (pH-5.0), and it was kept at 4°C for further studies. The same separation procedure was carried out for all three types of tannase fermentation.

Ammonium sulphate precipitation was done according to Englard and Seifter (1990). Ammonium sulphate (Nice chemicals, Cochin, Kerala) required to precipitate tannase was optimized by its addition, at varying levels of concentrations (50, 60, 70, 75, 80, and 100% saturation), to the crude extracts. To precipitate the protein, ammonium sulphate was slowly added initially at 50% saturation to the crude extract while keeping in ice with gentle stirring. The addition of ammonium sulphate was done under constant stirring at 4°C for 30 min and then stirring was continued for another 30 min. After this the mixture was kept to settle for 3 h at 4°C. After complete suspension of ammonium sulphate, the solution was kept at 4°C for overnight. Precipitated protein was collected by centrifugation at 10000 rpm for 15 min at 4°C. The precipitate was resuspended in minimum quantity of 0.05 M citrate buffer (pH5). To the supernatant ammonium
sulphate, required for next level of saturation, was added and the procedure mentioned above was repeated. This precipitation process was continued up to 90% ammonium sulphate saturation.

4.1.1.2. Dialysis

The precipitate obtained after ammonium sulphate precipitation was further dialyzed against (Sigma Aldrich) 0.05 M citrate buffer (pH=5). The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis tube (Sigma-Aldrich) was first treated to remove the humectants and protectants like glycerin and sulphur compounds present in it and to make the pores of the tube more clear. The pretreatment involved washing of the tube in running water for 3-4 h, dipping in 0.3 % (w/v) sodium sulfide at 80ºC for 1 min, further washing with hot water (60ºC) for 2 min followed by acid wash in 0.2 % (v/v) sulphuric acid. Finally the tube was rinsed with distilled water. Dialysis was conducted over night and the buffer was changed several times to increase the efficiency of the dialysis. The precipitated protein was dialyzed in the pretreated dialysis tube for 48 h at 4ºC with several changes of buffer and assayed for tannase activity, protein content, and specific activity. Yield was calculated. The treated tube retained most of the proteins of molecular weight 12 kDa and more.

4.1.1.3. Gallic acid estimation

Gallic acid is a product conversion of tannic acid by tannase enzyme. Gallic acid (tannase assay) was determined by the modified method of Sharma et al. (2000). Formation of chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4-ketothiazolidine). A standard curve was prepared using gallic acid.
i. The enzyme reaction mixture was prepared by the addition of 0.01M methyl gallate in 50 μl of 0.05 M citrate buffer, and 10μl of crude enzyme. Substrate blanks were maintained with buffer without substrate.

ii. The reaction mixture was incubated at 30ºC for 5 min.

iii. 60 μl of 0.667 % methanolic rhodanine was added again incubated at same conditions.

iv. 40 μl of 0.5 M Potassium hydroxide was added and then incubated again for 5 min.

v. Diluted 5 times with distilled water.

vi. The pink colour developed was read at 520 nm using UV-visible spectrophotometer (UV 3600; Shimadzu-Japan). A set of blanks and controls were maintained.

One unit of tannase activity is defined as the amount of enzyme required to liberate 1μM of gallic acid /min under defined conditions. Enzyme activity was expressed as U/ml.

4.1.1.4. Protein estimation

Protein content was estimated according to the method of Lowry et al. (1951), using Bovine Serum Albumin (BSA) as the standard. OD was measured at 660 nm and expressed in milligram per millilitre (mg/ml).
Reagent

(a) Sodium carbonate in 0.1 N sodium hydroxide 2.0 % (w/v)

(b) Cupric sulphate in distilled water 0.5 % (w/v)

(c) Solution of sodium potassium tartrate in distilled water 1.0 % (w/v)

(d) *Working reagent: 100 ml of reagent (a) added with 1 ml each of reagent (b) and reagent (c)

(e) 1:1 Folin and Ciocalteau's phenol reagent diluted with distilled water
* Prepared fresh before use

4.1.1.4.1. Estimation

An aliquot of 100 µl of the sample was made upto 1 ml with distilled water and added -to 5 ml of freshly prepared working reagent (d), mixed thoroughly, and incubated for 10 min. 0.5ml of reagent (e) was added and incubated for 30 min and appeared as blue color followed by measuring the absorbance at 660 nm in a UV-Visible spectrophotometer (UV 3600 Shimadzu, Japan).

4.1.1.5. Concentration of tannase by ultra filtration

Tannase clarified was further concentrated by ultra filtration. Clarified extract was concentrated to 50 ml by ultra filtration using membranes (Amicon ultra, Millipore) with a molecular weight cutoff of 30,000 Da followed by 100,000 Da.
4.1.1.6. Gel Filtration chromatography

Purification of enzyme protein by Gel filtration chromatography was performed using sephadex G100 superfine (Sigma-Aldrich) column.

4.1.1.6.1. Preparation of column

a. A fraction of 1 g of Sephadex G-100 superfine (Sigma-Aldrich) was suspended in 15-20 ml distilled water, allowed to hydrate for 3 h at 100°C in a water bath, and fine particles were removed by decantation.

b. Hydrated gel suspension was degassed under vacuum to remove the air bubbles.

c. Filled the glass column with eluent (0.05M Citrate Buffer pH 5) without air bubble.

d. Prepared Gel suspension was carefully poured into the column without trapping air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column.

e. Column was stabilized by allowing two times the bed volumes of eluent to pass through the column bed in descending eluent flow.

4.1.1.6.2 Sample preparation and application on the column

An aliquot of 2.0 ml of crude extract was applied to the prepared Sephadex-G100 superfine column (70 x 2 cm). Care was taken to make sure that the sample was completely free of un dissolved substances. After the complete
entry of sample in to the column the proteins were eluted using 0.05 M citrate buffer, pH-5 at a flow rate of 50 drops per tube in 2 minute flow. Tannase activity was assayed and protein content was estimated.

