

## Isolation and biochemical characterization of termite gut bacteria from Alagar Hills Reserve Forest, Dindigul District, Tamil Nadu

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### Abstract

Termites play a vital role in cellulose degradation through their symbiotic gut microorganisms, particularly bacteria. The present study aimed to isolate and characterize gut bacteria from *Cryptotermes* sp. collected from Alagar Hills Reserve Forest, Tamil Nadu. Gut bacterial isolates were cultured on nutrient agar, and four distinct bacterial strains were obtained. Growth analysis based on optical density measurements at 480 nm revealed that strain-2 exhibited the highest growth rate among the isolates. Colony morphology, including color, shape, elevation, margin, and size, was recorded, and all isolates were found to be motile. Gram staining showed that strains 1, 2, and 4 were Gram-positive bacilli, whereas strain 3 was a Gram-negative rod. Biochemical characterization indicated positive reactions for Methyl Red–Voges Proskauer (MR–VP), oxidase, and urease tests in all four strains, while all were negative for starch hydrolysis. Catalase activity was positive in strains 1, 3, and 4 but negative in strain 2. The study demonstrates the presence of diverse bacterial communities in the gut of *Cryptotermes* sp., comprising three Gram-positive and one Gram-negative bacterium, highlighting their potential role in termite digestion and cellulose degradation.

**Keywords:** Gram staining, Termites gut, pure culture, *Cryptotermes* sp., Gram negative bacteria

### Introduction

Termites are eusocial insects belonging to the order Blattodea and are among the most important decomposers in terrestrial ecosystems. They play a crucial role in carbon and nutrient cycling by degrading lignocellulosic materials such as wood, leaf litter, and soil organic matter. Their remarkable ability to digest cellulose is facilitated by a complex symbiotic association with diverse gut microorganisms, including bacteria, archaea, and protists, which produce cellulolytic and ligninolytic enzymes (Korsa *et al.*, 2023; Arab *et al.*, 2024) [7,1]. Termites are broadly classified into lower and higher termites based on their gut symbionts, with lower termites harboring flagellate protists and higher termites relying primarily on prokaryotic microbial communities for lignocellulose degradation (Gile, 2024) [5].

The termite gut functions as a highly specialized bioreactor, where microbial communities collaborate with the host to efficiently convert plant biomass into simpler metabolites that serve as energy sources. Recent metagenomic studies have revealed that termite gut microbiota possess diverse carbohydrate-active enzymes involved in cellulose, hemicellulose, and lignin degradation, highlighting their ecological and biotechnological importance (Marynowska *et al.*, 2023; Wang *et al.*, 2024) [8,11]. Furthermore, termite-associated microorganisms represent a valuable source of novel enzymes with potential applications in biofuel production, waste management, and industrial biotechnology. Among wood-feeding termites, species of *Cryptotermes* are particularly interesting due to their ability to survive exclusively on lignocellulosic substrates. Therefore, the isolation and characterization of gut-associated bacteria from termites can provide insights into microbial diversity and contribute to the discovery of

cellulolytic microorganisms with potential industrial applications.

### Materials and Methods

#### Collection and identifications of termites

Termite specimens were collected from four different sites in the Alagar Hills Reserve Forest, Tamil Nadu, India. The termites were obtained from dry wood habitats and transported in sterile 100 mL plastic containers to the Zoology Research Laboratory, V.H.N. Senthikumara Nadar College. Specimens were identified using standard morphological taxonomic keys and maintained under laboratory conditions for subsequent experimental studies.

#### Extraction of termite gut contents

Termites were surface sterilized with 70% ethanol, and the heads were removed aseptically. The bodies were crushed to obtain gut homogenates, which were serially diluted and used for the isolation of gut-associated bacteria.

#### Isolation and Characterization of Termite Gut Bacteria

Termite gut homogenates were serially diluted and cultured on nutrient agar plates at 37°C for 48 h. Distinct colonies were purified by repeated streaking and maintained on nutrient agar slants at 4°C and preserved in 20% glycerol at –20°C for long-term storage. Colony morphology was recorded, and bacterial motility was assessed using the hanging drop method (Arfah *et al.*, 2019) [2].

#### Gram staining

Gram staining was performed using a HiMedia Gram Staining Kit to determine the Gram reaction of the bacterial isolates. Smears prepared from 24 h-old cultures were heat-fixed and stained sequentially with crystal violet, Gram's

iodine, decolorizer, and 0.5% safranin. The stained slides were examined under a light microscope using the oil immersion objective, and the isolates were classified as Gram-positive or Gram-negative based on their staining characteristics.

