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Screening the diversity of cellulolytic bacteria inhabiting the gut of subterranean termite *Coptotermes sp.* and their control using *Azadirachta indica* leaf extract

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ABSTRACT

Termite control is essential to mitigate the damage they cause worldwide. It can be achieved by targeting the celluloytic bacteria that thrive within the termite gut. Therefore, the aim of the present study was to isolate and characterize the cellulose-degrading bacteria from the gut of the wood termite, *Coptotermes sp.* The cellulose degrading bacteria was isolated from the gut of the termite using the medium containing cellulose as the only carbon source. Three different bacteria were isolated and their cellulolytic activity on CMC-containing media was confirmed by the congo red clearing zone assay. Based on the morphological and physiological characteristics the isolates were identified as *Bacillus* sp., *Enterobacter* sp., and *Micrococcus* sp. Of the three isolates *Bacillus sp.* exhibit maximum cellulolytic activity. *In vitro* antibacterial efficacy of neem (*Azadirachta indica*) leaf extract against the cellulolytic bacteria was analysed. All the three isolates were found to be susceptible to the neem leaf extract. In addition to bactericidal activity termicidal potential of the leaf extract was determined by no-choice bioassay method.

Key words: *Termite control, Coptotermes sp., Cellulolytic bacteria, Azadirachta indica, Filter paper assay*

Introduction

Termites are an aggressive, invasive species that has caused billions of dollars of damage across the world for the past 50 years. Termites are a double-edged sword; they are integral to ecological imbalance, yet their interaction with human habitats and agriculture results in harm. Termites are reported to be notorious due to the digestion potential of cellulose, a complex carbohydrate found in the plant cell (Govorushko, 2018). This ability is largely attributed to the cellulolytic bacteria residing in their gut. The enzyme cellulase produced by the bacteria facilitates the breakdown of cellulose into simpler sugars that termites can metabolize (Azhar *et al.,* 2024). Termite control can be achieved by suppressing the proliferation and functionality of cellulolytic bacteria that reside within gut. Conventional chemical approaches have been used traditionally to control termites, concerns over their environmental and health effects have spurred the quest for more sustainable alternatives (Verma *et al.,* 2009). Employing plant extracts stands out as a potential approach for controlling termites and cellulolytic bacteria (Alamu and Ewete, 2023; Kumar and Upadhyay, 2021). Available literature illustrates that plant extracts have potential antimicrobial efficiency. Notably, extract derived from Rosemary (Nieto *et al.*, 2018), Moringa (*Moringa oleifera*), Guava (*Psidium guajava*) and Olive (*Oleaeuropaea* L) were reported to have antimicrobial activity (Hemeg *et al.*, 2020). Leveraging plant extracts to control termite gut bacteria offers a promising avenue for more sustainable, alternative, eco-friendly, and targeted termite management strategies (Kumar and Upadhyay, 2021). Furthermore, extracts from Neem (*Azadirachta indica*) and Basil (*Ocimum sanctum*) have exhibited antibacterial activity, suggesting their potential for exploration in termite control tactics (Wylie and Merrell, 2021). Research on the use of Neem leaf extract could provide valuable perspectives as an alternative approach for termite control. The present work was designed to investigate the efficiency of neem leaf extract on the control of wood termite and gut cellulolytic bacteria.

Materials and Methods

Termite Collection

Termites that live in woody materials were collected from backyards of the houses in Madurai, Tamil Nadu, India. The termites were collected in a sterile plastic container and immediately transferred to the laboratory. The termites were identified based on its morphological characters as described by Roonwal and Chhotani (1987).

Isolation of Cellulolytic Bacteria from termite gut

Ten termite workers were briefly surface sterilized with 70% ethanol followed by sterile distilled water. The guts were removed under aseptic conditions by grasping the thorax with one forceps and pulling the final segment of abdomen with another forceps. The extracted guts were homogenized in sterile phosphate buffer saline. Homogenate was serially diluted and the aliquots (100 µl) from each dilution were spread onto 1% carboxymethyl cellulose (CMC) agar medium. The plates were incubated at 37°C for 24h and observed for the bacterial growth. Later, the plates were flooded with 1% (w/v) congo red and stained for 10-15 minutes and destained with 1M NaCl to visualize the hydrolysis of cellulose (halo zone) around bacterial colony. Cellulolytic potential of the isolate was assessed using cellulolytic index (CI) formula. Bacterial colonies that posses clear zones were sub-cultured repeatedly to obtain pure culture and maintained in nutrient slant flooded with 5 % glycerol for future analysis (Gupta *et al.*, 2012; Peristiwati *et al.*, 2018;).