4.1.1.7. Calculation of Yield of Protein, Yield of Enzyme Activity, Fold of Purification.

Yield of protein and enzyme activity of each fraction obtained during purification is the percentage activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract as the case may be.

\[
\text{Yield of Protein} = \frac{\text{Total Protein content of the fraction}}{\text{Total Protein content of the crude extract}} \times 100
\]

\[
\text{Yield of activity} = \frac{\text{Total activity of the fraction}}{\text{Total activity of the crude extract}} \times 100
\]

\[
\text{Fold of Purification} = \frac{\text{Specific activity of the fraction}}{\text{Specific activity of the crude extract}}
\]

\[
\text{Specific Activity} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein activity (U/mg)}}
\]

4.1.2. Characterization of tannase from A. niger CEPC 11

Purified protein was further characterized and biochemical properties such as molecular mass determination by SDS-PAGE, Native PAGE, MALDI-TOF, pH and temperature stability, effect of oxidizing agents, reducing agents, organic solvents, metal salts and enzyme kinetics were checked as described in the following sections.
4.1.2.1 Determination of the molecular weight of tannase from *A. niger* CEPC 11

Ammonium sulphate precipitated sample and active fractions collected after gel filtration chromatography were electrophoresed by SDS-PAGE in a 10% poly acrylamide gel according to the method of Laemmlli (1970). Protein bands were detected by Coomassie blue staining along with a standard protein ladder (Amnion Bioscience, Bangalore, India).

4.1.2.2. Sample preparation of Native PAGE

Added 100 µl of 1 X sample buffer to purified sample or 20 µl of 2X sample buffer and 10 µl of 50% sucrose to 30 µl liquid sample, mixed well and 30 µl sample and 5 µl marker mix was loaded on to the gel.

4.1.2.2.1. Procedure:

1. Cleaned and assembled the gel plates

2. Resolving gel - Added all the components (Appendix 1) except APS in to a beaker, mixed gently and finally added APS. Immediately poured the mixture into the cast and a layer of water over the gel, and allowed to solidify at least for half an hour

3. Stacking gel - Added the components of stacking gel, except APS, into a beaker, mixed gently, and finally added APS. Poured the contents into the cast above the resolving gel and immediately inserted the comb between the glass plates. Allowed it to solidify at least for 30 min.
4. Gel was placed in the electrophoresis apparatus, and then the upper and lower reservoirs were filled with reservoir buffer for Native-PAGE

5. The gel was pre run for 1 h at 80 V

6. Loaded the gel with the protein sample

7. The gel was run at 80 V till the sample entered the resolving gel

8. When the dye front entered the resolving gel, increased the current to 100 V

9. Stopped the current when the dye front reached 1 cm above the lower end of the glass plate

10. Removed the gel from the cast and stained for at least 1 h in the staining solution

11. Destained till the bands became clear and observed under a transilluminator.

4.1.2.2.2. Sample preparation for SDS PAGE

Added 100 µl of 1X sample buffer to purified and lyophilized sample, mixed well, boiled for 5 min in a water bath, cooled to room temperature, and 30 µl sample and 5 µl low molecular weight markers were loaded on to the gel.

4.1.2.2.3. Procedure

Procedure followed for electrophoresis and staining was essentially the same as described in section 4.1.2.2.1 with the exception that the reservoir buffer used was that of SDS-PAGE.
4.1.2.3. Identification of tannase by MALDI-TOF-MS

The molecular mass of the purified tannase was also analyzed in the linear mode by matrix assisted laser desorption ionization-time of flight (Shimadzu Biotech Axima CFR plus, Power: 180, Laser repeat rate/10Hz). Identification of tannase by MALDI-TOF was performed by after the purification obtained by gel filtration chromatography. MALD-TOF-MS analyses of the purified tannase fraction were carried out on aliquots containing about 10pm of protein samples mixed matrix alpha cyano 4-hydroxy cinnamic acid with 0.1% Trifluoro acetic acid (TFA), spotted in the ratio of 1:3 onto MALDI sample plate and dried under ambient conditions.

4.1.3  Gallic acid confirmation.

Tannase catalyzes the hydrolysis of tannic into glucose and gallic acid. In this chapter, studied the by product of gallic acid from tannase enzyme using Aspergillus niger. The enzyme was purified and the product (gallic acid) was confirmed by FT-IR, HPLC and HPTLC.

4.1.3.1. Fourier-Transform Infra red Spectroscopy (FT-IR Spectroscopy).

Fourier-Transform infra red spectroscopy is a technique that provides information about the chemical bonding or molecular structure of materials whether organic or inorganic. The parameters used in FT-IR analysis were: spectral range 4000-500cm⁻¹, resolution 4cm⁻¹ The extracted gallic acid sample was subjected to FT-IR spectroscopic analysis (Shimadzu, Japan), equipped with
KBr beam splitter with DTGS (Deuterated triglycine sulphate) detector (7800-350cm-1).

4.1.3.2. High Performance Thin Layer Chromatography (HPTLC)

The samples were spotted in the form of bands of width 4 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60 F-254 (10 x 10 cm size) using a Camag Linomat-5 (Switzerland). The slit dimension was kept at 6 mm x 0.45mm and 10 mm/s scanning speed was employed. Plate was developed in a presaturated twin trough glass chamber and the length of the chromatogram run was 80 mm. Subsequent to the scanning, thin layer chromatographic plates were air dried. Densitometric scanning was performed on Camag TLC scanner -3 in the –absorbance mode at 305 nm and operated by CATS software (V 3.15, Camag). The sample was performed at Cashew Export Promotion council of India Laboratory, Kollam, Kerala.

4.1.3.3. High Performance Liquid Chromatography (HPLC)

The Sample was loaded at a concentration of 1 mg/ml to the HPLC (Waters, USA). Sample was diluted in Acetonitrile:Water (50:50). Injection volume was 50μl. Absorbance was monitored at 265 nm and flow rate maintained at 1 ml/min. The sample was applied to symmetry column 184.6x 250mm having Autosample and eluted with a linear gradient of acetonitrile containing 0.1 % TFA (trifluoroacetic acid). The sample and standard gallic acid were run identically. The reaction was performed at Cashew Export Promotion council of India Laboratory, Kollam, Kerala.
4.1.4. Determination of the optimal temperature of activity for the *A. niger* from tannase enzyme.

The effect of temperature on tannase activity was studied by incubating the enzyme at a range of 25 to 60°C; using 0.1 M methyl gallate as substrate in 0.2 M acetate buffer. Optimal temperature was defined as the temperature at which maximum gallic acid hydrolysis occurred, detected by increase in activity.