### Biochemical characterization of bacterial isolates

Biochemical characterization of the bacterial isolates was carried out according to the procedures described in Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994) [6]. The isolates were subjected to various biochemical tests, including the Methyl Red (MR) test, Voges-Proskauer (VP) test, catalase test, oxidase test, urease test, and starch hydrolysis test. The results obtained from these tests were used for the preliminary identification and characterization of the bacterial isolates.

### Determination of bacterial growth curve

Bacterial growth was determined by inoculating the isolates into 20 mL of nutrient broth and incubating at 37°C with shaking at 120 rpm. Optical density (OD) was measured at 480 nm at 2 h intervals using a UV-Visible spectrophotometer. The growth curve was constructed by plotting OD values against incubation time according to the method of Arif *et al.* (2012) [3].

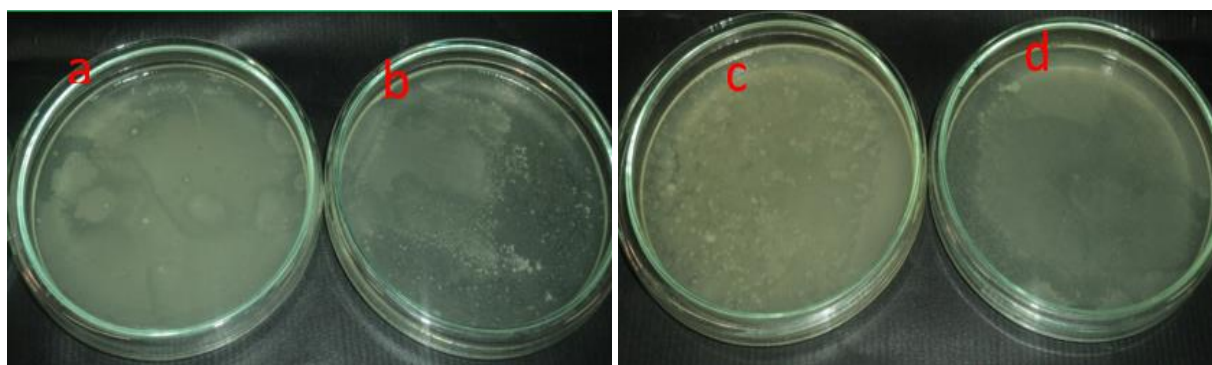
## Results and Discussion

### Identification of Termites

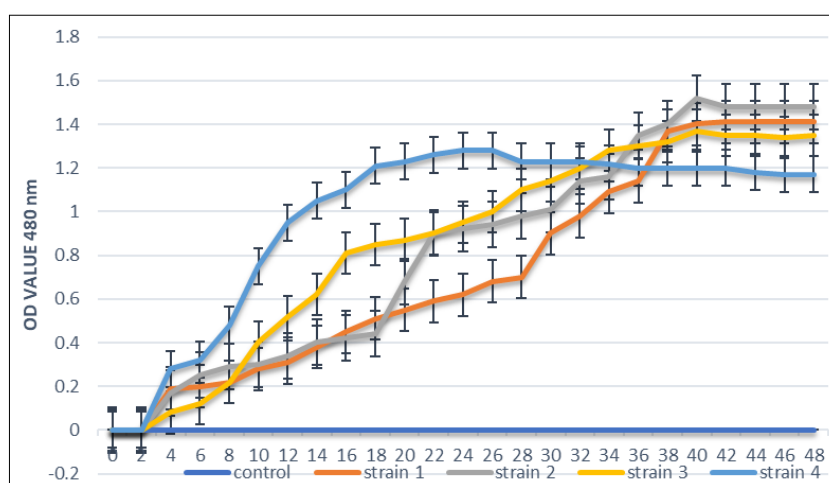
Termites collected from four locations within the Alagar Hills Reserve Forest were observed inhabiting both dry and decaying wood substrates. Based on their nesting behavior, habitat preference, and morphological characteristics, the specimens were identified as *Cryptotermes* sp. (Family: Kalotermitidae). The identification was supported by diagnostic features typical of drywood termites, including their habit of nesting entirely within wooden structures, compact and robust body form, strong mandibles adapted for wood feeding, moniliform (bead-like) antennae, short appendages, and relatively slow movement. Species of *Cryptotermes* are known to establish colonies within a single piece of wood and exhibit morphological adaptations associated with a wood-dwelling lifestyle (Arab *et al.*, 2024; Canello *et al.*, 2023) [1,4].