Identification of Cellulolytic Bacteria

The cellulolytic bacteria were characterized phenotypically by observing cell size and shape, colony color, arrangement, type of edge and opacity. Cell shape and appearance of the cell post-gram staining was determined through microscopic examination (Gerhardt *et al.*, 1994). Furthermore, the isolate was identified based on biochemical tests according to Bergey's manual of determinative bacteriology (Brenner *et al.*, 2005).

Preparation of plant bio-pesticide

For the plant bio-pesticide preparation, maceration method suggested by Punitha and Saral (2024) was followed with minor modification. Thirty grams of *Azadirachta indica* plant leaves was macerated in 100 ml of sterile distilled water and placed in a closed container for 24 hrs. The mixture underwent frequent agitation using an orbital shaker for the initial six hours and was then allowed to settle for an additional 18 hours. Using Whatmann filter paper, the extract was filtered and stored at 4°C. The extract was then reconstituted in sterile distilled water to obtain different concentrations by the equation below.

$M1V1 = M2V2$

Where

M1 – the concentration of the stock solution

V1 – the volume of stock solution

M2 – the concentration of the dilute solution

V2 – the volume of the dilute solution.

Antibacterial activity

Antibacterial activity of the plant extract was assessed using the agar well diffusion method on Muller Hinton Agar (MHA) plates as suggested by Manandhar *et al.* (2019) with slight modification. The test organisms were inoculated in nutrient broth and incubated overnight at 37 °C. The turbidity of a bacterial suspension was adjusted to match the 0.5 McFarland standards, which is roughly equivalent to 1.5×10^8 CFU/mL. Wells were made created in the inoculated media using a sterile cork borer (6mm). Wells were filled with 20 µl of different concentration of the plant extract and one well served as a control by adding 20 µl of sterile distilled water. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24 hours at 37 °C. After incubation, the plates were observed for the formation of a clear zone around the wells, indicating antimicrobial activity.

Termite control

Filter paper discs were immersed in the plant extract for 2 minutes. Filter papers immersed in solvent was used as control. All filter papers were air-dried in fume hood for 1 hour. The no-choice bioassay adopted in this study was a slightly modified method from a previous report (Acda, 2014). Six Petri dishes (90×15 mm, Borosil, India) was prepared by filling each with 25g of sterile sand moistened with distilled water. Treated filter paper discs were placed on the sand. Three replicates were maintained for each test. A total of 10 workers were introduced directly into the center of each petri dish. The plates were kept in an incubator and maintained in dark at 26±2°C with relative humidity of 65±5 %. The mortality of the termites was recorded every 24 hours. All dead and moribund termites were removed from each Petri dish during the counting. The percentage of termite mortality was corrected using Abbott's formula (Abbott, 1925).

$$P = Po–Pc / 100 – Pc \times 100$$

Where:
P: corrected mortality (%).
Po: observed mortality (%).
Pc: control mortality (%).

Results and Discussion

Based on the morphological characteristic features, the termites collected were identified as *Coptotermes sp.* (Isoptera: Rhinotermitidae). *Coptotermes* species were reported to cause severe damage to wood materials. Additionally, their aggressive foraging behavior disrupts agricultural practices and damage crops. Overall, the impact of *Coptotermes* sp. encompasses ecological, economic and structural concerns, emphasizing the importance of implementing efficient termite management strategies. Three different cellulolytic bacteria were isolated from the termite gut. In addition to their growth on CMC agar, the cellulolytic activity was confirmed by the formation of clear halo zones around the colonies on the addition of congo red dye. The zone of inhibition exhibited by different isolates is presented in Table 1. Butera *et al.* (2016) determined cellulolytic activity of bacteria isolated from the gut of the wood-feeding termite *Reticulitermes lucifugus* adopting the same procedure.

Table 1. Clear zone diameter exhibited by the isolates on CMC agar after congo red staining.

Isolates	Zone of Inhibition (mm)
Bacillus sp.	14 mm
Enterobacter sp.	11 mm
Micrococcus sp.	17 mm

The isolates were initially screened based on the colony morphology and growth characteristics. Further, the isolates were subjected to morphological and biochemical analysis and the results are presented in Table 2. Morphological and physiological characterization of the isolates revealed that they were *Bacillus* sp., *Enterobacter* sp., and *Micrococcus* sp. The gram staining procedure revealed that two isolates stained gram positive and one isolate stained gram negative. Thanh Giao (2021) isolated five different bacteria having high CMC degradation efficiency from the termite collected from Vinhlong province, Vietnam. Cellulolytic *Bacillus subtilis* was isolated from the termite, *Microcerotermes* sp. (Taechapoempol *et al.*, 2010).

