Pest Management in Horticultural Ecosystems
Vol. 32, No.1 pp 80-86 (2026)
DOI Number : 10.5958/0974-4541.2026.00009.3

Diversity and functional profiling of culturable cellulolytic bacteria in wood-feeding termites (Order: Blattodea)

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ABSTRACT: Termites are a large and diverse group of phytophagous insects that feed on cellulose-rich organic materials. During the decomposition of organic matter, they rely on symbiotic microorganisms, particularly bacteria and protozoa present in their digestive tract, to facilitate cellulose degradation. This study aims to identify the cellulolytic bacteria from the gut of the wood feeding termite species based on sequences of 16S ribosomal RNA (rRNA) gene. Berg's agar media supplemented with carboxymethyl cellulose (CMC) was used to culture the cellulose degrading bacteria. Cellulolytic activity was confirmed based on clear zone formation and the cellulolytic index on Berg's agar media using Congo red assay. Total nine isolates of bacteria from the gut of wood feeding termites showed cellulolytic activity. The highest cellulolytic index was recorded in *Bacillus cereus* (CI- 1.31), followed by *Bacillus subtilis* (CI-1.09). The high cellulolytic index in these isolates is likely owing to their ability to secrete greater amounts of cellulases, which degrade the amorphous regions of cellulose in the CMC substrate.

Keywords: Termite, Gut symbiont, Bacteria, Cellulolytic, *Bacillus*

INTRODUCTION

Termites (Order: Blattodea) are a large and diverse group of soil macrofauna, which are most abundant in tropics and subtropics (Brune, 2014). These phytophagous insects feed on cellulose-rich organic materials such as wood, twigs and leaves, playing a vital role as decomposers in terrestrial ecosystems. However, certain species are also recognised as economically important pests, causing damage to crops in agricultural and horticultural systems and wooden structures (Rana *et al*., 2021). Termites contribute up to 95 per cent of the insect biomass in tropical soils (Breznak and Brune, 1994). Although only a small proportion of the approximately 3000 described species are considered as pests, their impact on plant health and structural materials can be significant under favourable conditions. At the same time, termites contribute to nutrient recycling by breaking down dried plant material, thereby enhancing soil fertility through improvements in its physical and chemical properties (Arif *et al*., 2018; Sugimoto *et al*., 2000).

Termites are considered serious pests in several horticultural crops, particularly in tropical and subtropical regions. They attack roots, stems, cuttings, seedlings and woody tissues of fruit crops, plantation crops, ornamental plants and vegetable crops, leading to wilting, drying and, in severe infestations, plant death. Important horticultural crops such as mango, citrus, coconut, grapevine, pomegranate and several nursery plants are reported to suffer from termite damage (Rana *et al*., 2021). Damage is often more severe under dry soil conditions and in stressed plants, where termites initially feed on dead organic matter and later invade living tissues (Logan *et al*., 1990). Apart from causing direct economic losses through reduction in plant vigour and yield, termite infestation also predisposes plants to secondary infections and structural weakening. Globally, termites are estimated to cause billions of dollars in economic losses annually in agriculture, horticulture, forestry and structural materials (Rust and Su, 2012). Therefore, understanding termite biology and their associated microorganisms is important not only from an ecological perspective but also for developing sustainable termite management strategies in horticultural ecosystems.

In the process of decomposing organic matter, termites utilize microorganisms to degrade cellulose, such as bacteria and protozoa in the digestive tract, especially in the hindgut. In non-Termitidae termites, protozoa predominate over bacteria, whereas in Termitidae termites, bacteria are more dominant than protozoa (Breznak, 2014). These microbial symbionts

play a crucial role in host nutrition, survival, and may also influence termite adaptability and persistence as pests in different ecosystems.

