Assessment of the entomopathogenic nematode bacteria against the termite, *Microtermes mycophagus* D. (Isoptera: Termitidae)

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**Abstract**

The capability of symbiotic bacteria of entomopathogenic nematodes against the termite, *Microtermes mycophagus* D. (Isoptera: Termitidae), was assessed. Different fractions of Pakistani isolates of entomopathogenic bacteria viz., *Xenorhabdus indica* strain (Pak.S.B.50), *X. indica* strain (Pak.S.B.56), *X. stockiae* strain (Pak.S.B. 65), and *X. steinernematis* strain (C.B.10) were assessed against *M. mycophagus* by direct contact method (spraying method) and sand assay in laboratory conditions. Mortality response of cell-free filtrates after 24 h at 20 °C for *X. indica* (Pak.S.B.50), *T*₂ = *X. indica* (Pak.S.B.56), *T*₃ = *X. stockiae* (Pak.S.B. 65), and *T*₄ = *X. steinernematis* (C.B.10) ranged (88.3–100%) as 33 ± 9.34, 98.33 ± 6.22, 88.33 ± 7.22, and 100.00% ± 0.00, respectively. In the case of sand assay, the most effective treatment was *T*₄, where (100%) mortality rate was recorded 24 h post application of B.S. (bacterial suspension) (4 × 10⁴ CFU/ml) and CFF (cell-free filtrate) (100 μl/10 ml) at 20 and 25 °C.

**Keywords:** Entomopathogenic bacteria, *Xenorhabdus*, Termite, *Microtermes mycophagus*, Bacterial fractions, Biocontrol

**Background**

The termite, *Microtermes mycophagus* D. (Isoptera: Termitidae: Macrotermitinae), is a cosmopolitan pest of wood and wood products that can be distinguished by its colonial behavior. Colony members are distinctly varied morphologically, i.e., propagative (king and queen), soldiers, and workers. The head termites, the king and queen, are sexually functional but pheromonal regulation that is responsible for the caste production is only produced by the queen (Noirot and Noirot-Timothee, 1970). Wingless individuals, workers or soldiers, are usually non-reproductive males or females. Soldiers play a major role to defend the colony and represent 1/10th of the population of a colony (Bignell and Eggelton, 1998). Termites are highly devastating and cause damage to furniture, buildings, trees, and agricultural crops, such as cereals, oil crops, pulses, sugar-cane, fruits, and vegetables. Estimated losses by this pest are about US$22 billion annually across the world (Govorushko, 2011). The genera viz., *Microtermes*, *Odontotermes*, and *Termes*, are the most prevalent termites in Pakistan (Manzoor and Naeem, 2010).

Chemicals control applications to the wood or to the soil have been determined time by time. Chemical fumigants containing methyl bromide, sulfuric fluoride, or a combination of carbon dioxide and methyl bromide is the suitable procedure of eradicating dry wood termites. Biocontrol agents are environment friendly and proficient in working but its cost effective feature is debatable. Abiotic factors such as warm and moist favored by subterranean termites, which promote epizootics, also have the potential for biological control (Verma et al., 2009). Few studies have reported the potential of entomopathogenic nematodes (EPN) to control termites. EPN exposure to termites...
resulted in significant response of parasitization (47% after 4 days) and 100% mortality after 12 days under lab conditions. Fujii (1975) gained 96% mortality results of C. formosanus within 7 days after treating with infective-stage Steinernema carpocapsae (Weiser) (Steinernematidae) in laboratory analysis. Mortality rate, more than 95%, was documented within 3 days by Georgis et al., (1982) for both Reticulitermes sp. and Zootermopsis sp. after laboratory exposure to S. carpocapsae; further termites were also serve as vectors for EPN that take back to their colonies. S. carpocapsae has shown high rates of infection to Nasutitermes costalis and R. flavipes obtained under laboratory conditions (Laumond et al., 1979 and Trudeau, 1989).