Figure 1 represents the antibacterial activity of the neem extract against the cellulolytic bacteria by

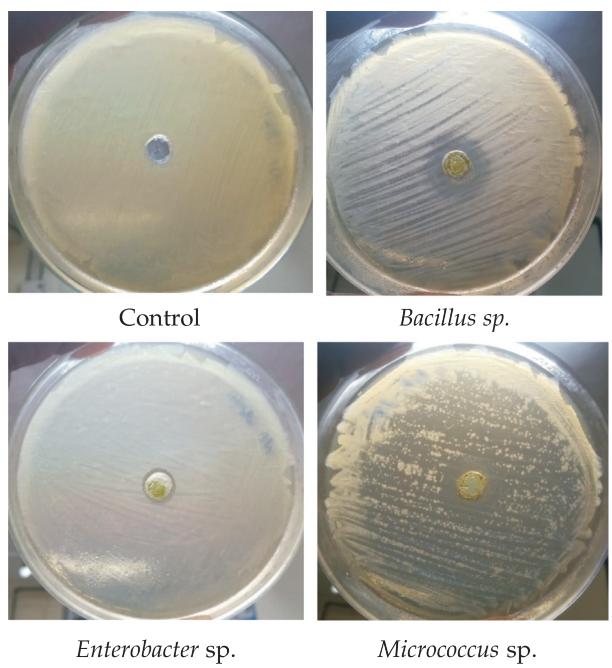

Fig. 1. Antibacterial efficiency of neem leaf extract (25 µg/ml) against cellulolytic bacteria.

agar well diffusion method. The leaf extract showed dose dependent inhibition of the cellulolytic bacteria isolated from the gut. It is evident from the figure 2 that with increase in concentration the inhibitory efficiency was found to be greater. Of the three isolates, *Enterobacter* was found to be highly susceptible to neem leaf extract followed by *Micrococcus* species and *Bacillus* species. The lowest concentration of neem leaf extract tested did not elicit inhibitory efficiency towards *Bacillus* sp. It may be due to the spore forming nature of the *Bacillus* sp.

In addition to antibacterial potential the neem extract exhibited a dose dependent mortality of termites under *in vitro* conditions following no-choice filter paper bioassay (Fig. 3). Exposure of termites to higher concentration of *Azadirachta indica* leaf exract

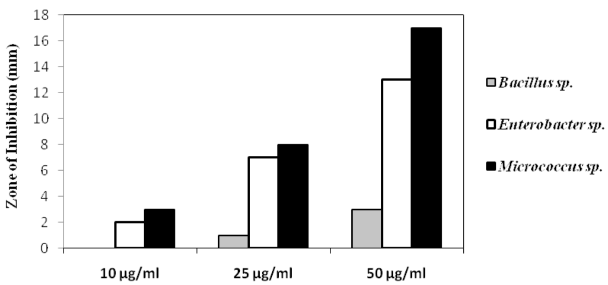

Fig. 2. Anti-bacterial efficiency of different concentration of neem extract against cellulolytic bacteria:

Control Treated

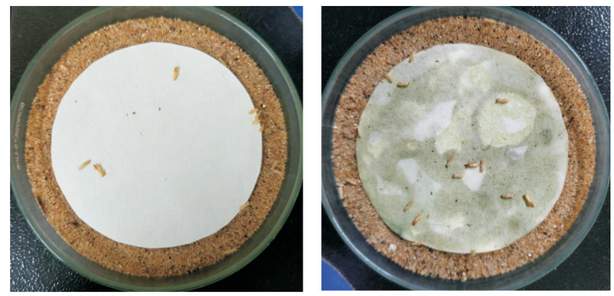

Fig. 3. No choice Filter Paper Bioassay:

Table 2. Morphological and biochemical Characteristics of the isolates:

Characteristics	*Bacillus sp.*	*Enterobacter sp.*	*Micrococcus sp.*
Cell morphology	Rod shaped	Rod shaped	Cocci
Motility	+	+	-
Gram reaction	+	-	+
Spore formation	Circular and subterminal	No spore formation	No spore formation
Colony morphology	Circular, Median, White, Flat	Circular smooth, Large, Pale White, Flat	Pale white, smooth and shiny
Utilization of			
Citrate	+	+	-
Carboxymethyl cellulose	+	+	+
Hydrolysis of			
Casein	+	+	+
Starch	+	+	+
Gelatin	+	-	+
Acid fermentation			
D-Xylose	+	+	+
D-Glucose	+	+	+
Monnitol	+	+	+
Cellobiose	+	+	+
Urea	-	-	-
Catalase	+	+	+
Oxidase	±	—	+
Methyl red	-	+	—
Voges-Proskauer	+	+	+
Nitrate reduction	+	+	+
Galactose	±	+	-
Lactose	+	+	+
Sucrose	+	+	+
Fructose	+	+	+
H2S production	+	±	-