### Isolation and Identification of Gut Bacteria

Termite gut homogenates were serially diluted and cultured on nutrient agar to obtain pure bacterial isolates (Fig. 1). Four distinct bacterial strains were isolated and purified by repeated streaking. Growth curves of the isolates were determined by measuring optical density at two-hour intervals in nutrient broth (Fig. 2). Cellulolytic bacteria were identified using carboxymethyl cellulose (CMC) agar medium after incubation at 37°C for 48 h, following the method of Arif *et al.* (2012) [3].



**Fig 1:** Isolation of bacteria a) Site-1 b) Site-2 c) Site-3 d) Site-4



**Fig 2:** Bacterial growth curve

The colony characteristics of the four bacterial isolates, including colour, shape, elevation, margin, and size, were recorded on nutrient agar plates (Table 1). The isolates exhibited distinct morphological features comparable to

those reported for termite-associated bacteria by Oktiarni *et al.* (2021) [9]. Motility analysis using the hanging drop method revealed that all four isolates were motile, consistent with previous observations by Arfah *et al.* (2019) [2].

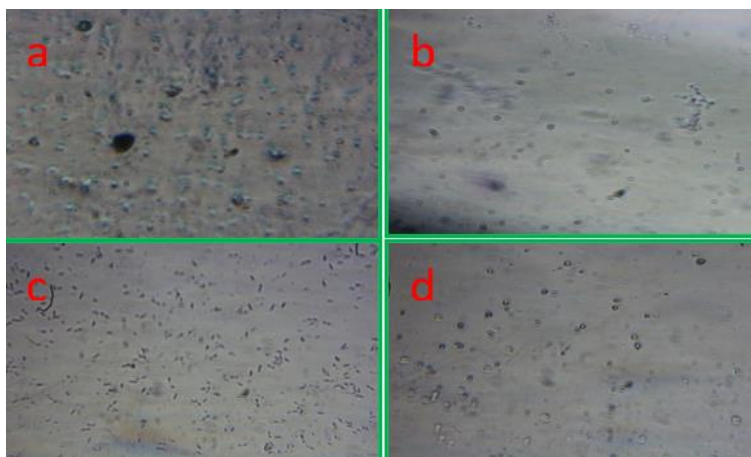
**Table 1:** Morphological characteristics of isolated bacteria

| Sl.No | Isolated strains | Shape     | Elevation | Margin   | Colony | Colour             |
|-------|------------------|-----------|-----------|----------|--------|--------------------|
| 1.    | Strain-1         | Irregular | Raised    | Undulate | Colony | White              |
| 2.    | Strain-2         | Irregular | Raised    | Undulate | Colony | White              |
| 3.    | Strain-3         | Irregular | Raised    | Undulate | Colony | White- transparent |
| 4.    | Strain-4         | Irregular | Raised    | Undulate | Colony | White              |

### Gram Staining

Gram staining differentiated the four bacterial isolates into Gram-positive and Gram-negative groups (Fig. 3). Strains 1, 2, and 4 were identified as Gram-positive bacilli, exhibiting a purple colour after crystal violet staining, whereas Strain 3

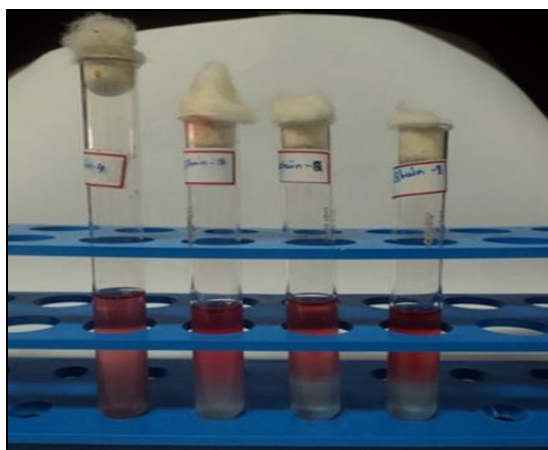
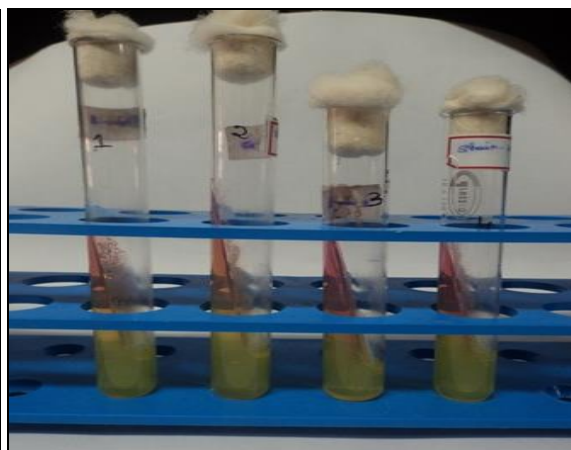
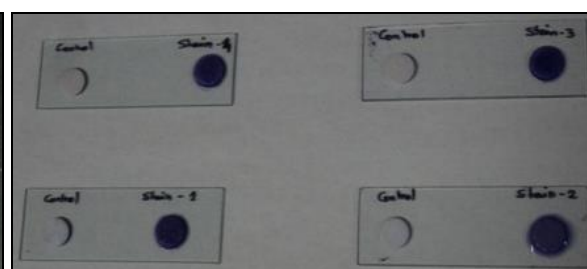
was identified as a Gram-negative rod, showing a red/pink colour following counterstaining. Similar observations of both Gram-positive and Gram-negative termite gut bacteria have been reported by Peristiwati *et al.* (2018) <sup>[10]</sup>.