To digest cellulose, termites have to provide cellulolytic enzymes, *i.e.,* cellulase produced by the termite itself and by the termite symbionts (Nakashima *et al*., 2002). Cellulose is a linear polymer of glucose linked through beta-1,4-glycosidic linkages. These linkages are hydrolysed by cellulase, which also plays a role in recycling the polysaccharides. Cellulase consists of various enzymes such as endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.74) and beta-glucosidase (EC 3.2.1.21) (Morana *et al*., 2011).

Carboxymethyl cellulose (CMC) is widely used as a suitable cellulosic material for detecting endo-acting cellulases, as its modified structure lowers the crystallinity of native cellulose and enhances the availability of amorphous regions for enzymatic action. Endoglucanases (CMCase) act on these regions by randomly cleaving internal β-1,4-glycosidic linkages within the cellulose chain, which leads to a significant decrease in both the degree of polymerization and the viscosity of the substrate (Zhang *et al*., 2006).

Cellulolytic enzymes from microorganisms also have many potential biotechnological and industrial applications. Cellulases are required in large quantities because of their application in many industries, such as textiles, detergent, food, animal feed, bio-fuel, paper and pulp, pharmaceutical and waste management (Mandels, 1985; Bhat, 2000; Park and Park, 2001). Isolating the possible strains is the first stage in creating an industrial method for producing an enzyme (Kasana *et al*., 2008). In addition, understanding cellulolytic gut bacteria of wood feeding termites may provide insights into the microbial mechanisms underlying cellulose degradation, which are important for termite survival and may have implications in developing novel approaches for termite pest management. In this context, the present study focuses on the isolation and screening of cellulolytic gut bacteria from wood feeding termites.

MATERIALS AND METHODS

Collection and identification of termite samples

Wood feeding termites *Odontotermes feae, O. yadevi, O. redemanni* and *Nasutitermes indicola* were collected from Kodagu and Chikkamagaluru districts of Karnataka, and brought to the insect molecular biology laboratory, Department of Entomology, College of Agriculture, GKVK, Bangalore, during the year 2024-25, in sterile plastic boxes. Live termites were collected for microbial isolation and boxes were labeled with the host name, location and collection date. Live worker castes were utilized for bacterial isolation within 24 hours of sampling (Sakolvaree and Deevong, 2016). Soldier caste was used for the termite species identification, utilising the keys provided in Fauna of India and Adjacent Countries Volume I by Roonwal and Chhotani (1989), Volume II by Chhotani (1997) (Santhrupthi *et al*., 2024).

Isolation of bacteria

A selected number of worker termites (3-4 individuals) were taken from the collected samples and surface sterilized with 70 per cent ethanol for one minute, followed by 0.1 per cent sodium hypochlorite, and then rinsed 2-3 times in autoclaved water to remove any contaminants adhered to the surface of the specimens. The entire gut was dissected using sterile forceps, needle and micro scissor under aseptic condition. Gut samples were pooled to ensure a sufficient amount of material for microbial analysis. The dissected guts were homogenized in a micro centrifuge tube using a sterile micro pestle with 1 ml of phosphate buffer saline (PBS) solution (pH 7.4) and then serially diluted till 10^{-7}. An aliquot (100 µl) from each dilution was plated on nutrient agar (NA) medium and spread using a sterile glass spreader. Then, petri plates were wrapped with the para-film and incubated at 32 °C for 24 to 48 h in biological oxygen demand (BOD) incubator (KEMI®).

Morphological characterization and purification of isolated bacteria

After incubation, plates with bacterial growth were observed for morphological characters *viz*., color, shape, margin, opacity, elevation, branching and gram stain. Colonies displaying distinct characteristics not seen on other plates were selected and transferred onto fresh nutrient agar plates for further purification (Kavitha *et al*., 2014). Pure cultures were added to autoclaved nutrient broth in pre-sterilized test tubes with appropriate labels. The tubes were incubated at 37°C for 24 hours in a BOD incubator until the clear broth turned turbid, indicating bacterial growth and multiplication.

