

Molecular characterization of bacterial biocontrol agents and their chitinase genes from tea soil

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Received 06 May 2015; revised 15 September 2017

In tea, bacterial biocontrol agents *viz.* *Bacillus* and *Pseudomonas* and an enzyme like chitinase from these bacterial strains are used to control tea pests and pathogens. However, literature on molecular identification of the same is quite scarce. In this study, *Bacillus* and *Pseudomonas* strains isolated from tea soil samples, were systematically identified by 16S rRNA sequencing. Molecular characterization of bacteria was carried out to identify the species of different level chitinase producing bacteria and diversity among them. Further, chitinase gene was characterized from these bacteria to understand the gene diversity among different bacterial chitinase that has potential application in controlling the plant pests and pathogens. Sequence analysis of 16S rRNA and chitinase gene sequences was made among thirteen *Bacillus* and five *Pseudomonas* species submitted in NCBI Genbank.

Keywords: 16S rRNA, Chitinase producer, NCBI Genbank, Phylogenetic relationships, Tea pests

The genus *Bacillus* and *Pseudomonas* encompasses a variety of species exhibiting a wide range of physiological, metabolic diversity and DNA base composition. Each consists of more than 100 species that have similar characteristics, and identifying them is difficult¹. Each species have similar characteristics, hence morphological and physiological identification is challenging. These bacterial biocontrol agents are widely used to control main tea pest/pathogens which include grey blight disease^{2,3}, blister blight disease⁴ and red spider mite⁵. But, the species of the same is still unclear due to inaccuracy in identification as well as the expensive and tedious nature of the process involved⁶. It requires a molecular level confirmation of bacterial species and their diversity. In recent years, PCR based methods have been widely applied in bacterial systematics⁷. The recent development of gene amplification and sequencing, especially that of the 16S rRNA gene sequences of bacteria, has simplified the detection and identification of specific bacteria as well as constructing bacterial phylogenetic relationships⁸. The information content of the 5' end of the 16S rRNA gene is sufficient for identification of

most bacterial species. The 16S rRNA gene is now used as a framework for the modern classification of many bacteria⁹.

Microbes show the higher chitinase productivity than plants and animals¹⁰. The diversity of chitinolytic bacteria *viz.* *Bacillus*, *Pseudomonas*, *Streptomyces*, *Serratia* and *Aeromonas* genera are frequently found in rhizosphere soil¹¹. Recently, chitinase from the bacteria is used as a biocontrol agent against many plant pests and pathogens¹²⁻¹⁵. Studies on the lytic activity among the biocontrol agents have focused largely on characterization of the enzyme systems capable of degrading the insect cuticle and peritrophic membrane components and also cell wall of fungi, in which chitinases are the most intensively studied¹⁶.

The insect moulting enzyme chitinase degrades the chitin, which is the principle structural component of outer skeleton (50% of the cuticle made up of chitin), foregut, hindgut and the midgut lining of peritrophic membrane of insects. Such degradation causes significant damage to the peritrophic membrane structure and restricts the insects' ability to feed and consequently leads to death or disrupts the cuticle which subsequently causes abnormal moulting. As chitinolytic enzymes play an important role in the growth and development of insects and nematodes, chitinase could prove to be a safer biocontrol agent¹⁶.

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The mode of action of chitinase is the same in all bacterial isolates but their nucleotide and amino acid structures are different. Once the species of different chitinase producing bacteria are identified, their chitinase genes can be characterized to understand the chitinase gene diversity.

Materials and Methods

Bacterial strains and culture conditions

About 113 bacterial strains including *Bacillus* and *Pseudomonas* were obtained from the plant pathology division, UPASI Tea Research institute, Valparai, which were isolated from the various soil samples, collected at various elevations in different agro climatic regions (The Nilgiris, The Anamallais, High Range, Central Travancore, Wayanad, Karnataka). Each bacterial strain was revived on Nutrient Agar (NA) medium.