+ - Positive; - - Negative; ± - variable; ND – Not detected;

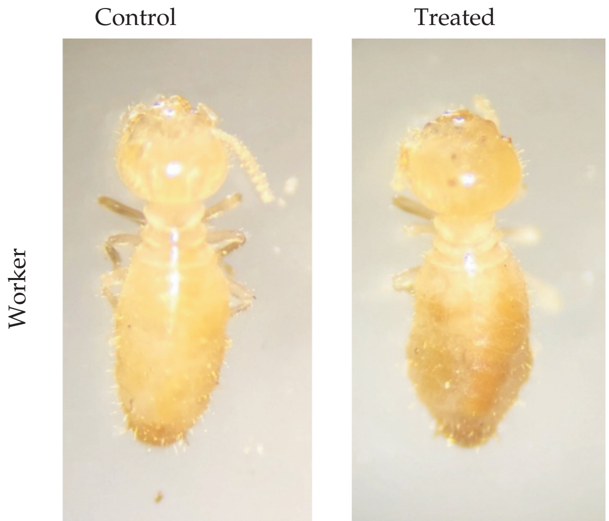

Fig. 4. Morphometric difference of the worker termite before and after treatment with neem leaf extract (50 µg/ml).

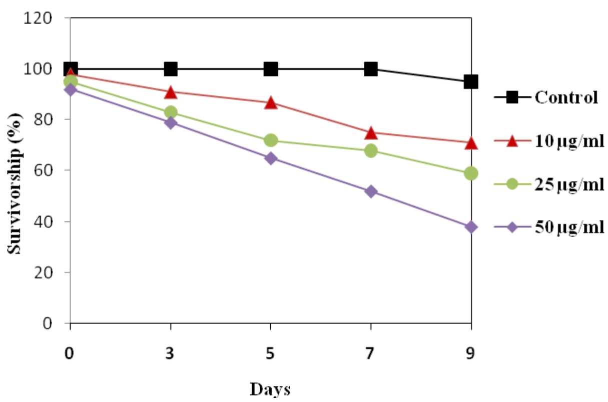

Fig. 5. Survival of termite on the exposure to different concentration of neem leaf extract.

(50 µg/ml) found to inhibit the feeding of the insect leading to the death. Morphological changes on termite before and after the treatment are shown in the figure 4. Shrinkage of the body and change in the colour of the insect was observed in. the larvae on exposure to the highest concentration of *A. indica* leaf extract (Fig. 4). Alavijeh *et al.*, (2014) reported morphological changes in the termite *Microcerotermes diversus* on exposure to Eucalyptus oil. At day 7 on the exposure of termites to higher concentration of neem leaf extract shows 52% mortality (Fig. 5). Our results illustrates *A. indica* leaf extract have bactericidal activity of the termite gut microbiota thereby inhibit the digestive potential leading to the death of the termites. The susceptibility of termites to *Azadirachta indica* leaf extract illustrates a new and noval control measures to be adopted in future.

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References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology.* 18: 265-267.

Acda, M.N. 2014. Repellent Effects of Annona Crude Seed Extract on the Asian Subterranean Termite Coptotermes gestroi Wasmann (Isoptera: Rhinotermitidae). *Sociobiology*. 61(3). https://doi.org/10.13102/sociobiology.v61i3.332-337

Alamu, O.T. and Ewete, F.K. 2023. Termites infestation on different Eucalyptus wood species and control using natural oil from plants. *European Journal of Wood and Wood Products.* 81(6): 1535-1542. https://doi.org/10.1007/s00107-023-01987-8

Alavijeh, E.S., Habibpour, B., Moharramipour, S. and Rasekh, A. 2014. Bioactivity of *Eucalyptus camaldulensis* essential oil against Microcerotermes diversus (Isoptera: Termitidae). *Journal of Crop Protection*. 3(1): 1-11.