Isolation of genomic DNA and amplification of 16S rRNA gene

Genomic DNA was isolated from bacterial cultures grown in nutrient broth following the sucrose

buffer method with minor modifications (Saha *et al*., 1989). The amplification of 16S rRNA was carried out by using universal primers, 16S rRNA forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SrRNA reverse (5′- ACGGCTACCTTGTTACGACTT -3′). Polymerase chain reactions were performed with 25 µl of PCR mixture (ProFlex) with an initial denaturation at 94 °C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, annealing for 45 sec at 59 °C with an extension for 1.5 min at 72 °C followed by final extension for 10 min at 72 °C and kept hold at 4 °C until further processing. The PCR amplified products were subjected to agarose gel electrophoresis on a 1 per cent gel and documented with a gel documentation system (Molecular Imager Gel Doc XR+, BioRad, USA and Software - Version 5.2.1).

16S rRNA Sequence analysis

The amplified PCR products were sent for nucleotide sequencing to Eurofins Genomics India Pvt. Ltd. Bangalore, using the Sanger dideoxy sequencing method. The obtained forward and reverse sequences were aligned, edited, and assembled into a contig sequence using BioEdit software (version 7.2), and the resulting sequences were compared with those in the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) for identification. The 16S rRNA gene sequences with maximum similarity were deposited in GenBank database and accession numbers were obtained.

Test for cellulolytic activity

Berg's Agar plates (For 100 ml, 0.2gm $NaNO_3$, 0.05gm $MgSO_4$, 0.005gm K_2HPO_4, 1mg $FeSO_4$, 2 mg $CaCl_2$, 0.2 mg $MnSO_4$, 2gm agar) were prepared with 0.1% carboxymethyl cellulose (CMC) to serve as a substrate for cellulolytic activity. Bacterial isolates were streaked onto these Berg's agar (Berg *et al*., 1972) plates and incubated at 37°C for 48 hours. Following incubation, the plates were flooded with 3 per cent Congo red solution and allowed to stain for 20 min. Excess stain was removed by washing the plates with 1M sodium chloride (NaCl) solution. Cellulolytic activity was indicated by the appearance of clear zones or halo zones, around the colonies against the red background of Congo red. The cellulase enzyme activity of each isolate was quantified using an enzymatic index, calculated by using the equation below (Ferbiyanto *et al*., 2015).

$$\text{Cellulolytic Index (CI)} = \frac{\text{Diameter of zone of clearance - Diameter of bacterial colony}}{\text{Diameter of bacterial colony}}$$

RESULTS AND DISCUSSION

Morphological characterization and Cellulolytic screening

A total of 41 Bacterial species were isolated from four wood feeding termite species. The cellulolytic activity of the isolates was assessed based on the principle of the CMC agar assay, where carboxymethyl cellulose serves as a substrate for cellulase enzymes. Cellulase-producing bacteria hydrolyse CMC, resulting in the formation of clear zones around the colonies upon staining with Congo red, which binds to intact cellulose (Teather and Wood 1982).

When all the obtained bacterial isolates were tested for cellulolytic activity, nine species showed positive result for CMC assay. Three isolates (34C, 29E, 34H) were from *Odontotermes redemanni,* two isolates (25B, 25BF) were from *Nasutitermes indicola*, two isolates (20S, 25S) were from *Odontotermes feae* and one isolate (26D) was from *Odontotermes yadevi*. Among them five were gram positive and four were gram negative. Morphological characterisations of the cellulolytic bacteria are presented in Table 1.