Isolation and screening of chitinase secreting bacteria

Single colony of each strain was inoculated in 10 mL of nutrient broth (NB) containing 1.5% colloidal chitin and allowed to grow for 24 h in a shaker at 37°C. Then it was centrifuged at 10000 g for 15 min. The supernatant was collected and used as the enzyme source. The chitinase activity was determined using colloidal chitin as a substrate following the Sun *et al*¹⁷ method. The reaction mixture was prepared by mixing 0.5 mL enzyme solution and 0.5 mL of 1% colloidal chitin in 1 mL McIlvaine buffer (100 m mol/L citric acid, 200 m mol/L sodium phosphate) at pH of 5.8. The mixture was incubated for 30 min at 36°C using a shaking water bath and the reaction was stopped by incubating on boiling water for 10 min. The amount of reducing sugars released in the supernatant was measured by a method¹⁷ using dinitrosalicylic (DNS) acid reagent, and the absorbance was measured at 540 nm using spectrophotometer (Amersham Biosciences). One unit (U) of chitinase activity was defined as the amount of enzymes that liberated 1 µmol N-acetyl glucosamine (GlcNAc) from colloidal chitin per minute. GlcNAc served as a standard.

Based on the chitinase assay, 18 biocontrol agents were grouped as low, moderate and high chitinase producers (Table 1). These bacterial cultures were revived on nutrient agar plates and incubated overnight at 37°C. Single colony of all bacterial cultures were inoculated in NB, grown at 37°C for overnight with vigorous shaking (200 rpm) for genomic DNA isolation.

Extraction of genomic DNA from pure cultures for PCR

About 100 mL of bacterial culture in each strain grown overnight in nutrient broth was centrifuged at

7000 rpm for 10 min at 37°C. The supernatant was decanted completely and pellets were frozen at -20°C for 2 h and thawed for 3 min at 37°C. The suspension in 10 mL lysis buffer (0.15 M NaCl, 0.05 M sodium citrate buffer) was incubated with 200 µL of lysozyme (10 mg/mL) at 37°C for 60 min. The cells were further lysed with 500 µL of 20% SDS by gentle shaking for 5 min. Thereafter, 15 mL extraction buffer was added [2.5 mL of 5 M sodium perchlorate and 12.5 mL of chloroform: isoamylalcohol mixture (24:1)]. These preparations were incubated at -20°C for 2 h and allowed to thaw at room temperature by gentle shaking for 30 min and subsequently centrifuged at 5000 rpm for 10 min. The supernatant was precipitated with an equal volume of isopropanol and washed with 70% ethanol. The DNA was air dried and dissolved in 1 mL TE buffer¹⁸. After spectrophotometric quantification, the DNA was further analysed on 0.8% agarose gel.

PCR amplification of 16S rRNA and chitinase gene

The 16S rRNA gene was amplified from the bacterial isolates with the Forward primer (5'-GAGTTTGATCCTGGCTCAG-3') and Reverse primer (5'-AGAAAGGAGGTGATCCAGCC-3'). Amplification was performed in a total volume of 25 µL containing 100 ng of genomic DNA, 1 µL of forward and reverse primer (40 pmol/µL), 200 mM of each dNTP (Fermentas), 1 unit of Taq DNA polymerase and 1X Taq buffer [10 mM Tris-HCl

Table 1 — Origins of the bacterial strains and 16S rRNA sequence GenBank accession numbers

Bacterial species	Strain	Isolation locality	GenBank access no.
<i>P. putida</i>	AWRH40	The Anamallais	KJ934368
<i>P. putida</i>	J-6	Koppa	KJ934369
<i>P. putida</i>	J-11	Koppa	KJ934370
<i>P. fluorescens</i>	MP-13	Meppadi	KJ934371
<i>P. monteilii</i>	BCM-8	Munnar	KJ934372
<i>B. cereus</i>	C-13	Coonoor	KJ934373
<i>B. subtilis</i>	MP-35	Meppadi	KJ934374
<i>B. subtilis</i>	C-6	Coonoor	KJ934375
<i>B. subtilis</i>	J-7	Koppa	KJ934376
<i>B. subtilis</i>	J-16	Koppa	KJ934377
<i>B. subtilis</i>	J-18	Koppa	KJ934378
<i>B. cereus</i>	BC-10	Valparai	KJ934379
<i>B. thuringiensis</i>	BC-15	Valparai	KJ934380
<i>B. cereus</i>	BC-7	Valparai	KJ934381
<i>B. amylo-liquefacience</i>	MP-11	Meppadi	KJ934382
<i>B. subtilis</i>	BC-9	Valparai	KJ934383
<i>B. amylo-liquefacience</i>	BCM-1	Munnar	KJ934384
<i>B. amylo-liquefacience</i>	MP-18	Meppadi	KJ934385

(pH 8.8)], 50 mM KCl, and 0.08% Nonidet P40 and 2.5 mM MgCl₂:Fermentas). The contents was mixed thoroughly and centrifuged for 5 s at 5000 rpm. The PCR reaction mixtures, after incubation at 95°C for 5 min as an initial denaturation, were cycled 30 times through the following temperature profile: denaturation for 30 s at 94°C; annealing for 15 s at 56°C; and extension for 1.5 min at 72°C with final extension for 5 min at 72°C.

