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ISOLATION AND CHARACTERIZATION OF TANNASE PRODUCING BACTERIA FROM THE GUT OF *GRYLLOTALPA KRISHNANI*

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ABSTRACT

Gryllotalpa krishnani mainly eats the degradable food waste, so the insect gut which may contains an interesting diversity of microorganism not usually found elsewhere. The tannase enzymes have been reported for its wider applications in food, feed, beverage, pharmaceutical, and chemical industries. In the present study,microorganism were isolated from the gut of *Gryllotalpa krishnani* and characterized for tannase activity. A total number of 40 bacterial strains were isolated and checked for tannase activity. Among them, 4 strains produced high level of tannase activity in plate assay. Further, to confirm the identify of bacterial isolates strains were subjected biochemical and 16S rDNA sequencing analysis. The results confirmed the identity if the isolates belonging to *Enterobacter cloacae* (15), *Bacillus subtilis* (25), *Enteroinbacteriaceae* bacterium (39) and *Bacillus cereus* (40). The four bacterial strain where subjected for the substrate utilization. Interestingly, all the isoaltes showed clearing zone upto 2% tannic acid. Tannic acid degrading microbiota detected in the present study may endow the insect with some ecological advantages by enabling them to overcome the anti-nutritional effects of plant tannins.

Keywords: Tannase enzyme, FT-IR, Insect, *Gryllotalpa krishnani*

INTRODUCTION

Insects are one of the most diverse groups of living organisms on earth (**Chapman, 2006; Erwin, 1982**). Due to their diverse behaviors and feeding habits almost no terrestrial food source can escape the consumption by one or more insect species. Despite the diversity the highly interdependent and well regulated symbiotic interactions with microorganisms seem to be an important common property for different insect species (**Brauman *et al.*, 1992**). The role of microorganism is insects has been recognized by the distribution of microbial community in the insect gut has been reported by (**Campbell, 1989**). Insect gut microorganism plays an important role in the host nutrition, development, resistance to pathogens and reproduction (**Moran *et al.*, 2005**). Insect anchorage gut microbial communities range from simple to complex community (**Handelsman, 2005; Liburn *et al.*, 2001; Vasanthkumar *et al.*, 2006**). The gut community study gives the understanding about the function of microbial consortium and insect biology (**Leadbetter *et al.*, 1999**).
In some cases, loss of microorganisms often effect in unusual development and reduces the survival of the insect host (**Fukastu, 2002**). Some specific roles of microorganism in the insect guts have been disclosed, including cellulose and lignocelluloses digestion, etc., the bacteria in the insect gut of some specialized niche feeders, such as termites and aphids, have attracted wide attention because of the microbial digest enzymes achieving particular biochemical transformations (**Brauman *et al.*, 1992; Chen, 1997; Warnecke *et al.*, 2007**). Though, relatively little is known about insects feeding on foliage, where no strict symbiotic interaction has been proposed so far. In fact, most of them are herbivores (**Carter, 1984; Daly, 1998**) and their gut content (food bolus) is not sterile (**Dillon, 2004**). Indigenous insect gut bacteria of insects have been found to detoxify harmful secondary metabolites (**Morrison *et al.*, 2009**) and to protect the host against the colonization of pathogens. The mole cricket genus *Gryllotalpa* belongs to subfamily of *Gryllotalpinae*, in total, 65 species of *Gryllotalpa* has been recorded (**Ma, 2010**). In India five species of *Gryllotalpa* namely *G. africana, G. hirusta, G. minuta, G. orientatlis* and *G. ornata* are known (**Chandra, 2011**). Further studies on the Indian *Gryllotalpa* gut microbes are needed (**Chopard, 1969**). Tannase (tannin-acyl-hydrolase, E.C. 3.1.1.20) is an industrially important microbial enzyme that catalyses the hydrolysis of ester bonds (galloyl ester of an alcohol moiety) and despised bond galloyl ester of gallic acid, ester bonds involving a screen microorganism from different sources, for novel and competent tannase scattered with the tannin degrading enzymes for their benefit (**Raghuwanshi *et al.*, 2011**). Gallic acid, the major hydrolytic product of tannic acid, is used in food, cosmetics, adhesives and in the synthesis of potent antioxidant, propyl gallate (**Aithal and Belur, 2013**) such as bacteria, yeast and filamentous fungi are known as tannase producers. A foremost problem in the utilization of fungal strains for industrial applications is that degradation by fungi is relatively slow and difficult to genetic manipulation because of their genetic complexity (**Beniwal *et al.*, 2013**).
The major role of tannase producing insect gut microbes such as bacteria like *Citrobacter* sp, *Klebsiella pneumoniae* and *Enterobacter* sp. Microorganism such as fungi, yeast also efficiently producing tannase enzymes more significant due to their potential role in the tannase is also largely applied in textile and tannery industries for treatment of their effluents, to overcome serious environmental pollution (**Arijit *et al.*, 2014; Jian *et al.*, 2014**) and widely used in the fine chemical industry, pharmaceutical, leather industry and food industries. So far, the main application of the tannase is instant tea, corn liquor, as well gallic acid production from plant materials high in gallotannins. Tannin acyl hydrolase is also used as clarifying agent in juice and flavoured coffee soft drinks (**Aguilar, 2001; Banerjee, 2007; Belmares *et al.*, 2004**). The rumen microbial population presents a rich and, until recently underutilized source of novel enzymes with tremendous potential for industrial application (**Kang *et al.*, 2015**). The aim of the present study is to identify the bacteria producing tannase from the insect gut of *Gryllotalpa krishnani*.