Table 1: morphological characteristics of cellulolytic bacterial isolates

Isolate code	Colour	Opacity	Margin	Shape	Elevation	Branching	Gram staining
34C	Whitish	Opaque	Irregular	Circular	Flat	No	Positive
25B	Off White	Opaque	Irregular	Irregular	Flat	No	Positive
29E	White	Opaque	Irregular	Circular	Flat	No	Positive
34H	White	Opaque	Irregular	Circular	Flat	No	Positive
25BF	Creamy white	Opaque	Smooth	Circular	Raised	No	Positive

26D	Red	Opaque	Smooth	Circular	Raised	No	Negative
20S	Greyish-white	Opaque	Smooth	Circular	Raised	No	Negative
30S	Cream to white	Opaque	Smooth	Circular	Raised	No	Negative
25S	White	Translucent	Smooth	Circular	Raised	No	Negative

Molecular identification of cellulolytic bacteria

Amplification of the 16S rRNA gene from the selected isolates yielded amplicons of approximately 1500 bp, confirming successful PCR amplification. Sequence analysis using BLAST revealed 100 per cent query coverage with closely related sequences in the GenBank database (Table 2), indicating that the entire length of the obtained sequences aligned with reference sequences without gaps, thereby supporting the reliability of sequence-based identification (Camacho *et al.,* 2009).

Based on sequence similarity analysis, isolates 34C and 25B were identified as *Bacillus cereus* and *Bacillus subtilis*, respectively. The corresponding 16S rRNA gene sequences were deposited in the GenBank database, and accession numbers PV809875 and PV819332 were assigned to isolates 34C and 25B, respectively.

Cellulolytic activity and functional hierarchy

The nine cellulolytic isolates exhibited varying degrees of hydrolysis on CMC agar, as reflected in their cellulolytic indices. The CI values ranged from 0.31 to 1.31. *Bacillus cereus* showed the highest cellulolytic index (CI = 1.31), followed by *B. subtilis* (CI = 1.09), indicating strong extracellular cellulase production (Fig. 1). *Bacillus tequilensis* (CI = 0.86) and *Acinetobacter* sp. (CI = 0.76) also displayed relatively high activity, while *Enterobacter cloacae* (CI = 0.69) and *Bacillus paramycoides* (CI = 0.67) showed moderate cellulolytic potential. In contrast, *Serratia marcescens* (CI = 0.31), *Bacillus stercoris* (CI = 0.32) and *Trabulsiella odontotermitis* (CI = 0.32) produced the smallest hydrolysis zones, suggesting low or limited cellulase activity under the tested conditions.

In the present study members of the genus *Bacillus* constituted the dominant cellulolytic bacteria isolated from the gut of wood feeding termites (*Odontotermes feae, O. yadevi, O. redemanni* and *Nasutitermes indicola*) representing five out of nine isolates. This dominance is consistent with the several other culture dependent investigations of termite gut microbiota. Previous studies on subterranean and wood feeding termites such as *Coptotermes* sp., *Odontotermes* sp. have similarly reported *Bacillus* spp. (*e.g., B. cereus, B. subtilis, B. pumilus, B. licheniformis*) as the most frequently obtained cellulolytic isolates, often exhibiting the largest clearing zones on CMC agar and highest CMCase activities among the obtained strains (Christy *et al.*, 2024; Boontanom and Chantarasiri, 2021).

Table 2: Percent similarity based on BLASTn analysis of the 16S rRNA gene sequence

Isolate code	Closest species (Accession number)	Query coverage (%)	*% Similarity
34C	*Bacillus cereus* strain VBE11 (MG027688)	100	98.00
25B	*Bacillus subtilis* strain Maf-Sh832 (OR230097)	100	99.55
29E	*Bacillus paramycoides* strain D1 (OR477310)	100	98.00
34H	*Bacillus stercoris* strain 23865 (OR454011)	100	97.71
25BF	*Bacillus tequilensis* strain HYM41 (KT982221)	100	99.55
26D	*Serratia marcescens* strain SADG7 (PP527159)	100	99.62
20S	*Trabulsiella odontotermitis* strain OLH2 (MH542271)	100	98.00
30S	*Acinetobacter* sp. strain ZJW-6 (MZ206177)	100	96.75
25S	*Enterobacter cloacae* strain ps-5 (KU353685)	100	98.49

*% Similarity indicates the percent identity based on BLASTn analysis of the 16S rRNA gene sequence against the NCBI GenBank database.