4.1.5. Determination of the optimal pH of activity for the *A. niger* from tannase enzyme

The effects of pH on enzyme activity was determined over a pH range of 3-8; using methyl gallate as the substrate prepared in acetate buffer (pH 4-6) and Phosphate Buffer (pH 7) and Tris Buffer (pH 8). Optimal pH was defined as the pH at which maximal enzyme activity was obtained in the assay.

4.1.6. Temperature stability of tannase enzyme of *A. niger*

Thermal stability of tannase enzyme was determined by incubating the enzyme sample at various temperatures ranging from 30-80°C over a total period of 24 h, and the enzyme assay was conducted after 1h, 2h, 4h, 6h, and 24h. Enzyme activity of the sample kept at 4°C was taken as control. Residual activity of enzyme was calculated as described under section 4.1.9.6.

4.1.7. Carbohydrate content

The carbohydrate content of the enzyme was determined by phenol sulphuric acid method using glucose as standard (Dubois et al. 1956). An aliquot
of 0.1 ml of enzyme was diluted to 1ml with distilled water and 1ml of phenol was added to it. 5 ml of 96 % sulphuric acid was added, mixed well and kept at 28°C for 20 min. Absorbance was read at 490 nm.

4.1.8. Substrate Specificity and Kinetic Constants

The substrate specificity of tannase was evaluated with tannic acid and catechin (+) as substrates at 1000 ppm concentrations, prepared in citrate buffer (pH 5.5) respectively. Enzyme activity was expressed in (U/ml). The enzyme kinetic constants KM and Vmax were determined by incubating the enzyme in the enzyme reaction buffer containing different concentrations of tannic acid (0.1 to 1%) at a pH of 5.5 for 30 min at 30°C, and enzyme activities were then calculated by using the software Graphpadprism5.0. The initial velocity data was plotted as the function of the concentration of substrate by the linear transformation of the Michaelis-Menten equation.

4.1.9. Effect of various metal salts, detergents and organic solvents, reducing agents and inhibitors on enzyme activity

4.1.9.1. Effect of various metal salts on tannase enzyme activity from A. niger

Effect of various metal salts on enzyme activity was evaluated by incubating the enzyme along with different concentrations (5, 10, 15 and 20 mM) of various metal salts in the enzyme reaction mixture for 1h followed by measuring the residual enzyme activity. The metal salts studied included sodium chloride, calcium chloride, magnesium sulphate, zinc sulphate, potassium sulphate, cobalt chloride, manganese chloride, ferric chloride, sodium molybdate,
barium chloride and cadmium sulphate which contributed the metal salts Na+, Ca\(^{2+}\), Mg\(^{2+}\), Zn\(^{2+}\), K\(^{+}\), Co\(^{2+}\), Mn\(^{2+}\), Fe\(^{3+}\), Cu\(^{2+}\), Mo\(^{6+}\), Ba\(^{2+}\), Cd\(^{2+}\) respectively in the enzyme reaction mixture, at a 1:1 (v/v) ratio, for 1 h, followed by an estimation of the residual activity.

4.1.9.2. Effect of various detergents on tananse enzyme activity from *A. niger*

The Effect of various detergents such as triton X-100, tween-80 and tween-20 (w/v) on enzyme activity was determined by conducting enzyme assay in the presence of each detergent and residual activity was calculated. After incubation of enzyme in different concentration of each detergent 0.2, 0.4, 0.6, 1% for 1 h, assay was conducted.

4.1.9.3. Effect of organic solvents on tananse enzyme activity from *A. niger*

Impact of various organic solvents on enzyme activity was evaluated by incubating the enzyme with each organic solvent for 1 h and 24 h and the residual activity was calculated. The organic solvents studied included acetone, formaldehyde, Tetro hydro furan and carbon tetra chloride at 10 % (v/v).

4.1.9.4. Effect of various Inhibitors on tananse enzyme activity from *A. niger*

Effect of enzyme inhibitors Sodium dodecyl sulphate and Sodium deoxycholate on tannase activity was evaluated. Each inhibitor solution was prepared at 1mM level and mixed at 1:1 (v/v) ratio with tannase enzyme. Residual tannase activity was calculated as described in section 4.1.9.6.
4.1.9.5. Effect of various reducing agents on tananse enzyme activity from \textit{A. niger}

Activity and stability of the enzyme in the presence of reducing agents were studied by incubating the enzyme solution with 10 \% (v/v) of $\beta$-mercaptoethanol for 1 h and the residual activity was estimated as described under section 4.1.9.6.

4.1.9.6. Residual Activity

Residual activity is the enzyme activity of the sample with respect to the original enzyme activity of the control sample and is expressed in percentage.

\[
\text{Residual activity} = \left( \frac{\text{Activity of sample (U/ml)}}{\text{Activity of the Control (U/ml)}} \right) \times 100
\]

4.1.10. Thermal stability of cashew testa by TGA analysis

Thermo Gravimetric analyser (TGA) analysis was done for the investigation of the thermal-oxidative properties from cashew industry waste cashew testa (husk). Thermal-oxidative stability studies were carried out using thermo analytical measurements (TG). Thermo analytical measurements were carried out using a SHIMADZU TGA-50H thermo gravimetric analyser. Thermo gravimetric (TG) measurements were performed at a scanning rate of 10º C/min. Samples of approximately 5 mg were heated from 5º C to 600º C. The measurements were carried out in a nitrogen atmosphere (20 ml/min).
All the experiments were performed in triplicate and average values were reported with standard deviation. All the experimental data were statistically analyzed using Microsoft Excel.

4.2. Results

4.2.1.1 Ammonium sulphate precipitation

Fractional precipitation with ammonium sulphate showed initial fractionation up to 50% ammonium sulphate addition and removed some of the non-enzyme proteins. Tannase was precipitated at and above 90% saturation with ammonium sulphate (Fig.4.1). Only 75% yield was obtained. On further dialysis of the precipitated enzyme protein, did not show any clear bands on PAGE and hence it was inferred that only minimum recovery of the enzyme is possible by this technique.