MATERIAL AND METHODS

The insect was collected in wet soil with near to a kitchen waste, Salem district, Tamil Nadu, India. The soil was buried up to 10-15 cm depth by a digger and a colony of this species was found beneath the soil. The insect was collected dissection in our laboratory conditions (latitude: 11.6500 °N, longitude: 78.1600 °E; elevation: 154 ft (46.7 m).
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Strains and growth conditions

These strains were originally isolated from or and culture were grown on medium for **tryptone soya agar.** All bacteria were incubated at 37° C in 24hrs.

Culturing of the gut microbiota and tannase plate assay

The dissected gut was suspended in 10mM sterile phosphate-buffered saline (PBS) **Sambrook *et al.*, (1989)**. The guts were sonicated (50/60 Hz,117 V, 1.0 Amps; Branson Ultrasonics, Danbury, CT) for 30 Sec, macerated with a plastic pestle, and vortexes at medium speed for 10 sec to separate bacterial cells from the gut suspension were cultured immediately on nutrient agar plates and plates were incubated for 24 hours at 37 °C. Screening of the potent tannase producing bacterial isolates were screened for tannase activity by hydrolysis tannin test. After incubation plate was flooded with thereafter, the isolation of tannase-producing bacteria was carried out on nutrient agar plates supplemented with tannic acid (2%; filter sterilized). Addition of tannic acid to nutrient agar forms tannin–protein complex; cleavage of this complex by bacteria producing tannase forms a zone around the colonies.

Genomic DNA extraction of bacteria

The genomic DNA was extracted from the isolated bacteria colonies were by using the slightly modified protocol described **Broderick *et al.*, (2004)** the 12hrs cultures of bacterial isolates were taken in the micro centrifuge tube. The tube was centrifuged at 10,000rpm for 10min. the pellet was collected and 90 µl of 10% SDS was added. The tubes were incubated at 37 °C for 1 hrs. After incubation, addition 150 µl of 5M NaCl was added prior to the addition of 100µl of 10% Cetyltrimethyl ammonium bromide (CTAB). The sample was mixed thoroughly and incubated at 65 °C kept in a water bath for 30min, after incubation, add phenol, chloroform and isoamylalcohol in the ration of 25:24:1 (Vol/Vol/Vol). The tube was centrifuged at 13,000rpm for 15min and the aqueous layer was separated into a fresh tube. Then it was precipitated with 70% ethanol and centrifuged at 7000rpm for 5min. Pellets were suspended in 30µl of TE buffer. The DNA sample was separated according to their molecular weights under electrophoresis system. Finally the DNA band was visualized under gel documentation system (Lark, Germany). The DNA concentration was determined by measuring the absorbance at the ratio 260/280nm and the DNA suspension was stored -20 ºC it is used for further analysis.