Azhar, S., Aihetasham, A., Chaudhary, A., Hussain, Z., Rahat Abdul Rehman, Abbas, G., Sulaiman Ali Alharbi, Mohammad Javed Ansari and Samina Qamer, 2024. Cellulolytic and Ethanologenic Evaluation of *Heterotermes indicola*'s Gut-Associated Bacterial Isolates. *ACS Omega*. https://doi.org/10.1021/acsomega.3c10030

Brenner, D.J., Krieg, N.R., Staley, J.T. and Al, E. 2005. *Bergey's manual of systematic bacteriology / Vol. 2, The, Proteobacteria / Don J. Brenner Pt. B, The gamma proteo bacteria.* Springer.

Butera, G., Ferraro, C., Alonzo, G., Colazza, S. and Quatrini, P. 2015. The gut microbiota of the wood-feeding termite Reticulitermes lucifugus (Isoptera; Rhinotermitidae). *Annals of Microbiology*. 66(1): 253-260. https://doi.org/10.1007/s13213-015-1101-6

Hemeg, H.A., Moussa, I.M., Ibrahim, S., Dawoud, T.M., Alhaji, J.H., Mubarak, A.S., Kabli, S. A., Alsubki, R.A., Tawfik, A.M. and Marouf, S.A. 2020. Antimicrobial effect of different herbal plant extracts against different microbial population. *Saudi Journal of Biological Sciences.* 27(12): 3221-3227. https://doi.org/10.1016/j.sjbs.2020.08.015

Gerhardt, P., Murray, R.E., Wood, W.A. and Krieg, N.R. 1994. *Methods For General And Molecular Bacteriology.* ASM Press, Washington, DC.

Govorushko, S. 2018. Economic and ecological importance of termites: A global review. *Entomological Science.* 22(1): 21-35. https://doi.org/10.1111/ens.12328

Gupta, P., Samant, K. and Sahu, A. 2012. Isolation of Cellulose-Degrading Bacteria and Determination of Their Cellulolytic Potential. *International Journal of Microbiology.* 1-5. https://doi.org/10.1155/2012/578925

Manandhar, S., Luitel, S. and Dahal, R.K. 2019. *In vitro* Antimicrobial Activity of Some Medicinal Plants against Human Pathogenic Bacteria. *Journal of Tropical Medicine*. 1-5. https://doi.org/10.1155/2019/1895340

Nieto, G., Ros, G. and Castillo, J. 2018. Antioxidant and Antimicrobial Properties of Rosemary (*Rosmarinus officinalis* L.): A Review. *Medicines.* 5(3): 98. https://doi.org/10.3390/medicines5030098

Roonwal, M.L. and Chhotani, O.B. 1989. Fauna of India-Isoptera (Termites). Vol. I. *Zoological Survey of India* Publication, Calcutta. 420-461 pp.

Taechapoempol, K., Sreethawong, T., Rangsunvigit, P., Namprohm, W., Thamprajamchit, B., Rengpipat, S. and Chavadej, S. 2010. Cellulase-Producing Bacteria from Thai Higher Termites, Microcerotermes sp.: Enzymatic Activities and Ionic Liquid Tolerance. *Applied Biochemistry and Biotechnology.* 164(2): 204-219. https://doi.org/10.1007/s12010-010-9128-4

Thanh Giao, N. 2021. Investigating Pesticide Use and Management in King Orange (Citrus nobilis) Farming in Vung Liem District, Vinh Long Province, Vietnam. *International Journal of Environment, Agriculture and Biotechnology.* 6(1): 210-215. https://doi.org/10.22161/ijeab.61.25

Kumar, S. and Upadhyay, R.K. 2021. Anti-termite potential of various bio-organic constituents with special reference to family Asteraceae. *World Journal of Pharmaceutical Research.* 10(3): 1109-1149. https://doi.org/10.20959/wjpr20213-19977

Peristiwati, Natamihardja, Y.S. and Herlini, H. 2018. Isolation and identification of cellulolytic bacteria from termites gut (Cryptotermes sp.). *Journal of Physics: Conference Series.* 1013: 012173. https://doi.org/10.1088/1742-6596/1013/1/012173

Punitha, U. and Saral, A.M. 2024. Nickel oxide nanoparticles from Sargassumwightii: Synthesis, Characterization, and biomedical applications. *Results in Chemistry.* 7: 101289-101289. https://doi.org/10.1016/j.rechem.2023.101289

Verma, M., Sharma, S. and Prasad, R. 2009. Biological alternatives for termite control: A review. *International Biodeterioration & Biodegradation.* 63(8): 959-972. https://doi.org/10.1016/j.ibiod.2009.05.009

Wylie, M.R. and Merrell, D.S. 2022. The Antimicrobial Potential of the Neem Tree *Azadirachta indica*. *Frontiers in Pharmacology.* 13: 891535. https://doi.org/10.3389/fphar.2022.891535