The present study aimed to assess the efficacy of symbiotic bacteria Xenorhabdus species as biopesticide against the termite, M. mycophagus, under laboratory conditions.

Materials and methods
Collection of termite
The termite (M. mycophagus) was collected from different infested trees in the premises of the University of Karachi, Karachi (24° 56’ 21.833” N, 67° 7’ 14.869” E), Pakistan.

Bacterial culture (isolation of bacteria from insect hemolymph)
Entomopathogenic nematodes (EPN) were obtained from the storage unit, maintained by Prof. Dr. Shahina Fayyaz at NNRC, University of Karachi, Karachi, Pakistan. All nematodes were propagated in last instar larvae of the greater wax moth, Galleria mellonella L., using the method of Dutky (1974). Infective juveniles were collected by White traps (White, 1927), harvested, and stored in sterilized distilled water at 10–15 °C for no more than 2 weeks before they were used.

Isolation of bacteria from insect hemolymph
To isolate bacteria from hemolymph, G. mellonella larvae were inoculated by EPN (Table 1) 100 IJs in a Petri dish lined with moistened filter paper. After 48 h the dead larvae were surface sterilized by 75% ethanol for 15 min; then, the cadavers were passed through the flame for further sterilization. Cadavers were dissected with sterilized scissors at the second foot, a loop full of hemolymph streaked onto NBTA agar medium (Akhurst, 1980). The streaked plates were incubated in the dark at 28°C for 48 h for the development of primary colonies. For further purification, single colonies of bacteria were sub cultured on new plates of agar medium. Then, single colony was transferred to the nutrient broth (0.81 broth + 61 ml water) and kept it for incubation on shaking bath for 2 days at 150 rpm ND 28 °C. The bacterial suspension was used for bioassay.

Table 1

| Strains | Bacterial symbionts | EPN | Accession no. | Authority* |
|--------|---------------------|-----|--------------|------------|
| Pak.S.B.50 | Xenorhabdus indica | Steinernema abbasii | MF498486 | Shahina F. & Salma J. |
| Pak.S.B.56 | Xenorhabdus indica | S. pakistantense | MF521953 | Shahina F. & Salma J. |
| Pak.S.B. 65 | Xenorhabdus stockiae | S. siamkayai | MF521964 | Shahina F. & Salma J. |
| Pak.C.B. 10 | Xenorhabdus steinernematis | S. maqbooli | KJ097324 | Shahina F. & Salma J. |

*Accession no. authorized by these persons

Biochemical analysis of bacterial isolates
The pure cultures of different species of Xenorhabdus were subjected to biochemical test through API 20E test kit of Biomerieux Ltd., USA.

Effect of different application method for controlling the termite
Pakistani isolates of entomopathogenic bacteria viz., Xenorhabdus indica (Pak.S.B.50), X. indica (Pak.S.B.56), X. stockiae (Pak.S.B. 65), and X. steinernematis (C.B.10) were assessed against M. mycophagus by direct contact method (spraying method) and sand assay in a laboratory experiment.

Spray method
Heavy infested branches of different trees were selected and kept in a plastic shopper after cutting with a hammer or cutter and brought in to the laboratory. Plastic containers about the size of 8 × 6 in. lined with wax at the edges were used for the experiment. Six-inch pieces of tree branches carrying approximately 50 termite individuals were placed in each container and sprayed with 20 ml of each treatment separately. Each container was sealed with a parafilm and each set of experiment incubated at different temperatures 20, 25, and 30 °C. Different EPB formulations were examined viz., B.S. (bacterial suspension) (4 × 10⁴ CFU/ml), CFF (cell-free filtrate) (100 μl/10 ml), B.R. (bacterial residue) (100 μl/10 ml) of T₁ (Xenorhabdus indica (Pak.S.B.50)), T₂ (X. indica (Pak.S.B.56)), T₃ (X. stockiae (Pak.S.B. 65)), and T₄ (X. steinernematis (C.B.10)). This combination was replicated 3 times with control treatment, which was sprayed only with water. Mortality rate was assessed each after 24, 48, and 72 h.