PCR amplification of 16S rDNA gene

The selected bacterium was identified on the basis of its 16S rDNA sequence. DNA from the bacterial cells was isolated using QIAamp DNA Purification Kit (Qiagen, Japan) and electrophoresed in agarose gel. Fragment of 16S rDNA gene was amplified by PCR upto 30 cycles (using the following profile: initial denaturation, 95 ^{o}C for 2min; final denaturation, 94 ^{o}C for 30s; annealing, 52 ^{o}C for 30s; extension,72 ^{o}C for 90s; final extension,72 ^{o}C for 10min). Amplified PCR product was purified using Qiagen Mini elute gel extraction kit (Qiagen, Japan). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 27F AGAGTTTGATCCTGGCTCAG and 1492 R GGTTACCTTGTTACGACTT primers using BDTv3.1 Cycle sequencing kit on (ABI3730xl) Genetic Analyzer (**Maity *et al.*, 2011**). A single discrete PCR amplicon band of 1500bp was observed when resolved on 1.2% agarose gel.

Phylogenetic analysis

The reference sequences required for comparison were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank). The aligned sequences were then manually checked for gaps in each row and saved in molecular evolutionary genetics analysis (MEGA) format using MEGA v.2.1 software. Pairwise evolutionary distances were computed using the Kimura 2-parameter model (**Kimura, 1980**). To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method. The bootstrapped dataset was used directly for constructing the phylogenetic tree with the MEGA program or for calculating multiple distance matrixes. The multiple distance matrix obtained was then used to construct phylogenetic trees using the neighbor-joining method of **Saitou and Nei (1987)**. All of these analyses were performed using MEGA v.2.1 (**Kumar *et al.*, 2004**).

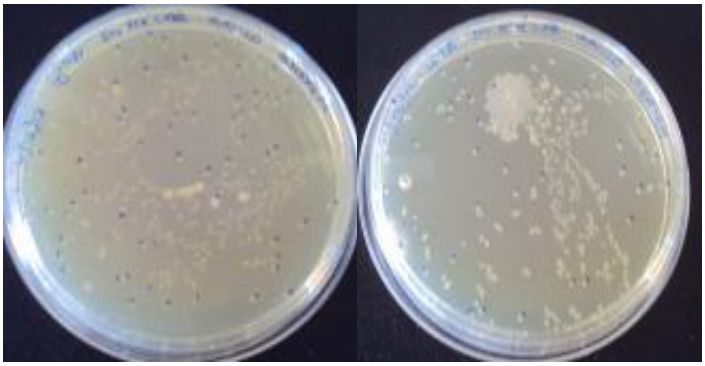
FT-IR analysis

In determining the possible functional groups FT-IR analysis was performed using Perkin Elmer's most power, which is used to detect the characteristic peaks and their functional groups. The vibration pattern that appears in the infrared spectra provides information about the chemical functional group of the sample. Tannase enzymes prepared and 500µl of 1% tannin was mixed together and incubated at 37 °C for 30min. A fraction of sample was encased directly in sample holder and spectra were scanned from 500-4000cm^{-1}.

