6. SUMMARY

Insects are the most successful group of animals, both in terms of diversity and survivability in various ecological niches. The microbiota within their guts plays important roles by engaging in beneficial and pathological interactions with these hosts. Microorganisms colonize the insect gut through food and play a significant role in digestion and metabolism. Microbes are considered as a source of good and robust enzymes which can be used at higher temperature with greater stability. In our study, the mole cricket genus *Gryllotalpa krishnani* belongs to subfamily of Gryllotalpinae was used to explore the tannase producing bacteria. A total of 45 isolates with tannase activity were obtained from the gut contents of *G. krishnani* by plating on tannic acid supplemented medium. Among these, 5 isolates were able to show significant growth and hydrolyzing property on direct plating. These 5 isolates namely TAH 6, TAH 13, TAH 36, TAH 38 and TAH 41 were found to be positive for visual detection method as indicated by a color change of the medium from yellow to brown color using tannic acid as the substrate. However, TAH 41 strain was able to resist 1 % tannic acid and zone of hydrolysis (diameter) was higher than the other bacterial strains. Secondary screening of these isolates was subjected liquid culture analysis, in this experiment TAH41 showed maximum activity (2.128 U/ml). Bacterial isolate TAH 41 was identified as *Enteobacter cloacae* based on various morphological characters, biochemical characterization and 16S rDNA sequencing analysis. BLASTn analysis showed that our strain *Enterobacter cloacae* (KX156583) 98% homology with the existing sequence.
Process optimization for maximum tannase production was now attempted from *Enterobacter cloacae*. To begin with, under submerged conditions, these bacteria produced only 2.128 U/ml of tannase under initial unoptimized conditions of medium. The culture conditions of *Enterobacter cloacae* were optimized for tannase enzyme production. The tannase enzyme production was found to be maximum 3.871 U/ml at an initial pH 6.0, at 50°C in 18 h incubation and 100 rpm with glucose as carbon source and ammonium nitrate as nitrogen source. HPLC analysis showed the degradation of tannic acid to gallic acid and glucose by *E. cloacae* tannase enzyme. Further FT-IR analysis also confirmed the degradation of tannic acid to gallic acid and glucose. The polyacrylamide gel electrophoresis and zymogram analysis revealed that tannase was a monomer with molecular weight of ~ 45kDa protein.

Tannase enzyme purification was carried out employing ammonium sulphate precipitation followed by two step chromatography techniques. Precipitation with ammonium sulphate 60% saturated condition give satisfactory tannase enzyme activity further passed through 50 kDa cutoff membrane. Later filtrate was passed through ion exchange chromatography and sephadex G 100 column, the purified tannase was yielded 29.97% and 20.25% with purification fold 1.45 and 1.96 respectively. The purified enzyme showed a single band of molecular weight about ~45 kDa in SDS-PAGE and zymogram analysis. The isoelectric point of this tannase was determined with thin-layer gel isoelectrofocussing, using Ampholine pH range 3.0 to 10.0, and the results indicated that it was glycosylated and pI was 7.0. Purified tannase enzyme again confirmed the size was ~45 kDa with 2D gel electrophoresis and MALDI-TOF/MS analysis. HPLC analysis was performed purified tannase
enzyme from *E. cloacae*, based on their retention time and spectra it was confirmed as gallic acid and glucose by product. TLC analysis also confirmed gallic acid as a single product of tannic acid hydrolysis detected as single spot on plates. *R*<sub>f</sub> value of the sample was laid closer to that of standard gallic acid. NMR and FT-IR analysis also confirmed the identity of gallic acid. Through circular dichroism the secondary structure prediction showed the presence of alpha helix, extended strand and random coil and no beta structures, the purified tannase enzyme was a globular protein. The morphology of purified tannase was ranged from 50 to 300 nm diameter with an average of about 120 nm, 4~11 nm in height was analyzed by atomic force microscopy.