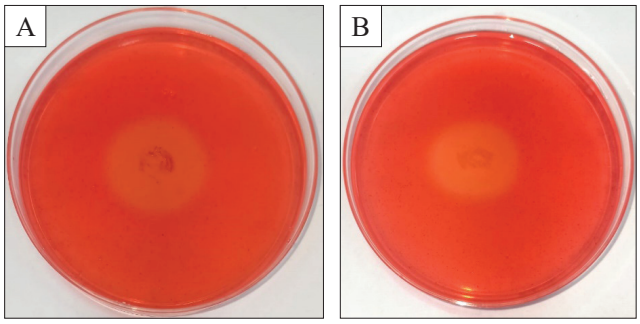

Fig. 1. Cellulolytic activity of isolate A. 34C and B. 25B on CMC agar media

Bacillus spp. produce a diverse array of extracellular glycoside hydrolases (including endoglucanases, exoglucanases and β-glucosidases) that hydrolyse both soluble (CMC) and crystalline cellulose (Lynd *et al*., 2002; Gupta *et al*., 2012). For instance, *B. subtilis* is known for secreting thermostable and alkali-tolerant cellulases suitable for industrial applications, while *B. cereus* isolates have demonstrated high endoglucanase activity and efficient degradation of lignocellulosic biomass under laboratory conditions (Asha and Sakthivel 2014; Wang *et al*., 2024). These enzymatic traits likely provide a competitive advantage to certain strains in cellulose-rich environments such as the guts of wood feeding insects.

In addition to their enzymatic capacity, *Bacillus* spp. may contribute to cellulose degradation in the termite gut through their ecological role as opportunistic and rapidly growing bacteria capable of utilizing partially degraded plant polymers generated during lignocellulose breakdown. By secreting extracellular cellulases, these bacteria are capable of contributing to the hydrolysis of amorphous cellulose and releasing soluble sugars such as cellobiose and glucose, as demonstrated by cellulolytic bacterial isolates from termite guts, including *Bacillus* spp. (Wenzel *et al.*, 2002). Such functional contributions are consistent with observations from termite gut studies that highlight cooperative lignocellulose degradation involving multiple microbial taxa.

The rapid growth of *Bacillus* spp. on standard laboratory media further biases their recovery in culture-dependent approaches compared to more fastidious, strictly anaerobic symbionts (*e.g*., certain *Treponema* or *Fibrobacter* relatives) that dominate culture-independent metagenomic surveys of wood feeding termite guts (Warnecke *et al*., 2007).

CONCLUSION

The high cellulolytic potential observed in the *Bacillus* isolates (e.g., *B. cereus* and *B. subtilis*) indicates their relevance in cellulose degradation within termite gut systems and highlights their potential for industrial applications such as bioethanol production, pulp processing and agricultural waste valorisation. From a pest management perspective, these findings also provide insights into the microbial mechanisms supporting termite nutrition and survival, which may serve as potential targets for developing novel control strategies. However, the reliance on aerobic cultivation may have underestimated the diversity of obligate anaerobic microbes that contribute significantly to in situ cellulose degradation. Future studies integrating metagenomics, meta transcriptomics and targeted anaerobic culturing will enable a more comprehensive understanding of the cellulolytic microbial community and aid in identifying key functional interactions relevant to both biotechnology and termite management.

ACKNOWLEDGEMENT

The authors would like to acknowledge the Department of Entomology, GKVK, University of Agricultural sciences Bangalore, for their support.

AUTHORS CONTRIBUTION

SAB carried out the experimental work, performed data analysis and wrote the manuscript. SB contributed to conceptualization, supervision and critical review of the manuscript. A, AKC and KP contributed to review and editing. All authors read and approved the final manuscript.

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MS Received: 28 February 2026
MS Acceptance: 20 June 2026