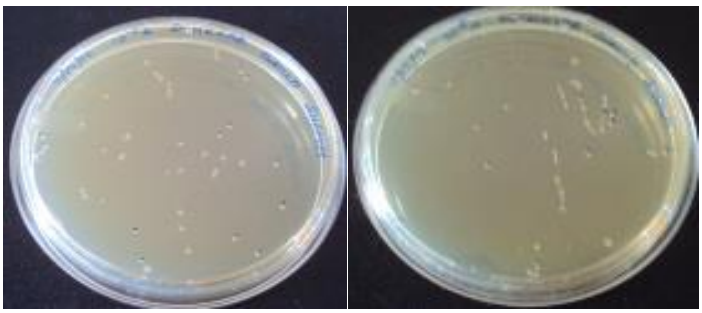
RESULTS

G. krishnani was dissected in the laboratory conditions and plated in the nutrient agar. Enumeration of microbial flora in the GI tracts of *G. krishnani* studied revealed that in general the number of cultured aerobic bacteria was higher in the gut region (Figure 1). Maximum number of microbiota 8.2×10^{6} CFU/ml was found in the gut region of *G. krishnani*. Forty bacterial strains shows tannase activity and further screening revealed 4 strains with different morphological appearances showed more tannase activity (Figure 2). The tannase secreting microbes were found growing at a fast pace despite the presence of antimicrobial tannic acid in the media. The four tannase producing bacteria were subjected to different concentration of tannic acid. The entire four strains showed clearing zone in 0.5%, 1%, 1.5% and 2% of tannic acid and the results confirmed that bacterial strains were also able to resist 2% tannic acid (Figure 3). *G. krishnani* is the mainly concerned as much to seek in its digestive systems for the search of beneficial microbes which is responsible for the digestive a large amount of tannase producing microbes.

Figure 1 Dissection of insect *Gryllotalpa krishnani* midgut



A **B**



C **D**

Figure 2 Enumeration of gut microbes of different dilution from insect *G. krishnani* maintained at 37 °C. Serial dilution of bacterial growth A-10^{4}/ ml ; B-10^{5}/ml; C-10^{6}/ml and D-10^{7}/ml .

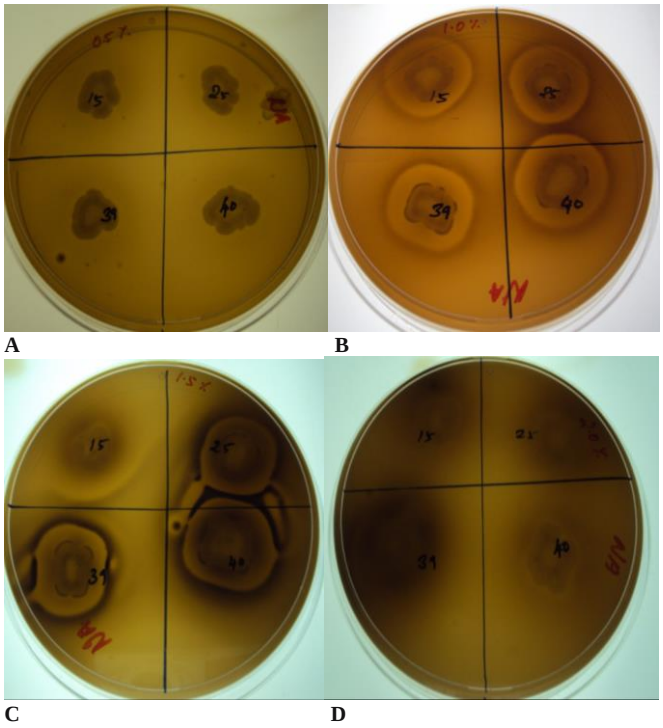


Figure 3 Efficiency of tannase-producing bacteria in different concentration tannic acid. A-0.5% Tannic acid; B-1.0% Tannic acid; C-1.5% Tannic acid; D-2% Tannic acid