Sand barrier assay
A set of 50 termite individuals was placed in 6 × 6 in. Petri dish lined with a filter paper. For sand assay, a
thin film of autoclaved sand was spread over the filter paper and then the termites were placed. Twenty milli-
liters of different formulations B.S. (bacterial suspension) \((4 \times 10^8 \text{ CFU/ml})\), CFF (cell free filtrate) \((100 \mu\text{l}/10 \text{ ml})\), B.R. (bacterial residue) \((100 \mu\text{l}/10 \text{ ml})\) of \(T_1\) (\(Xenorhabdus indica\) (Pak.S.B.50)), \(T_2\) (\(X. indica\) (Pak.S.B.56)), \(T_3\) (\(X. stockiae\) (Pak.S.B. 65)), and \(T_4\) (\(X. steinernematis\) (C.B.10)) along with 1 ml of 2% Tween 80 (as emulsifier) were dropped on the sand layer under the laminar flow cabinet. Plates were sealed by a parafilm and incubated at different temperatures 20, 25, and 30 °C. Each treatment had 3 replicates. Control treatment only contained water. Mortality rate was assessed after 24, 48, and 72 h of exposure.

### Table 3 Influence of different bacterial fractions on the mortality of termites in relation to temperature and time duration by spray method

| Treatment                  | Conc. | Mortality% ± SD |
|----------------------------|-------|-----------------|
|                            |       | 20°C 24 h 48 h 72 h | 25°C 24 h 48 h 72 h | 30°C 24 h 48 h 72 h |
| \(T_1\), \(Xenorhabdus indica\) (Pak.S.B.50) | B.S.  | 83.66 ± 7.44a 100 ± 0.00c | 80.66 ± 6.98a 100 ± 0.00c | 71.66 ± 6.44a 78.66 ± 6.75a 78.66 ± 7.83a |
|                            | CFF  | 88.33 ± 9.34a 100 ± 0.00c | 87.33 ± 7.45a 100 ± 0.00c | 77.33 ± 8.54a 82.33 ± 7.35a 82.33 ± 8.41a |
|                            | B.R.  | 34.66 ± 5.34b 45.66 ± 5.41b | 32.33 ± 7.33b 45.66 ± 6.76b | 24.66 ± 5.20b 29.66 ± 5.67b 33.33 ± 5.75b |
| \(T_2\), \(Xenorhabdus indica\) (Pak.S.B.56) | B.S.  | 85.66 ± 7.34a 100 ± 0.00c | 80.33 ± 8.34a 83.33 ± 9.38a 92.33 ± 6.39c | 74.66 ± 6.78a 77.33 ± 6.78a 81.33 ± 6.39a |
|                            | CFF  | 98.33 ± 6.22c 100 ± 0.00c | 87.33 ± 8.32a 88.66 ± 7.57a 95.33 ± 9.34c | 78.33 ± 7.43a 80.33 ± 7.56a 82.33 ± 8.45a |
|                            | B.R.  | 35.66 ± 4.56b 37.33 ± 5.22b | 34.66 ± 8.55 b 35.33 ± 6.56b | 28.66 ± 4.56b 32.33 ± 4.87b 35.33 ± 5.43b |
| \(T_3\), \(Xenorhabdus stockiae\) (Pak.S.B. 65) | B.S.  | 85.66 ± 8.23a 100 ± 0.00c | 78.66 ± 8.23a 73.66 ± 9.26a 78.97 ± 7.87a | 69.66 ± 6.87a 72.66 ± 9.45a 79.66 ± 8.09a |
|                            | CFF  | 88.33 ± 7.22a 100 ± 0.00c | 88.33 ± 7.22a 100 ± 0.00c | 72.33 ± 8.49a 75.33 ± 7.54a 82.33 ± 9.34a |
|                            | B.R.  | 33.66 ± 4.22b 35.33 ± 6.22b | 29.33 ± 5.35b 30.66 ± 5.67b | 25.66 ± 3.86b 35.33 ± 6.22b 38.43 ± 4.10b |
| \(T_4\), \(Xenorhabdus steinernematis\) (C.B.10) | B.S.  | 100 ± 0.00c | 100 ± 0.00c | 100 ± 0.00c | 100 ± 0.00c | 100 ± 0.00c |
|                            | CFF  | 100 ± 0.00c | 100 ± 0.00c | 100 ± 0.00c | 100 ± 0.00c | 100 ± 0.00c |
|                            | B.R.  | 36.33 ± 3.43b 39.33 ± 6.37b | 34.33 ± 3.03b 35.66 ± 4.67b | 26.33 ± 3.65b 27.33 ± 4.55b 28.33 ± 5.43b |
| Control                    | H2O   | 0.00 ± 0.00d | 0.00 ± 0.00d | 0.00 ± 0.00d | 0.00 ± 0.00d | 0.00 ± 0.00d |