**Table 4.1. Effect of Ammonium sulphate concentration**

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Enzyme Activity of Pellet (U/ml)</th>
<th>Enzyme Activity of Supernatant (U/ml)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>44.46</td>
<td>45.8</td>
<td>0.09</td>
</tr>
<tr>
<td>60</td>
<td>47.18</td>
<td>54.84</td>
<td>0.18</td>
</tr>
<tr>
<td>7</td>
<td>47.51</td>
<td>41.85</td>
<td>0.05</td>
</tr>
<tr>
<td>75</td>
<td>48.02</td>
<td>45.05</td>
<td>0.167</td>
</tr>
<tr>
<td>80</td>
<td>50.32</td>
<td>68.88</td>
<td>0.081</td>
</tr>
<tr>
<td>90</td>
<td>10.4</td>
<td>46.3</td>
<td>0.012</td>
</tr>
</tbody>
</table>
4.2.1.2 Solvent Precipitation

In the case of solvent precipitation with acetone; the precipitated fraction showed negligible activity indicating the unsuitability of this method for fractionation of this enzyme.

4.2.1.3. Dialysis

Dialysis of the crude enzyme fraction for removing tannic acid present in the crude extract did not show much influence, since only very less amount of tannic acid was removed through the dialysis membrane (10 kDa cut off).

4.2.1.4 Gel Filtration Chromatography

Two ml of Dialyzed concentrated sample was loaded on a gel filtration column and 11 fold purification was obtained. Results obtained for purification of tannase enzyme is summarized in Table 4.2.

Fig 4.1. Ammonium sulphate concentration of Pellet and Supernatant on enzyme activity
Table 4.2 Purification profile of the tannase extracted from *Aspergillus niger*

<table>
<thead>
<tr>
<th>Purification protocol</th>
<th>Volume (ml)</th>
<th>Total Protein (mg/ml)</th>
<th>Total activity (U/ml)</th>
<th>Specific Activity (U/mg of Protein)</th>
<th>Yield of protein (%)</th>
<th>Yield of Activity (%)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>100</td>
<td>27.594</td>
<td>25.29</td>
<td>0.92</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation (80%)</td>
<td>67</td>
<td>5.794</td>
<td>19.034</td>
<td>3.285</td>
<td>21</td>
<td>75.26</td>
<td>3.57</td>
</tr>
<tr>
<td>Dialysis</td>
<td>15</td>
<td>2.380</td>
<td>14.54</td>
<td>6.11</td>
<td>8.6</td>
<td>57.49</td>
<td>6.64</td>
</tr>
<tr>
<td>Gel Filtration chromatography by Sephadex G-100 superfine.</td>
<td>5</td>
<td>1.195</td>
<td>12.218</td>
<td>10.22</td>
<td>4.33</td>
<td>48.31</td>
<td>11.11</td>
</tr>
</tbody>
</table>

4.2.2. Characterization of tannase enzyme from *A. niger* CEPC 11

4.2.2.1. Determination of the molecular weight of purified tannase enzyme

The sample on analysis with SDS PAGE showed a single band on staining with coomassie Brilliant blue G250 indicating the homogeneity of the preparation. SDS PAGE analysis of purified enzyme performed under non reducing condition (without β-mercaptoethanol) yielded a single band of 89.9 kDa, (Plate.4.1) which indicated the presence of monomers.
Lane 1: molecular mass protein markers; Lane 2: purified tannase

Plate 4.1. Determination of the molecular weight of purified tannase enzyme by 10% SDS PAGE.

4.2.2. Native PAGE analysis of purified tannase

Results observed for the Native Polyacrylamide gel electrophoresis (Plate 4.2) of the purified sample without tannic acid showed a single band.

Plate 4.2. Native PAGE analysis of purified tannase.
MALDI-TOF-MS analysis of purified tannase

The MALDI-TOF-MS analysis (Fig 4.2.) of purified tannase also indicated the presence of a single peak at m/z 91, 525.81.

**Fig 4.2.** Molecular mass spectroscopic analysis of purified tannase.

Confirmation of Gallic acid

The gallic acid were confirmed through FT-IR, HPTLC and HPLC.

FTIR

The FT-IR absorption for gallic acid has strong bands at 1687.71 cm\(^{-1}\) and 1514.12 cm\(^{-1}\) representing the C=C bond (1900-1500 cm\(^{-1}\)), O-H bonds at 3217.27 cm\(^{-1}\) (3700-2500 cm\(^{-1}\)), C-C bond at 1448.81 cm\(^{-1}\) and 1336.42 cm\(^{-1}\). This correlates with the presence of these bonds in a commercially available gallic acid run as standard (Fig.4.3).
4.2.3.2. HPTLC analysis

The method as described in the present study, utilize silica gel 60F254 TLC plate as stationary phase and Toluene: Ethyl acetate: Formic acid: Methanol (6:6:1:0.1 v/v/v/v) as mobile phase gives good separation of Gallic acid at Rf =0.46, from the other components present in cashew testa. The TLC plate was visualized under UV light at 254 nm and the HPTLC fingerprinted photographed chromplate is shown in Plate 4.3. The calibration curve of Gallic acid was found to be linear (200 -1200 ng/spot) dependent on the concentration against area. The equation of best fitting line was $Y = 1509.653 \times 482.357$. 5% alcoholic FeCl$_3$ solution was used as a visualizing agent to visualize the presence of poly phenols (Gallic acid) and bluish black colored bands indicated their presence. The peak areas obtained for standard gallic acid were used as a standard to quantify the amount of gallic acid present in the cashew testa. The presence of gallic acid was observed at $R_f$ value 0.41 in Plate 4.4. However the other bands at $R_f$ value of 0.51 and 0.47 were poly phenols which gave bluish coloration with ferric chloride solution and were present in trace amounts. The calibration curve depicted in Figure 4.4 indicated excellent linear relationship with the correlation coefficient 0.99896.
Plate 4.3. Identification of Gallic acid by HPTLC analysis.

Plate 4.4. HPTLC analysis of Standard Gallic acid and Sample
Fig.4.4. Calibration curve of gallic acid

4.2.3.3. HPLC

HPLC analysis of the sample and standard gallic acid gave single peak at the same retention time (2.24 and 2.32) (Fig.4.5 & Fig.4.6) indicated the purity of extracted and purified sample

Fig. 4.5. High performance liquid chromatogram of sample
4.2.4. Carbohydrate content

The carbohydrate content of the purified enzyme was determined by phenol sulphuric acid method. It was found that the \textit{A. niger} tannase was glycosylated to an extent of 6.02%.