A sequence analysis of ribosomal operons is a method of choice to determine the phylogenetic relationship among organisms. 16S rRNA sequence analysis has been used to distinguish the species of the organism and to delineate the lineage. The 1.5kb amplified fragment was subjected to sequence analysis (Figure 4). The results confirmed the identity of isolates belonging to *E. cloacae* (15) *B. subtilis* (25), *E. bacterium* (39) and *B. cereus* (40). A phylogram was constructed based on the UPGMA with 1,000 bootstrap samplings. The analysis was performed with the reference sequences of the representative organism belonging to *E. cloacae* (3), *B. subtilis* (2), *B. cereus* (2) and *E. bacterium* (2) (Table 1). The phylogram was generated for thirteen sequences including our isolates which resulted as in four distinct clusters (Figure 5). However, the respective isolates shared their sequence similarity with their own groups but found distinct from the others. These results clearly suggest the prevalence of genetic diversity among the bacterial strains isolated from the gut of *Gryllotalpa krishnani*. Interestingly, the present investigation revealed that all four isolates were from the same environment but showed high degree of functional and genotypic diversity among them. Finally, it concludes that the highly similar organism we can keep as a reference model and further research to our target model.It shows that formation of two major clades one is grouped with *Enterobacter* genera and another one *Bacillus* genera. The FT-IR spectrum was used to identify the functional chemical group of the active components based on the peak value in the region of infrared radiation. FT-IR spectra of tannin after treatment with tannase enzymes of strain T1 *E. cloacae* and T2 *B. cereus* for 1 hrs at 37 ºC was preformed (Figure 6).

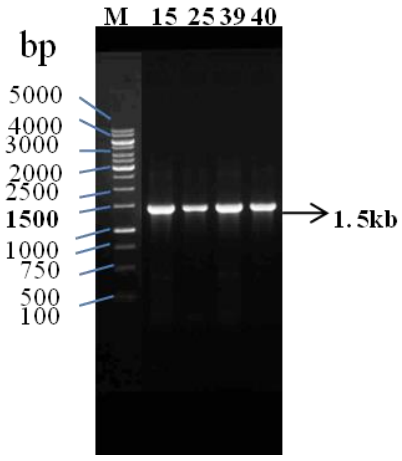


Figure 4 PCR amplification of 16S rDNA gene from insect gut microbes of *G. krishnani*.

Table 1 List of tannase producing bacteria identified by 16S rRNA sequence analysis

Strain No	Organism Name
15	*Enterobacter cloacae*
25	*Bacillus subtilis*
39	*Enterobacteriaceae bacterium*
40	*Bacillus cereus*

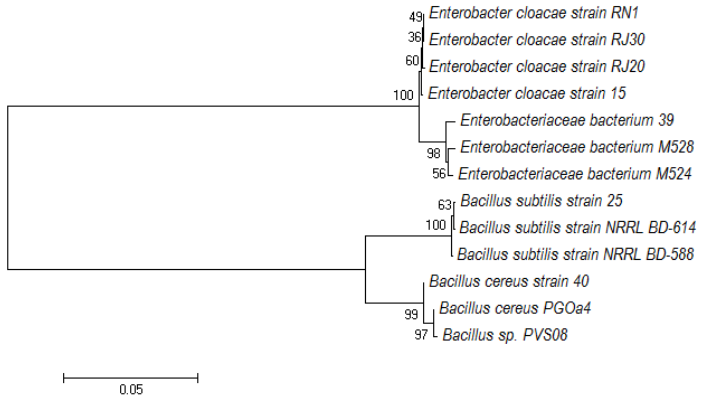


Figure 5 Phylogenetic tree based on 16S rDNA sequences for the gut microbes isolated from insect *G. krishnani* .
The tree was constructed using the neighbour joining method. To obtain confidence values, the original dataset was resampled 1,000 times using the bootstrap analysis method.