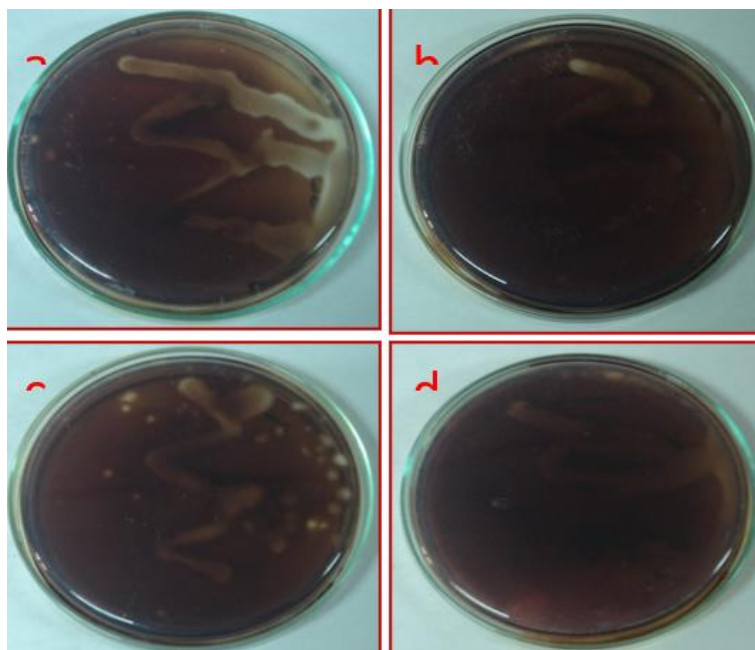
**Fig 3:** Gram staining techniques a) Strain-1 b) Strain-2 c) Strain-3 d) Strain-4

### Biochemical Characterization

The biochemical characteristics of the four bacterial isolates were determined using Methyl Red (MR), Voges-Proskauer (VP), Catalase, Oxidase, Urease, and Starch hydrolysis tests (Figs. 4–8). The isolates exhibited distinct biochemical

profiles, indicating differences in their metabolic and enzymatic activities (Table 2). Similar variations in biochemical characteristics among termite-associated bacterial isolates have been reported by Peristiwati *et al.* (2018) <sup>[10]</sup>.

**Fig 4:** Methyl red (MR), Voges-Proskauer (VP) tests**Fig 5:** Urease test**Fig 6:** Catalase test**Fig 7:** Oxidase test



**Fig 8:** Oxidase test

**Table 2:** Biochemical tests for isolated bacteria

| Sl.No | Isolated strains | Strain-1 | Strain-2 | Strain-3 | Strain-4 |
|-------|------------------|----------|----------|----------|----------|
| 1.    | MR & VP test     | Positive | Positive | Positive | Positive |
| 2.    | Catalase test    | Positive | Negative | Positive | Positive |
| 3.    | Oxidase test     | Positive | Positive | Positive | Positive |
| 4.    | Urease test      | Positive | Positive | Positive | Positive |
| 5.    | Starch test      | Negative | Negative | Negative | Negative |

## Conclusion

The present study successfully identified the termite *Cryptotermes* sp. and isolated four bacterial strains from its gut using nutrient agar medium. All isolates exhibited motility and were characterized based on morphological, Gram-staining, and biochemical analyses. The bacterial strains showed positive reactions for MR-VP, oxidase, and urease tests, while all were negative for starch hydrolysis. Gram-staining results revealed the presence of three Gram-positive bacteria and one Gram-negative bacterium. These findings demonstrate the diversity of gut-associated bacteria in *Cryptotermes* sp. and highlight their potential role in termite digestion and cellulose degradation.

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