4.2.5. Optimal temperature for tannase activity

The results presented in Table 4.3 and Fig. 4.7 indicated that the enzyme was active in the range of incubation temperature 25 - 60°C with maximal activity at 40°C (single level experiment). The enzyme was active up to 50°C and at temperatures above 80°C the enzyme activity declined. In fact, the tannase activity showed a linear increase along with increase in temperature, and the increase was rapid over a range of temperature varying from 32°C to 35°C.
### Table 4.3. Optimal temperature for tannase activity

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Temperature (°C)</th>
<th>Enzyme activity (U/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>25</td>
<td>40.32 ± 0.09</td>
</tr>
<tr>
<td>2.</td>
<td>30</td>
<td>69 ± 0.18</td>
</tr>
<tr>
<td>3.</td>
<td>35</td>
<td>75.2 ± 0.05</td>
</tr>
<tr>
<td>4.</td>
<td>40</td>
<td>80 ± 0.167</td>
</tr>
<tr>
<td>5.</td>
<td>45</td>
<td>65.4 ± 0.081</td>
</tr>
<tr>
<td>6.</td>
<td>50</td>
<td>54.5 ± 0.11</td>
</tr>
<tr>
<td>7.</td>
<td>55</td>
<td>50.2 ± 0.105</td>
</tr>
<tr>
<td>8.</td>
<td>60</td>
<td>39.8 ± 0.16</td>
</tr>
</tbody>
</table>

*Values are mean of 3 replicates with ±SD

**Fig 4.7.** Optimal temperature for tannase activity
4.2.6. Thermo stability of tannase at different temperatures

It is evident from the results presented in Fig.4.8 for the temperature stability studies conducted with tannase it is inferred that the enzyme has thermal stability over a broad range of temperatures (20-60°C) (Table 4.4) for one h since more than 50 % of the relative enzyme activity was retained after incubation. It may be suggested that the enzyme is thermostable up to 40- 60°C.

Table 4.4 Thermo stability of tannase at different temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>1hour</th>
<th>2 hour</th>
<th>4 hour</th>
<th>24 hour</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>40.99</td>
<td>23.12</td>
<td>18.13</td>
<td>11.17</td>
<td>0.739</td>
</tr>
<tr>
<td>30</td>
<td>68.56</td>
<td>40.0</td>
<td>25.0</td>
<td>11.33</td>
<td>0.532</td>
</tr>
<tr>
<td>40</td>
<td>80.52</td>
<td>60.7</td>
<td>32.2</td>
<td>11.5</td>
<td>0.486</td>
</tr>
<tr>
<td>50</td>
<td>53.79</td>
<td>48.45</td>
<td>29.6</td>
<td>10.66</td>
<td>0.619</td>
</tr>
<tr>
<td>60</td>
<td>50.68</td>
<td>33.23</td>
<td>23.3</td>
<td>10.2</td>
<td>0.712</td>
</tr>
<tr>
<td>70</td>
<td>39.46</td>
<td>29.8</td>
<td>15.5</td>
<td>9.8</td>
<td>0.491</td>
</tr>
<tr>
<td>80</td>
<td>27.32</td>
<td>24.12</td>
<td>12.3</td>
<td>9.1</td>
<td>0.864</td>
</tr>
</tbody>
</table>

Fig.4.8 Thermo stability of tannase at different temperatures
4.2.7. Optimal pH for tannase activity

Results depicted in Fig. 4.9 indicated that the tannase has optimum pH 5.5 for maximal activity (Table 4.5). In general, the tannase was active over a pH range of 3.0-8.0 and an increase in pH from 6.0 to 7.0 led to proportionate decrease in enzyme activity. Moreover more than 60% of enzyme activity was recorded at pH in the range between 4.0-6.0 and the stability of the pH was range from 4.0-6.0 (Fig 4.10).

**Table 4.5** Optimal pH for tannase activity

<table>
<thead>
<tr>
<th>Sl No</th>
<th>pH</th>
<th>Enzyme activity (U/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>57.7 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>64.1 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>76.0 ± 0.107</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>78.2 ± 0.10</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>81.1 ± 0.024</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>69.8 ± 0.012</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>48 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>36.2 ± 0.091</td>
</tr>
</tbody>
</table>

*-Values are mean of 3 replicates with ±SD
Fig 4.9. Optimal pH for tannase activity.

Table 4.6 pH stability of tannase enzyme

<table>
<thead>
<tr>
<th>Sl No</th>
<th>pH</th>
<th>Enzyme activity (U/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>57.166 ± 0.088</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>64.33 ± 0.105</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
<td>80.91 ± 0.167</td>
</tr>
<tr>
<td>4</td>
<td>6.0</td>
<td>72.81 ± 0.167</td>
</tr>
<tr>
<td>5</td>
<td>7.0</td>
<td>48.14 ± 0.432</td>
</tr>
<tr>
<td>6</td>
<td>8.0</td>
<td>29 ± 0.232</td>
</tr>
</tbody>
</table>

* Values are mean of 3 replicates with ±SD
Fig 4.10. pH stability of tannase enzyme

4.2.8. Kinetic Studies

Kinetic parameters $K_M$ and $V_{max}$ of the tannase were estimated by determining the initial reaction rate of tannase at various concentrations of tannic acid as the substrate and the data obtained is presented in Fig. 4.11. $K_M$ and $V_{max}$ were estimated by plotting the initial velocity data as the function of the concentration of substrate. Kinetic parameters were obtained from the Lineweaver-Burk plot (Fig.4.12) and then were recalculated by solving the Michaelis-Menten equation using Graphpad prism software 5.0. $K_M$ and $V_{max}$ were recorded as 0.1133 M (substrate concentration) and 44.79 $\mu$mol/min respectively.
Fig. 4.1. Kinetics of tannase at different tannic acid (substrate) concentration

Fig 4.12. LB plot for tannase kinetics.