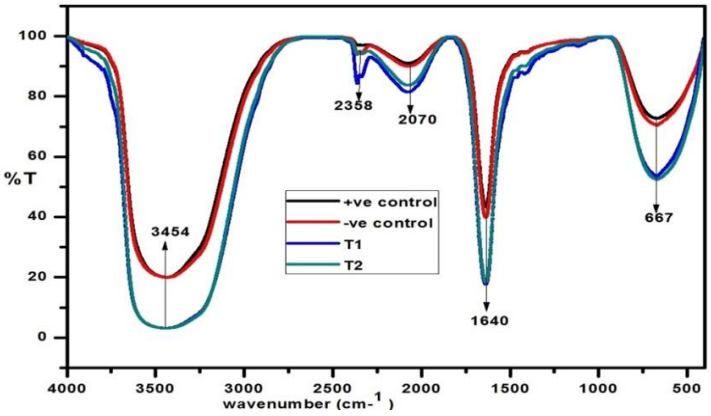


Figure 6 FT-IR analysis of tannin degradation

DISCUSSION

The insect gut of animals exhibiting a natural adaptation to dietary resources containing tannins could harbor valuable microbes and enzymes as augment livestock production (**Singh *et al.*, 2008**). Tannins have several important biological activities, such as a mechanism of defense against diseases caused by fungi, bacteria, and viruses. Bitter taste of tannin helps to protect plant tissues from the attack of insects and herbivores (**Arijit *et al.*, 2014**). Moreover, gut anaerobes have also been isolated from non-ruminants which can dissociate hydrolysable tannins HT-protein complexes, but not the complexes of proteins with condensed tannins (**McSweeney *et al.*, 2001**). *E. ludwigii* GRT-1 isolate was found to grow as scattered cells when grown on culture media containing different levels of tannic acid (**Nelson *et al.*, 1995; Goel, 2007**).Tannin-degrading bacteria such as *Streptococcus caprinus, Streptococcus gallolyticus, Streptococcus macedonicus, Selenomonas ruminantium* has been earlier (**Brooker *et al.*, 1994; O'Donovan, 2001; Goel, 2005; Hiura *et al.*, 2010)**.Tannase activity is reported in *Enterobacter* sp isolated from soil. The fiber-degrading bacteria and fungi usually adhere to the surface of plant cell walls, and a lack of understanding of how to exploit this process may be one of the reasons for the difficulty in establishing inoculant microorganisms in the rumen (**Wang *et al.*, 2013**). The study suggests that the tannase could be useful to synthesize molecules of pharmaceutical interest, and that tannase and the *Enterobacter* sp, itself could be used to protect grazing animals against tannins (**Sharma, 2011**). This is the first report on tannase activity of *E. cloacae* (15), *B. subtilis* (25) and *E. bacterium* (39), *B. cereus* (40) isolated from gut region *G. krishnani* insect. However, the present work is a preliminary study, and relevant

to screening of tannase plate assay microbial degradation of hydrolysable tannins (HT), and the physiological role of their metabolites in these animals requires further investigation. The FT-IR spectrum of tannin samples was described by five main modes with maximum absorbance peak near 3500, 2500, 2000, 1500, 500cm-^{1}. The peaks at 3454 cm-^{1} am O-H bond stretch which the peaks at 1640 cm-^{1} recognized to be amide C=O stretch group peaks at 2070 cm-^{1}, 2358 cm-^{1}, corresponded to the overflow of aromatic and carboxyl group 667 cm-^{1} substitutions in aromatic rings. The variations in the absorbance at 3454, 2358, 2070, 1640, 667 state that the tannase enzymes degradable to tannin in the tannin compound (**Hoong *et al*., 2009**). The FT-IR result showed control tannin treated to tannin with enzymes exhibited a different pattern. This assumption is logical when one realizes that most industrial enzymes will necessarily have originated from the small percentage that has been cultured because of the poor ability to culture environmental microorganisms (**Yeung, 2012**).The result show that tannase producing bacteria have been degraded tannins substrate to simple molecules of activity was notably presented in digestive system of many insects (**Terra *et al*., 1996**). In insects the digestive of food by gut associated bacteria has been demonstrate in crickets mole, germ free crickets have much less hydrolytic enzymes activity a conventional cousins and these bacteria help in utilization a wide range of food substrate (**Santo Domingo *et al*., 1998**).

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. With regards to tannase much of it activity, especially in the insect world, seems get to be explored.

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