Chapter 7

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A potential tannase producing fungus was selected among 6 tannase producers isolated from soil samples collected from different cashew factories located in Kollam, Kerala. The fungus was identified as Aspergillus niger and was deposited in MTCC culture collection with accession number 5898. The selected fungus was subjected to ribotyping using partial 18S rRNA gene, for confirming its identity, which could be amplified and sequenced. Partial sequence of the 18S rRNA gene was submitted to GenBank (Accession number: KM516789). Present study indicated scope for utilizing cashew testa as a potential substrate for tannase production due to its high content of hydrolysable tannin.

Different natural substrates were tried as carbon source, both independent and in combination with tannic acid, for tannase production by A. niger CEPC 11. Various bioprocess parameters affecting tannase production by the fungus, under different modes of fermentations like submerged fermentation and solid state fermentation were optimized towards maximal enzyme production. Process parameters important in tannase production were identified by Plackett and Burman design and the parameters with significant effects on enzyme production were optimized statistically employing Box-Behenken method. The optimum values of parameters obtained through RSM were cashew testa (23%), Di-KHPO4 (3.40mM), Sodium Chloride (0.47mM) and temperature (32-35°C).
Enzyme purification was carried out employing ammonium sulphate precipitation followed by chromatography. 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. The enzyme concentrate obtained after ultrafiltration through ultra filtration membrane showed a single band of about 89.9 kDa in SDS PAGE. In native PAGE it shows 66.6 kDa. Phenolsulphuric acid assay used to determine carbohydrate content in tannase enzyme indicated to 6.02%.

Effect of pH on purified tannase was determined in different buffers from pH 3 to pH 8. Maximum activity was observed at pH 5.5 while the stability of the enzyme was retained at pH 4.0-6.0 up to 24 h of incubation and hence the enzyme is considered to be active. Effect of temperature on the activity of purified tannase was determined at various incubation temperatures (10°C to 60°C) and 40°C was found to be the optimum temperature although the enzyme was active over a wide range of temperatures. Maximum temperature stability was also observed at 30-50°C up to 24 h. The Km and Vmax were estimated by plotting the initial velocity data as the function of the concentration of the substrate. Km and Vmax were recorded as 0.1133 M (substrate concentration) and 44.79 μmol/min respectively. MALDI-TOF-MS analysis of purified tannase also 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.

The gallic acid produced by *A. niger* was identified by its Rf value in thin layer chromatography (0.46), and HPTLC. Presence of C=C, CC, OH bonds identified by FT-IR represented the bonds present in the gallic acid structure. Significant peaks obtained for the test sample in HPLC and HPTLC analysis were very much same obtained with the commercial sample of gallic acid, confirming the identity of gallic acid produced during fermentation. All these analysis were confirmed that gallic acid was produced as a byproduct during the hyrolysis of tannic acid.

More than 75% of the enzyme required the presence of metal salts activators to express the full catalytic activity. Results obtained suggested that K⁺, Mo⁶⁺, Mn²⁺, Cu²⁺ and Ca²⁺ inhibit the enzyme activity. Tannase activity was significantly enhanced by Ba²⁺ and Na⁺, which correlates with factors influencing enzyme production significantly. The effects of organic solvents on tannase activity were determined. Acetone and tetrahydrofuran increased the enzyme activity where as formaldehyde and carbon tetra chloride found to be decreased enzyme activity. The oxidizing agent, acetone enhanced the tannase activity enormously upto 10 % (v/v) concentration. Inhibition of enzyme activity was recorded at higher concentrations of detergents like triton X, tween 80. Complete inhibition of tannase activity was observed in the presence of sodium deoxy cholate and β-mercapto ethanol.
TGA is a thermal analysis technique used to quantify mass changes in a material as a function of heat. Result observed that, the tannin samples from cashew testa has the initial weight of 4.86mg (sample weight. When the heat was applied, the percentage of mass loss between the temperature of 29.47º C and the complete dissociation temperature at 60ºC 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 ºC no change in the mass loss was observed. TGA of tannic acid shows three thermal zones, the first one is appreciated between 30.9 and 180ºC with a mass loss at 93.35%. In the second level degradation was done at the temperature between 200 to 210ºC and in this section is seen a mass loss at 88.53% and the complete degradation was at 380-600ºC.