4.2.9. Substrate specificity

Substrate specificity for tannase were tested with tannic acid and catechin as substrates at 100ppm and 1000ppm concentration. High level of affinity was recorded with 0.1M concentration of tannic acid followed by 0.01M to catechin.
4.2.10. Effect of various metal salts, detergents, oxidising and reducing agents and inhibitors on enzyme activity

4.2.10.1. Effect of various metal salts on tannase activity

Among the various metal ions evaluated for their effect on tannase activity, barium chloride alone showed enhanced enzyme activity which however declined along with increase in concentration in the medium (Table 4.7). However there was no inhibition of enzyme activity even at the highest concentration tested. Thus maximum activity of 299.6 U/ml was recorded for 1mM concentration compared to other concentrations. Cobalt chloride and Potassium sulphate also supported enhanced enzyme activity at concentrations from 1mM to 10mM. While at higher concentrations there was a decline in enzyme activity. Whereas NaCl presented enhanced enzyme activity at concentration from 10mM to 20mM. In general more than 50% residual activity was recorded for Calcium chloride and magnesium sulphate at 10mM-20mM. In the case of Sodium molybdate the enzyme activity enhanced at increased level up to 15mM and declined in 20mM. Zinc sulphate increased the enzyme activity along with increase in the concentration. Cupric sulphate inhibited the enzyme activity at 20mM compared to other concentrations. FeCl₃ and Mn²⁺ caused enzyme inhibition at 1mM and increased activity from 5mM to 15mM and declined thereafter in 20mM.
### Table 4.7. Effect of various metal salts:

<table>
<thead>
<tr>
<th>Metal salts</th>
<th>Residual activity at different concentrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1mM</td>
</tr>
<tr>
<td>Sodium chloride (Na+)</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride (Ca^{2+})</td>
<td>30.66</td>
</tr>
<tr>
<td>Magnesium sulphate (Mg^{2+})</td>
<td>41.12</td>
</tr>
<tr>
<td>Zinc sulphate (Zn^{2+})</td>
<td>51.5</td>
</tr>
<tr>
<td>Cobalt chloride (Co^{2+})</td>
<td>89.9</td>
</tr>
<tr>
<td>Manganese chloride (Mn^{2+})</td>
<td>95.21</td>
</tr>
<tr>
<td>Ferric chloride (Fe^{3+})</td>
<td>60.4</td>
</tr>
<tr>
<td>Cupric sulphate (Cu^{2+})</td>
<td>52.8</td>
</tr>
<tr>
<td>Sodium molybdate (Mo^{6+})</td>
<td>50.9</td>
</tr>
<tr>
<td>Barium chloride (Ba^{2+})</td>
<td>299.6</td>
</tr>
<tr>
<td>Potassium sulphate (K^+)</td>
<td>61.77</td>
</tr>
</tbody>
</table>
4.2.10.2: Effects of various detergents on enzyme activity

Effect of various detergents were evaluated in the Table 4.8. Among the three detergents studied both Tween 20 and 80 and tritonX supported enhanced enzyme activity at certain levels of concentrations. Tween 80 supported maximal enzyme activity at 0.4% (399.0U/ml). It was also observed that enzyme activity increased along with increase in concentration in Tween 20 from 0.2% to 0.4%. Similarly triton X also supported enhanced enzyme activity along with increase in concentration from 0.2 % to 0.4 %. However at all other concentrations, decrease in enzyme activity was recorded.

Table 4.8. Effect of various detergents on enzyme activity

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 %</td>
</tr>
<tr>
<td>Triton X 100</td>
<td>122.47 u/ml</td>
</tr>
<tr>
<td>Tween 80</td>
<td>364.0 U/ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>60.2 U/ml</td>
</tr>
</tbody>
</table>

4.2.10.3 Effect of organic solvents on enzyme activity.

Effect of organic solvents acetone, formaldehyde, Tetrahydro furan and carbon tetra chloride on tannase activity was tested (Table 4.9). From the results it was find out that acetone (79.23%) have the maximum residual activity followed by tetrahydrofuran (33.40%), Formaldehyde (26.83%) and carbon tetra chloride (12.01%).
Table 4.9. Effect of organic solvents on enzyme activity.

<table>
<thead>
<tr>
<th>Organic solvents (10%)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>79.23</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>26.83</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>33.40</td>
</tr>
<tr>
<td>Carbon tetra chloride</td>
<td>12.01</td>
</tr>
</tbody>
</table>

4.2.10.4 Effect of Reducing agent and inhibitors on enzyme activity

The effect of reducing agent β-mercaptoethanol (1mM) on the purified tannase activity showed that the enzyme activity was inhibited (12.01%) at room temperature for 30 min incubation. The enzyme activity was significantly reduced by the enzyme inhibitor sodium deoxycholate (Table 4.10) retaining only 4.5%, activity.

Table 4.10. Effect of Reducing agent and inhibitors on tannase activity.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration (1mM)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing agent</td>
<td>Sodium deoxycholate</td>
<td>4.542</td>
</tr>
<tr>
<td>inhibitors</td>
<td>β-mercaptoethanol</td>
<td>12.01</td>
</tr>
</tbody>
</table>
4.2.11. Thermal stability of cashew testa by TGA analysis

TGA is a thermal analysis technique used to quantify mass changes in a material as a function of heat. The TGA traces and mass spectra obtained for the tannins and tannic acid heated at a rate of 5 °C cm$^{-1}$ are shown in the Figures 4.13 and 4.14, which showed the dependence of the mass loss of the samples expressed as percentage of the initial mass and temperature.

In Figure 4.13 the tannin samples from cashew testa had the initial weight 4.86mg (sample weight). When the heat was applied, the percentage of mass loss between the temperature of 29.47°C and the complete dissociation temperature at 60°C were 89.91%, and 5% respectively. After that, the mass was found to be in the steady state condition (main degradation was observed in this range). Finally, after 500°C no change in the mass loss was observed.

TGA of tannic acid in Figure 4.14 shows three thermal zones, the first one is appreciated between 30.9 and 180°C with a mass loss at 93.35%. In the second level degradation was done at the temperature between 200 to 210°C and a mass loss of 88.53% and the complete degradation was observed at 380-600°C.
**Fig 4.13:** Percentage of mass loss in cashew testa (*Anacardium occidentale* L).