The purified tannase was analyzed for the pH, temperature and stability of time, the maximum activity was observed at pH 6.0, 50°C for 60 min incubation time period. Purified tannase of *E. cloacae* is salt tolerant upto 3 M NaCl that retained 68% of its original activity after 24 h of incubation. The *K*<sub>m</sub> and *V*<sub>max</sub> were estimated by plotting the initial velocity data as the function of the concentration of the substrate. Tannic acid concentration in the range of 0.05–0.5 mM, *K*<sub>m</sub> was obtained as 3.0×10<sup>−3</sup> M with a *V*<sub>max</sub> 4.401 U/ml. Among the various inhibitors tested, methyl gallate and n-propyl gallate were found to be the strongest inhibitors of tannase but gallic acid could not inhibit the tannase activity. Significant loss of activity was observed in the presence of Triton 100-X followed by Tween 80, however no loss of activity with EDTA. Whereas sodium thioglycolate enhanced the tannase activity, Maximum inhibition of the enzyme occurred in the presence of Cu<sup>2+</sup> (25%), Fe<sup>2+</sup> (51%) followed by P<sup>3+</sup> (26%), Ba<sup>2+</sup> (35%), Hg<sup>2+</sup> (30%), Ag<sup>+</sup> (29%), Na+ (8%) and K+ (39%) at 5 mM concentration. The decreased activity in
the presence of divalent cations could be due to the non-specific binding or aggregation of the enzyme. The toxicological data suggest that tannase enzyme concentration upto 1800 µg/ml has no demonstratable ill effect on Green monkey kidney Vero cell, provide a potential basis of biotechnological attention which, given its GRAS (Generally Regarded As Safe) status, can be further exploited in food and feed sectors.

To clone the gene encoding tannase, PCR strategy was employed using degenerated primers deduced from the amino acid sequence of tannase gene. The amplification of genomic DNA with primer set F2-R2 yielded an amplicon size of approximately 1100 bp and 950 bp with the expected amplicon size. PCR product was cloned in pGEM-T vector and transformed with E. coli DH5α as host cell. The nucleotide sequence was converted to protein and analysed for the BLAST analysis. Deduced amino acid sequence was compared to the sequences of other tannase/feruloyl esterase characterized standard search algorithm (BLASTP). Analysis of the sequence of this 283aa revealed strong homology with the sequences tannase/feruloyl esterase in the GenBank and EMBL databases. The results showed resemblance in its amino acid sequence with the earlier reported tannase of bacteria. Conserved domain search (RPSBLAST) analysis confirmed the presence of catalytic domain of tannase/feruloyl esterase.

Evaluation for various applications revealed that this tannase could carry out various hydrolysis reactions and esterification for the synthesis of commercially important products. The tannase efficiently degrades tannic acid to gallic acid within 60 min of incubation (3.61 U/ml) and reduces the production of gallic acid (2.88 U/ml) at 75 min incubation. Experiments were also carried out to determine the
optimal pH and temperature for gallic acid production and it was observed that pH 6.0 and 50°C was found to be optimum. In food industry, tannase mediated biotransformation of black tea (CTC and Kangra orthodox) was investigated. Black tea infusion treated with tannase showed that both epigallocatechingallate (EGCG) and epicatechingallate (ECG) of tea catechins were hydrolysed by this enzyme into epigallocatechin (EGC) and epicatechin (EC), respectively, increase in gallic acid (GA) concentration. The tannase was also evaluated for its potential for tannery effluent treatment, results of the present study revealed that; there is a considerable reduction in the values of important parameters. The treatment of the effluent with the enzyme reduces the color. Significant reduction in the values of TDS, TSS, COD, chloride and tannin by the enzyme was observed. Hence, this tannase can be successfully exploited for the treatment of tannery effluent. Removal of stains of tea and betel leaves was examined on small pieces (5 x 5cm) of clean cloth. The results are presented in showed that, cloth pieces having tea and betel stain when washed in solution containing detergent and tannase showed best brightness as compared to other treatments. Increase in percentage reflectance of 61 and 72 as compared to 18.14 and 15.06 for betel and tea stains (control) was achieved.
7. CONCLUSION

Screening of gut microbiome from the insect G. krishnani revealed the presence of diverse group of microbes producing tannase enzyme. Tannase are a family of esterases that catalyze the hydrolysis of the galloyl ester bond in hydrolyzable tannins to release gallic acid. The tannase enzymes have reported for its wider applications in food, feed, beverage, pharmaceutical, and chemical industries. Nevertheless, the tannase enzyme possesses several interesting phenomenon to be explored, however little is known about them at the molecular level, including the details of the catalytic and substrate binding sites in the tannase enzyme. Hence, recent biotechnological intervention, genetic manipulation through bioinformatics prediction approaches must be made to enhance the functional property of tannase enzymes to exploit them for various industrial applications.