The chitinase gene was amplified from the bacterial DNA with the Forward primer ChiA_F2 (5'-CGT GGA CAT CGA CTG GGA RTW YCC-3') 5' end labelled with 5'-FAM and reverse primer ChiA_R2 (5'-CCC AGG CGC CGT AGA RRT CRT ARS WCA-3')¹⁹. PCR amplification was carried out in 25 µL volume included 10× PCR buffer (contains 25 mM MgCl₂), 2 µL dNTP mixture (0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP), 1 µL chitinase forward and reverse primer (10 µmol each), 1 µL template DNA (50-150 ng), 0.3 µL Taq DNA polymerase (3U/µL-Bangalore Genei Pvt. Ltd, Bangalore, India), and 16 µL distilled water. PCR conditions were further optimized for chitinase primers by systematically testing different annealing temperatures. Optimized reaction conditions used to amplify chitinase gene, included an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 5 min, annealing at 55°C for 15 s, and extension at 72°C for 3 min, and a final extension at 72°C for 5 min. About 5 µL of each PCR amplification mixture of 16S rRNA and chitinase was mixed with 2 µL of Blue/Orange 6x loading dye and analysed by 1.2% agarose gel electrophoresis.

Cloning, sequencing analysis and Phylogenetic Analysis

PCR amplified bands of 16S rRNA and chitinase gene for all the 18 isolates were excised from the gel and purified using Qiagen Q1A Quick Gel Extraction Kit (Qiagen, Inc., Germany) following the manufacturer's protocol. The purified PCR product was ligated into T/A cloning vector, pTZ57R/T (Fermentas, USA) and transformed into *E. coli* DH5α strain as per the manufacturer's protocol. The insert in the plasmid was sequenced with automated DNA sequencer with specific primers using the facility at Macrogen Inc. (Seoul, Korea). Sequences were edited and aligned using BioEdit 7.0. The BLASTn program (<http://www.ncbi.nlm.nih.gov/blast/>) was employed to identify similarities between the sequences obtained in this work and previously published data. Multiple alignments of the sequences were performed using

ClustalW, common gaps from all the selected sequences were removed and the alignment was checked manually for quality. The sequences were submitted to NCBI GenBank. The phylogenetic analysis was carried out using MEGA5 software²⁰.

Results

Investigation of chitinase activity and 16S rRNA, chitinase gene amplification

A total of hundred and thirteen bacterial strains from different origins were evaluated for their chitinase activity. Among them eighteen strains were selected in low, moderate and high chitinase producer to identify the species, diversity among them and for chitinase gene characterization (Fig. 1). Genomic DNA was recovered from all eighteen bacterial strains. Standardization of PCR conditions for 16S rRNA and chitinase gene amplification by checking different annealing temperature (50-60°C) using gradient PCR technique, desirable amplification has been achieved at the annealing of 56°C for 16S rRNA gene amplification and 55°C for chitinase gene amplification. The band of a 1500 bp fragment corresponding to 16S rRNA gene (Fig. 2) and 1800 bp

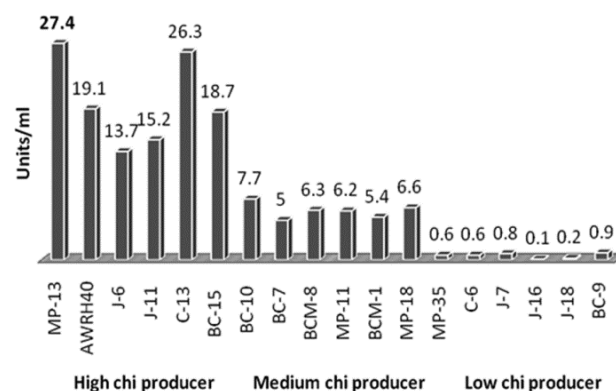


Fig. 1— Classification of bacterial strains based on chitinase assay

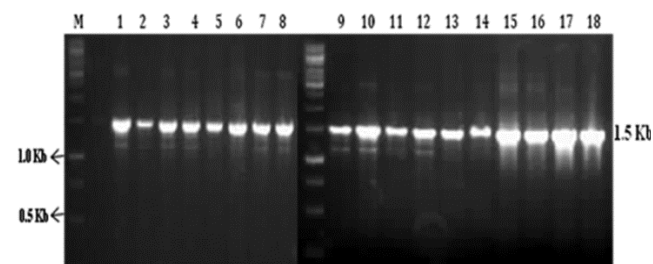


Fig. 2— PCR amplification of 16S rRNA gene from different bacterial species. [M = 1 Kb DNA ladder; Lane 1 to 18 16S rRNA gene amplification from different bacteria isolated from tea growing regions of southern India]