**Fig 4.14:** Percentage of mass loss in tannic acid.
4.3. Discussion

Conventionally Tannase concentration has been carried out by precipitation with salts or solvents. These techniques are simple, rapid, economical, and do not require sophisticated equipment. But they often lead to low recovery yield of enzymatic activity due to irreversible denaturation of protein. The precipitate formed at 50–90% saturation of (NH₄)₂SO₄, which showed a 4-fold increase in specific activity compared to the crude sample, was used for further purification employing Gel filtration chromatography. Elution profile from the Sephadex G-100 superfine column (2x70cm) furnished a single peak with tannase activity, which could be eluted with buffer containing 0.2M acetate buffer at pH 5.0. This step resulted in 48% tannase recovery (approx.11-fold purification) with a specific activity of 10.22 U/mg proteins. *Paecilomyces variotii* tannase purification by sulfate precipitation obtained 1.9-fold purification with a recovery yield of 34% (Battestin and Macedo. 2007b). Rajakumar and Nandy (1983) purified the enzyme by ammonium sulphate precipitation followed by DEAE-cellulose column chromatography and gel filtration on a Sephadex G-200 column. Lekha and Lonsane(1997) precipitated *Aspergillus niger*GH1 tannase with acetone. They obtained 6.2-fold purification but with a low recovery yield of 28%. Ma et al. (2015) studied the potential of aqueous two-phase extraction (ATPE) for the purification of tannase from *Aspergillus ficuum* Gim 3.6 and the best result of purification (2.74-fold) with an enzyme activity recovery of 77.17% was obtained in the system containing 17% (w/w) sodium citrate.
Ultra filtration is a pressure driven filtration of solutes through a membrane with a defined range of pore size and is used for purifying, concentrating and fractionating macromolecules (Brummer and Gunzer, 1987; Pohl, 1990). The molecules pass through the membrane and will be present in the ultrafiltrate. Larger molecules are retained and concentrated relatively to the starting solution. In the present study ultrafiltration proved very useful as a concentrating step. When 30 MW cut off membranes were used for concentrating the enzyme a total of 30 times concentration occurred for the sample. Even though the yield was very less in this case the enzyme was devoid of any other materials that forms complexes. This was then subjected to gel filtration chromatography with sephadex G-100 and get good result. Gel filtration appears to be an excellent and gentle technique to avoid inactivation of enzymes during purification (Scopes, 1987). It is a non denaturing separation technique, based on hydrodynamic volume and molecular size of the protein facilitates complete recovery of enzymes (Lillehoj and Malik, 1989). Ramirez-Coronel and co-workers (2003) purified *A. niger* tannase by preparative isoelectric focusing followed by ionic exchange (MonoQ column) and gel filtration chromatography (Sephadex G-100).

The purity was assessed by SDS-PAGE and the purified enzyme has molecular weight 89.9 kDa. It is discussed that tannase of *Aspergillus* strains have been reported to have a molecular mass of around 90-350 kDa (Ramirez-Coronel et al.2003). Lekha and Lonsane (1997) reported a molecular mass of 160,000 Da for the native enzyme produced by *A. niger* PKL104 as estimated by gel filtration; and under reduced conditions, a single protein band corresponded to a molecular mass of 42,000 Da, which indicated that the subunits are held together by
disulfide linkages that are disrupted on treatment with β-mercaptoethanol, thereby resulting in different electrophoretic motilities.

Hatamoto et al. (1996, 1997) cloned and sequenced the gene-encoding tannase, and a structural study of the enzyme subunit from A. oryzae gave the possibility to manipulate the producer systems to increase and to improve the levels of tannase activity. According to them native tannase consisted of four pairs of the two subunits, forming a hetero-octamer with a molecular weight of about 300,000 Da. Most of the available literature on tannase purification also indicated that the enzyme is composed of several subunits. There is only a slight band with the native page.

Mass spectrometry has been an established analytical technique in organic chemistry for many years. But the very low volatility of proteins made mass spectrometry useless for the investigation of these molecules. This difficulty has been circumvented by the introduction of techniques for effectively dispersing proteins and other macromolecules into the gas phase. These methods are called Matrix-assisted laser desorption/ionization (MALDI) and electrospray spectrometry. It is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules. In the present study MALDI-TOF-MS analysis of purified tannase indicated the presence of single peak at m/z 91, 525, thus definitely ruling out any possibility of multimeric protein being responsible for tannase activity (Plate Fig 4.2). Previously, Bacillus subtilis, K. pneumonia and Lactobacillus planetarium tannase were also found to be monomer with molecular
mass of 52, 50 and 46.5kDa, respectively. (Jana et al. 2013; Sivashanmugham and Jayaraman. 2011; Iwamoto et al. 2008).

A double extraction method of gallic acid from culture filtrate using ethyl acetate and diethyl ether has been reported in which the gallic acid produced was identified by its Rf value (0.698) obtained in thin layer chromatography and melting point of gallic acid (Karet al., 2003). The gallic acid produced by A. niger was identified by its Rf value in thin layer chromatography (0.43), and HPTLC. Ghosh et.al. (2011) recorded the Rf value of standard gallic acid as 0.90 where as the product of hydrolysis gave Rf values 0.89 and 0.88. In our study, gallic acid estimation gave the Rf value of 0.46 which was in conformity with the findings of Nainwal and Batsa, (2012) who reported 0.40 Rf value from leaves powder extracts of Droseraindica.

A comparative method with commercially available gallic acid was adopted in the identification methods. Presence of C=C, C-C, OH bonds identified by FT-IR represents the bonds present in the gallic acid structure (Fig 4.3). A significant peak obtained in HPLC analysis was supported by the same peak in the commercial sample of gallic acid (Fig 4.5). Polyphenols are considered as one of the important secondary metabolites produced by plants, and hence our HPTLC studies focused on the production of gallic acid using tannase from cashew industry waste.

All fungal and yeast tannases are glycoproteins with a variable content of carbohydrate ranging from 5.4 to 64% (Rodriguez-Duran et al. 2011). On the contrary, bacterial tannases seems not to present such posttranslational
modifications (Iwamoto et al., 2008; Skene and Brooker 1995). Tannase glycosylations consist primarily of neutral sugars like mannose, galactose, and hexosamines (Albertse 2002) and in the present study the carbohydrate content become 6.02%.

The effect of temperature on tannase activity was studied by incubating the enzyme at a temperature range of 25-60°C, using 0.1% tannic acid as substrate and 0.2M Acetate buffer (Fig 4.6). The optimal temperature previously reported for the maximal production of tannase in SSF was between 25 and 34°C for A. niger, A. acuelatus, Lactobacillus sp. and Paecilomyces variotii, (Mukherjee and Banerjee 2004, Sabu et al. 2006, Anwar et al. 2007, Banerjee et al. 2007, Battestin and Macedo 2007a). The optimum temperature for tannase activity was 40°C and optimum pH was determined as 5.5. The optimal pH for tannase production by Aspergillus species (A. foetidus, A. ruber, and A. niger,) in SSF has been found to be between 5.0 and 5.5 (Mukherjee and Banerjee 2004, Sabu et al. 2005a, Kumar et al. 2007).