DNA isolation was standardized for the strain and the degenerate primers were designed from the already available sequence of *Aspergillus sp.* tannase gene deposited at Genbank. Amplification of genomic DNA with degenerate primers forward TAN 2 and reverse TAN 2 yielded an amplicon (TAN2) of approximately 550 bp at an annealing temperature of 50ºC. The insert was further sequenced and confirmed as tannase gene. The comparison of nucleotide sequence information and the deduced amino acid sequence with the known proteins from Genbank indicated that this gene encode tannin acyl hydrolase. A total of 550 of tannase gene sequence was obtained from the genomic DNA and sequence analysis revealed an open reading frame consisting of 530bp (147 amino acids) of one stretch in +2 strand. The longest ORF shows homology to *Aspergillus* tannase. Genscan predicted a sequence of 147 amino acids, which is an internal exon as the
predicted peptide without introns. GOR secondary structure prediction indicates the presence of alpha helix, extended strand and random coil and no beta.

There are very limited studies of tannase enzyme in the molecular level, including the details of catalytic and substrate binding sites. 3D model is generated by MUSTER. Aligned length is 140 amino acids. Sequence identity by threading results is 0.325. Z score is 13.5 which were considered as a good template for modeling. The final model is generated by MODELLER v8. This is the first report of three dimensional structure of tannase from *Aspergillus niger*.

Fruit juice clarification is one of the important applications of tannase. One mL of tannase (10.22 U/mg) was used to clarify 10 mL of fruit juice. 27.36% of sample was clarified when treated with the purified enzyme having 10.22U/mg activity. The efficiency of partially purified enzyme on tea cream solubilisation was tested and 10.22U of enzyme could give 29.5% solubilisation with in 1h which could be increased by optimization of temperature, incubation time and concentration of the enzyme.

Tannase enzyme has applications in the treatment of tannery effluents. Tannery effluent was collected from the local leather industry and the physico-chemical parameters were estimated using standard methods. 5 ml of the tannery effluent was treated with 1 ml of purified enzyme (10.22 U/mg and checked for heavy metals such as Cadmium (Cd), Lead (Pb), Nickel (Ni) and Copper (Cu) using the Atomic Absorption Spectrophotometer (AAS). The result of the test shows that Lead (Pb), Cadmium (Cd), Nickel (Ni) is present at very low concentrations. In our present study we could observe an interesting result of
reduction of copper ions in the tannery effluent when treated with tannase. Copper (Cu) is present at a concentration of 1.05mg/L and when treated with tannase enzyme the value down upto 0.34mg/L. This may be due to the complex ion formation with the product gallic acid, which cannot be detected with AAS.

Fig.7.1 Schematic diagram showing major objectives of the present study

This is the first report on production of tannase by Aspergillus niger under SSF with cashew industry by-product cashew testa as the substrate. The use of residues is an alternative to solve pollution problems that can be caused by an incorrect environmental disposal. Cashew testa, (Cashew industry waste) which is a natural substrate has scope for use as the ideal source of tannin for maximal tannase production. This technology helps to reduce the environmental pollution caused by burning of waste cashew testa or shell by producing valuable enzyme from it. The left over solid substrate after fermentation can be sterilised, dried into fine powder and can be used as manure also. Hence no waste material is generated
from this technology. 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.

**Future Perspectives**

Scientists have developed various enzyme technologies for the production and purification of the enzyme at laboratory level. In contrast, the use of recent techniques of molecular biology has allowed the development of highly capable processes for production and recovery of the enzyme and it will displays in the future years. The results have been positive, but research is needed on basic and applied aspects of tannase, such as regulation of the enzyme, metagenomics, new expression systems, design of new bioprocesses using emerging large-scale development technologies, efficient and cost-effective downstream processing, new therapeutic development, and propose of new applications for the enzyme such as the production of antioxidants from waste materials or the bioremediation of tannery effluents. Also there is further scope on molecular structure elucidation and enzyme engineering towards a wide range of applications. Obviously there is option for cloning and expressing the gene encoding this tannase enzyme into a domesticated host and expression of the enzyme for industrial production.