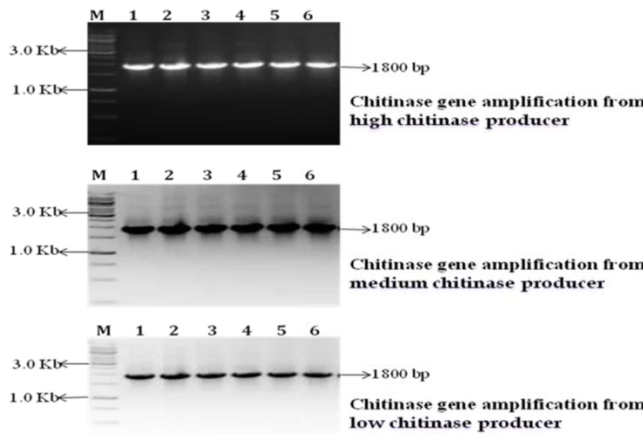


Fig. 3— PCR amplification of chitinase gene from different bacterial isolates. [M = 1 Kb DNA ladder; Lane 1 to 6 chitinase gene amplification from different level chitinase producing bacteria]

fragment corresponding to chitinase gene (Fig. 3) has been amplified under standardized PCR conditions. Amplification of the PCR products was confirmed in 1.2 % agarose gel using standard 1 Kb DNA ladder and excised from the gel, purified, cloned into pTZ57R/T vector and sequenced.

Sequence analysis of 16S rRNA

In the present study, the 16S rRNA gene sequences of the 18 bacterial strains were aligned and compared. Using BLAST-N searches. All strains were analysed and submitted to NCBI GenBank (Table 1). Primary screening of different chitinase producing bacteria and species identification of the same using 16S rRNA is essential to understand the molecular mechanism behind their control. The sequence analysis revealed that all six low chitinase producers were *Bacillus subtilis*. In the moderate chitinase producers, five were *Bacillus* which belonged to the *B. amyloliquefacience* group, *B. cereus* and one was *Pseudomonas* which belonged to the group *P. monteilii*. In high chitinase producers, four were *Pseudomonas* which belonged to the group *P. fluorescens*, *P. putida* and two were *Bacillus* belonging to *B. thuringiensis*, *B. cereus* group. The molecular identity of the high chitinase producing strain was confirmed as *P. fluorescens* based on the BLAST analysis of the sequences.

The identities of *Bacillus* and *Pseudomonas* isolates were determined by comparing them with high-scored rRNA sequences in BLAST-N searches. After judging similarity scores, separated *Bacillus* and *Pseudomonas* into 13 and 5 species, respectively. For example, all thirteen *Bacillus* showed a high degree of similarity among *Bacillus* species, such as *B. cereus* (99.9%), *B. subtilis* (99.0%), *B. thuringiensis* (98%),

B. amyloliquefacience (97%), *B. acidicola* (96%), *B. vallismortis* (96%) and *B. weihenstephainensis* (95%). Similarly all five *Pseudomonas* showed a high degree of similarity among *Pseudomonas* species, such as *P. putida* (99.0%), *P. fluorescens* (99.0%), *P. monteilii* (95%), *P. plecoglossicida* (94%), *P. thivervalensis* (94%).