**Note:** B.S. bacterial suspension \((4 \times 10^8 \text{ CFU/ml})\), CFF cell-free filtrate \((100 \mu\text{l}/10 \text{ ml})\), B.R. bacterial residue \((100 \mu\text{l}/10 \text{ ml})\), “-” experiment complete

The similar alphabets in rows and columns have shown non significant differences at \(p = 0.01\)
**Data analysis**

Data are expressed as means, standard deviation and the significance of mean differences was determined with Duncan’s multiple range test (SAS Institute, Cary, NC).

**Results and discussion**

**Biochemical analysis**

The biochemical test of *Xenorhabdus* species were assessed for the following features. Citrate utilization, esculin hydrolysis, catalase, meso-inositol fermentation, salicin fermentation, ribose fermentation, and lipase tween 80. *X. indica* Pak.S.B.50 showed 90–100% positive expression in all analysis, except for Esculin hydrolysis. *X. indica* Pak.S.B.56 contained Citrate utilization; Esculin hydrolysis and Lipase Tween 80 resulted in 90% positive expression in all analysis, except for meso-inositol fermentation. *X. indica* Pak.S.B.65 had 25–74% positive results for meso-inositol fermentation where all remaining factors were found to be 90–100% negative.

**Table 4** Influence of different bacterial fractions on the mortality of termites in relation to temperature and time duration by sand barrier assay method