Inoculation was carried out with spore or mycelia inocula, harvested from a liquid or solid medium often containing the inducer tannic acid to increase productivity in the subsequent culture (Aoki et al. 1976: Pourrat et al. 1982; Hadi et al. 1994). Oxygen appears to be an essential requirement for the biosynthesis of tannase (Yamada et al. 1968, Aoki et al. 1976, Pourrat et al. 1982 and Bajhomauf et al. 1994) presumably since insufficient oxygen inhibits growth and thereby tannase production. However, tannase synthesis by Lactobacillus plantarum was optimal in the absence of oxygen (Ayed and Hamdi 2002). Excessive oxygen
caused tannin oxidation resulting in reduced tannase synthesis. The moisture content, a crucial parameter during SSF. It was favorable at 62 and 72 for an *Aspergillus* sp and *Rhizopus* sp, respectively, cultivated on wheat bran (Chatterjee et al. 1996; Lekha and Lonsane 1997). Kar et al. (1998) claimed that a moisture level of 93 was optimal for tannase synthesis by their *R. oryzae* strain.

The kinetics of tannase biosynthesis and the modeling thereof has not been studied except by Van de Lagemaat (2001). A mathematical growth model for a batch SSF process for fungal tannase production was developed and tested experimentally. The unstructured model described the uptake and growth kinetics of *Pencillium glabrum* in an impregnated polyurethane foam substrate system containing tannic acid as main carbon source. In the present study KM and Vmax were recorded as 0.1133 M (substrate concentration) and 44.79 μmol/min respectively. The enzyme recorded a high level of substrate specificity in tannic acid when compared with Catechin. The values of kinetic constants (KM and Vmax) depend on the particular substrate used and the enzyme source. A wide range of values (2×10⁻⁵-1.03×10⁻³M) for KM and Vmax have been reported for tannases from several microorganisms (Bhardwaj et al. 2003, Rajkumar and Nandy 1983). Tannase from *A. niger* GH1 recorded KM and Vmax values of 0.41×10⁻⁴ M and 11.03 μmol/min, respectively, with methyl gallate as a substrate (Marco et al. 2009). The kinetic parameters of tannase self-immobilized on polyurethane particles were calculated to be 5 mM and 0.41×10⁻² mM/min for KM and Vmax. (Mata-Gomez et al. 2015)
Metal salts play important roles in the biological function of many enzymes. The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators. Those that participate directly in the catalytic mechanism usually exhibit anomalous physicochemical characteristics reflecting their entatic state.

At low concentrations, metal salts act as cofactors for many enzymes, thereby enhancing the catalytic activity of the enzyme, whereas high concentrations lead to a reduction in catalytic activity. This may be due to the partial denaturation of the enzyme as a result of the presence of excessive free ions in the enzyme extract. In the present study, K\(^+\), Mo\(^{6+}\), Mn\(^{2+}\), Cu\(^{2+}\) and Ca\(^{2+}\) inhibit the enzyme activity. The inhibitory effects of heavy metal ions are well documented in the literature (Valleeb and Ulmer 1972). Tannase from A. oryzae (Iibuchi et al. 1968) and P. chrysogenum (Rajkumar and Nandy 1983) were heavily inhibited by Zn\(^{2+}\) and Cu\(^{2+}\). Tannase from A. awamori reported that, (Beena et al. 2010) Mg\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Hg\(^{2+}\), Ba\(^{2+}\), Li\(^{2+}\), Cd\(^{+}\), and Al\(^{3+}\) inhibited the enzyme activity and A. niger was strongly inhibited by Cu\(^{2+}\). In the present study, tannase activity was significantly enhanced by Ba\(^{2+}\) and Na\(^+\), which correlates with factors influencing enzyme production significantly (Table 4.7).

Surfactants are substances that can alter the conditions prevailing at interfaces, and because of their amphiphilic property, they have a tendency to accumulate at interfaces and to adsorb onto surfaces. They can modify the surface tension by dispersing the proteins to the hydrophobic extreme of the peptide and interacting with the aqueous medium by another extreme. Surfactants can
denature the enzyme protein, and because of this reason, it is very important to
describe the effect of surfactants on the enzyme (Marco et al. 2009). In the present
study, enhancement in tannase activity were noticed in the presence of 0.4%
Triton X-100, higher concentrations of Tween-80, and at all concentrations of
Tween-20. Similar effects were also observed with a xerophilic fungus, A. niger
GH1 (Marco et al. 2009).

The effects of organic solvents on tananse activity were determined. Acetone and tetrahydrofuran increased the enzyme activity where as
formaldehyde and carbon tetra chloride found to be decreased enzyme activity
(Table 4.9). The former group of solvents may facilitate substrate availability at the active site of the enzyme and enhance the rate of catalysis. On the contrary, Xu et al. (1997) reported that the polar solvents reduce the catalytic efficacy by absorbing the essential water molecule from the enzyme. In later the effect of β-
mercaptoethanol also studied in the inhibition of enzyme activity.

TGA is a thermal analysis technique used to quantify mass changes in a material as a function of heat. Thermogravimetric analysis showed that the tannins exhibited a similar thermal stability pattern as that of tannic acid, which also have more depolymerization stages than tannins. In the present experiment, the thermal stability of tannase was determined between 10-60ºC for 24 h. In Fig 4.13Cashew testa the percentage of mass loss between the temperature of 29.47º C and the complete dissociation temperature at 60ºC were 89.91 %, and 5 % and tannic acid mass loss showed at 93.35% and 88.53% and the complete degradation was observed at 380-600ºC respectively, while in cashew gum decomposition temperature was around 240 ºC (Mothé and Freitas 2014).
Fig 4.15. Diagrammatic representation of characterization of Tannase

4.4 Conclusion

The present paper, reports the purification of tannase from A. niger. SDS-PAGE analysis indicated that the enzyme protein molecular mass was 89.9 kDa. Enzyme activity was stable up to the temperature of 40 °C. Similarly, the enzyme was active over a wide range of pH values and inhibited by increasing the substrate concentration. Gallic acid is also determined as an inter-mediatory by-product of this technology and it has under application in various industrial processing, especially in the food-processing and pharmaceutical industry. Since the enzyme is active over a wide range of pH and temperature it could find potential use in the food-processing industry. The economic production cost of tannase from cashew testa by SSF is cheaper compared to other production strategies. So the current work leads to new interesting prospects, which could be pursued to gain further insights into economic tannase production.