Sequence analysis of chitinase

Chitinase gene sequences for eighteen different bacterial isolates were aligned and compared. Using BLAST-N searches, chitinase gene sequences were analysed and submitted to NCBI GenBank under the accession numbers KM249880 to KM249888. BLAST analysis revealed that chitinase gene sequences from *P. fluorescens* MP-13 (High chitinase producing strain) showed 97% similarity with *P. fluorescens* chitinase gene (HM356022), chitinase gene sequences from *B. cereus* C-13 and *B. thuringiensis* BC-15 (High chitinase producing strains) showed 99% similarity with *B. thuringiensis* MR11 endochitinase gene (HQ418216), chitinase gene sequences from *P. putida* AWRH40, *P. putida*J-6 and *P. putida* J-11 (High chitinase producing strains) showed 99% similarity with *Pseudomonas* sp. BK1 chitinase gene (AY249148), chitinase gene sequences from *B. cereus* BC-7, BC-10 (Moderate chitinase producing strains) showed 99% similarity with *B. thuringiensis* chi74 gene (EF197878), chitinase gene sequences from *B. amyloliquefacience* MP-11, MP-18 and BCM-1 (Moderate chitinase producing strains) showed 99% similarity with *B. thuringiensis* chi74 gene (EF197878), chitinase gene sequences from *P. monteilii* BCM-8 (Moderate chitinase producing strain) showed 99% similarity with *Pseudomonas* sp. TxG6-1 chiC gene (GU724605), chitinase gene sequences from *B. subtilis* MP-35, C-6, J-7, J-16, J-18 and BC-9 (Low chitinase producing strains) showed 96% similarity with *B. subtilis* chitinase gene (AF069131).

Phylogenetic analysis

A phylogenetic tree was constructed based on 16S rRNA sequences obtained from the present study to determine the genetic diversity among different bacteria. On the basis of 16S rDNA sequence homology, 18 bacterial isolates were distributed among two clades of the tree (Fig. 4). The results of this phylogenetic analysis are shown in Fig. 4. The tree showed that, out of 18 isolates, 13 were different *Bacillus* species, including *B. cereus*, *B. thuringiensis*, *B. amyloliquefacience*, *B. subtilis* are coming in one glade, which could be discriminated phylogenetically

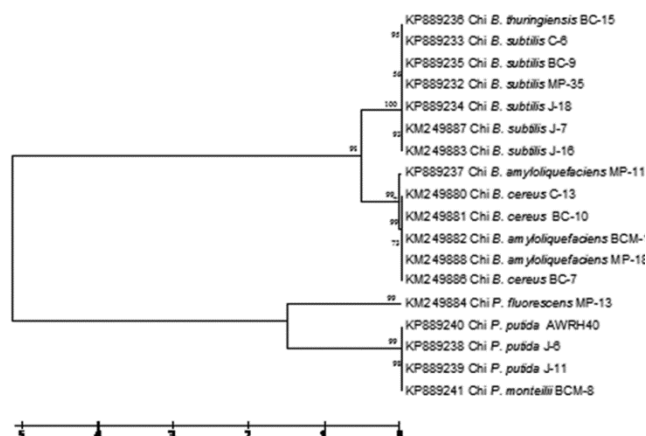


Fig. 4—Phylogenetic relationships of 16S rRNA sequences from chitinase producing bacterial strains. [The branching pattern, which is rooted by using *E.coli* (J01859.1) as the out-group, was generated by the Maximum Parsimony method based on an alignment of the 16S rRNA gene sequences. Bootstrap values are given for each node having 20% or greater support. Bar = 0.02 nucleotide substitution per site. Numbers in parentheses represent the sequence accession numbers in GenBank. Evolutionary analyses were conducted in MEGA5]

from the other five *Pseudomonas* species including *P. fluorescens*, *P. putida* and *P. monteilii* in another glade.

For the phylogenetic analyses, nucleotide sequences of chitinase from different bacteria were aligned using ClustalW program and the UPGMA method was used for building the tree (Fig. 5). The phylogenetic analyses were conducted to determine the chitinase gene diversity among different bacterial isolates. Phylogenetic analyses showed that chitinase gene sequences from the *Bacillus* isolates *viz.* *B. cereus*, *B. thuringiensis*, *B. amyloliquefaciens*, *B. subtilis* are clustered together with difference in their nucleotide sequence which could be discriminated phylogenetically from the chitinase gene sequences of *Pseudomonas* species including *P. fluorescens*, *P. putida* and *P. monteilii* in another glade.