| Treatment | Conc. | Mortality % |
|-----------|------|-------------|
|           | 20°C | 25°C | 30°C |
|           | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| **T1, Xenorhabdus indica (Pak.S.B.50)** | B.S. | 82.66 ± 100 ± | - | 85.66 ± 100 ± | - | 73.33 ± 76.66 ± | 80.66 ± | 6.98 ± 0.00c | 8.56 ± 0.00c | 5.98 ± 7.87a | 6.88a |
| | CFF | 87.33 ± 100 ± | - | 87.33 ± 100 ± | - | 78.33 ± 84.33 ± | 85.33 ± | 7.98 ± 0.00c | 9.12a | 7.35a | 8.41a |
| | B.R. | 33.66 ± 45.66 ± | 47.33 ± 64.5b | 32.33 ± 45.66 ± | 47.33 ± 7.33b | 23.66 ± 30.66 ± | 35.33 ± | 7.33b | 7.54a | 7.56a | 7.89a |
| **T2, Xenorhabdus indica (Pak.S.B.56)** | B.S. | 88.66 ± 100 ± | - | 80.33 ± 83.33 ± | 92.33 ± | 72.66 ± 78.33 ± | 85.33 ± | 7.34a | 9.38a | 6.78a | 6.39a |
| | CFF | 93.33 ± 100 ± | - | 88.33 ± 86.66 ± | 95.33 ± | 75.33 ± 82.33 ± | 85.33 ± | 7.76a | 7.56a | 7.89a | 8.45a |
| | B.R. | 37.66 ± 38.33 ± | 39.33 ± 4.56b | 34.33 ± 35.33 ± | 39.33 ± 5.34b | 26.66 ± 36.33 ± | 39.33 ± | 7.33b | 8.45a | 9.45a | 8.09a |
| **T3, Xenorhabdus stockiae (Pak.S.B.65)** | B.S. | 88.66 ± 100 ± | - | 78.66 ± 73.66 ± | 78.97 ± | 65.66 ± 75.66 ± | 75.66 ± | 7.33b | 8.45a | 9.45a | 8.09a |
| | CFF | 85.33 ± 100 ± | - | 86.33 ± 100 ± | - | 71.33 ± 75.33 ± | 84.33 ± | 7.72a | 8.49a | 9.54a | 9.34a |
| | B.R. | 34.66 ± 35.33 ± | 42.22b | 38.33 ± 4.22b | 41.0b | 30.33 ± 34.66 ± | 38.66 ± | 6.87b | 6.88a | 7.56a | 6.22b |
| **T4, Xenorhabdus steinernematis (C.B.10)** | B.S. | 100 ± | - | 100 ± | - | 83.66 ± 100 ± | - | 0.00d | 0.00d | 4.87b | 4.78b |
| | CFF | 100 ± | - | 100 ± | - | 66.66 ± 100 ± | - | 0.00d | 0.00d | 6.78a | 6.39a |
| | B.R. | 38.33 ± 40.33 ± | 44.33 ± 3.43b | 35.33 ± 36.66 ± | 37.66 ± | 26.33 ± 28.33 ± | 34.33 ± | 7.34b | 6.88a | 7.56a | 5.43b |
| Control | H2O | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± | 0.00 ± |

*B.S.* bacterial suspension (4 × 10⁶ CFU/ml), *CFF* cell-free filtrate (100 μl/10 ml), *B.R.* bacterial residue (100 μl/10 ml). *-* experiment complete.

The similar alphabets in rows and columns have shown non significant differences at *p* = 0.01.

**Effect of different application methods for controlling Microtermes species**

**Spray method**

All bacterial isolates were found to be significantly effective against termites by spray method. Different fractions of bacterial formulations showed significant differences of mortality rate (*P* < 0.001). Bacterial suspension and cell-free filtrates of all treatments (bacterial isolates) had the potential to control termites at 20 and 25°C even after 24 h, whereas the bacterial residue of all the bacterial isolates had least potential for controlling termites. Due to direct contact of formulations with termites, effective results were obtained within 24 h in most of the cases. Mortality response of cell-free filtrates after 24 h at 20°C in *Xenorhabdus indica* (Pak.S.B.50), *T2* (*X. indica* (Pak.S.B.56)), *T3* (*X. stockiae* (Pak.S.B. 65)), and *X. steinernematis* C.B.10 expressed 90–100% positive for all examined biochemical tests but negative 90–100% for Salicin fermentation (Table 2).
In previous studies, after 5 days of incubation (Dua, 2014). Four bacterial strains having termite-killing ability showed >80% mortality biocontrol against termites. Different bacterial strains along with two control strains have been evaluated as Bacillus subtilis (100% mortality found to be 100% after 24 h of application of T1 (Xenorhabdus indica (Pak.S.B.50)), T2 (X. indica (Pak.S.B.56)), T3 (X. stockiae (Pak.S.B. 65)), and T4 (X. steinernematidis (C.B.10)). Significant differences were observed between control and treatments (P < 0.001). Effectiveness of different formulations was dependent on the temperature and time duration. The most effective treatment was T4, where maximum percentage of mortality found to be 100% after 24 h of application by B.S. (4 × 10^4 CFU/ml); CFF (100 µl/10 ml) at 20 and 25 °C. After applying T1 (Xenorhabdus indica (Pak.S.B.50)), T2 (X. indica (Pak.S.B.56)), and T3 (X. stockiae (Pak.S.B. 65)) 100% mortality of termites was obtained after 48 h in B.S. (4 × 10^4 CFU/ml) and CFF (100 µl/10 ml), whereas all insects survived in control treatment (Table 4).