Discussion

In the present study, the 16S rRNA was used to identify the species of different chitinase producing bacteria and to analyze the diversity. High chitinase producing bacterial strain was identified as *Pseudomonas fluorescens* and the sequences showed maximum similarity (99%) with GenBank available 16S rRNA sequence of *P. fluorescens*. Further, this *P. fluorescens* used for isolation and characterization of the chitinase enzyme (~30 kDa) by our research group²¹. By using specific 16S rRNA gene, molecular characterization of functionally important beneficial microbes *viz.* *Bacillus*

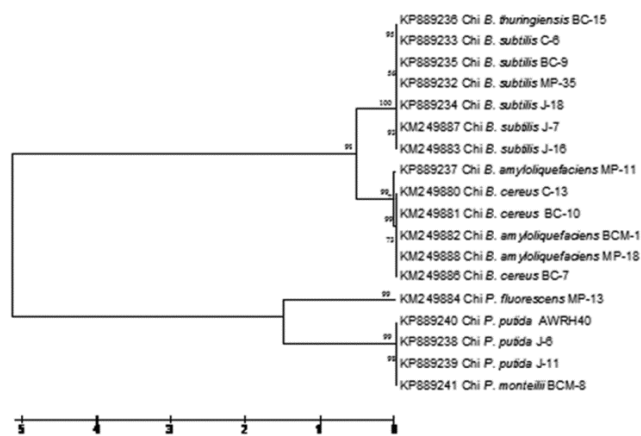


Fig. 5—Diversity characterization of chitinase gene sequences from different bacterial strains. [The evolutionary history was inferred using the UPGMA method. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 620 positions in the final dataset. Evolutionary analyses were conducted in MEGA5]

subtilis, *Pseudomonas fluorescens* and *Streptomyces noursei* have been identified from the gut of indigenous silkworm breeds²². In the present study, 18 different bacterial species were identified using 16S rRNA gene sequencing which were collected from different tea growing regions of southern India. Miranda *et al.*²³ reported species of the genus *Bacillus* recovered from marine sediments by conventional biochemical tests, sequencing analysis of 16S rRNA genes and tDNA-intergenic spacer length polymorphism (tDNAPCR). Later, Ki *et al.*²⁴ sequenced the 16S rRNA region of twenty *Bacillus* strains from diverse marine environments and based on the sequence analysis, separated the strains into 13 *Bacillus* genotypes and identified 9 different species.

The results of the phylogenetic analysis revealed that all of the clades of *Bacillus* and *Pseudomonas* were separated clearly with high phylogenetic resolution (Figs. 4 and 5) but shows relatively high level of sequence diversity between and within the targeted 16S rRNA and chitinase gene sequences of the *Bacillus* and *Pseudomonas* isolates. It is still generally accepted that the universal phylogenetic tree construction based on the 16S rRNA gene sequence²⁵. Phylogenetic analysis on the basis of 16S rDNA sequences provided better understanding in evaluation of genetic diversity of bacteria isolated from same and

different ecological niche; phylogenetic analysis of 500 bp of terminal region of 16S rDNA from cultivated strain has been found to show existence of large bacterial diversity²⁶.

Williamson *et al.*²⁷ was the first to conduct the molecular analysis of bacterial chitinase genes in soil DNA using denaturing gradient gel electrophoresis (DGGE) however no sequence information was provided following their profile analysis. Novel groups of chitinase genes in maize rhizosphere and bulk soil were identified by culture independent method and molecular diversity of bacterial chitinase genes also employed by terminal restriction fragment length polymorphism and sequence analysis²⁸. In the present study, different chitinase genes were identified from different bacterial isolates of tea rhizosphere and these chitinase genes are diversified in sequence level.

Among 113 bacterial strains, one strain was found to produce high level of chitinase enzyme and identified as *P. fluorescens* using 16S rRNA sequencing. In sugar beet, endochitinase dependent antagonism toward plant-pathogenic microfungi was observed by *P. fluorescens* and molecular characterization of the organism done by 16S rRNA²⁹. Parvathi *et al.*³⁰ characterized the most predominant *Bacillus* spp. like *B. pumilus*, *B. cereus* and *B. sphaericus* from the coastal environments of Cochin, India using 16S rDNA sequencing and biochemical assays and they concluded these study will help in identifying novel mechanisms of environmental survival, diverse metabolic activities, production of biotechnologically valuable enzyme like chitinase. Presently, we are focusing our research on molecular characterization of tea mosquito bug in tea plants³¹ and characterization of chitinase enzyme from *Pseudomonas fluorescens* MP-13²¹ to manage tea mosquito bug.

Acknowledgement

The authors are thankful to Dr. R Premkumar and his team, Plant Pathology Division, UPASI Tea Research Institute, Valparai, Tamil Nadu, India for providing bacterial cultures and assistance in culturing work on bacterial biocontrol agents.

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