Two different application methods were determined for their proficiency and significantly similar results obtained from both methods.

Different bacterial species have the capability to control termites. The effects of Bacillus thuringiensis subspecies was examined under laboratory conditions against Nasutitermes ehrhardtii (Castilhos-Fortes et al. 2002). They observed that B. thuringiensis subspecies kurstaki produced 80% mortality of termite species. B. thuringiensis proteins having insecticidal properties are highly specific as gut toxins and it has shown a superior safety in reference to the effectiveness for non-target organism (Sarwar, 2015).

Pseudomonas sp., P. maltophilia, Bacillus strains, and Paenibacillus sp., are reported to produce chitinase (Suyal et al., 2015; Verma et al., 2016a; Yadav et al., 2016). It was also reported that ten bacterial strains along with two control strains have been evaluated as biocontrol against termites. Different bacterial strains having termite-killing ability showed >80% mortality after 5 days of incubation (Dua, 2014). Four bacterial strains caused 100% killing at 10 days of observation. The cell-free culture filtrate of these cultures showed that the antagonistic substance was extracellular having protein properties. Bacterial strains of Bacillus subtilis KMB79 and Pseudomonas syxanthia KPM35 possessed proteolytic, chitinolytic, and lipolytic enzyme activities and caused 100% killing of termites (Yadav et al., 2016b).

In previous studies, X. nematophila has been proved as a potential candidate of biocontrol agent against termites (Hiranrongwera et al., 2007).

**Sand barrier assay**

In a sand barrier assay, Pakistani isolates of EPB were applied 20 ml of different formulations: B.S. (bacterial suspension) (4 × 10^4 CFU/ml), CFF (cell free filtrate) (100 µl/10 ml), B.R. (bacterial residue) (100 µl/10 ml) of T1 (Xenorhabdus indica (Pak.S.B.50)), T2 (X. indica (Pak.S.B.56)), T3 (X. stockiae (Pak.S.B. 65)), and T4 (X. steinernematidis (C.B.10)). Significant differences were observed between control and treatments (P < 0.001). Effectiveness of different formulations was dependent on the temperature and time duration. The most effective treatment was T4, where maximum percentage of mortality found to be 100% after 24 h of application by B.S. (4 × 10^4 CFU/ml); CFF (100 µl/10 ml) at 20 and 25 °C. After applying T1 (Xenorhabdus indica (Pak.S.B.50)), T2 (X. indica (Pak.S.B.56)), and T3 (X. stockiae (Pak.S.B. 65)) 100% mortality of termites was obtained after 48 h in B.S. (4 × 10^4 CFU/ml) and CFF (100 µl/10 ml), whereas all insects survived in control treatment (Table 4).

**Conclusion**

The present results proved that different bacterial fractions of Xenorhabdus species were found effective against termites in certain adequate conditions and can be a successful candidate for integration in termites’ controlling strategy.

**Abbreviations**

B.S.: Bacterial suspension; CFF: Cell-free filtrate; B.R: Bacterial residue

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**Authors’ contributions**

EYI planned the research experiment and performed biochemical analysis and mortality response. UAM and SFZA managed data and analyzed and interpreted the results. SR helped in writing the manuscript and reviewed the manuscript. SF provided the EPN material and supervised this research. All authors have read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

N/A

**Consent for publication**

N/A

**Competing interests**

The authors declare that they have no competing interests.

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