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Research article

Antimicrobial activity of a new entomopathogenic bacteria *Acinetobacter*

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ABSTRACT

The entomopathogenic nematodes (EPNs) are a nematode-bacterium complex. The symbiotic nematode-bacterial mutualism is highly specific. Novel EPN belonging to the family rhabditidae were reported for the first time from the Central Tuber Crops Research Institute. These new EPNs belonging to the *Rhabditis* sp. and their symbionts offer great scope for their exploitation of bio separation and identification of novel bioactive molecules. The present work is mainly focused on the antimicrobial activity of a new entomopathogenic bacteria *Acinetobacter*. The bacteria were isolated from infective third stage dauer juveniles of the nematode isolate collected from Vellayani, Thiruvananthapuram. The incubation time of the bacteria was standardized and cell free culture filtrate was prepared. The cell free culture filtrate was then separated into an organic and aqueous fraction. Antibacterial and antifungal activity of the organic fraction were tested. The organic fraction of 72 hr have highest antibacterial activity against *B.subtilis* with a zone diameter of 20 mm and lowest antibacterial activity with a zone diameter of 13 mm against *P. aeruginosa* and antifungal activity (29 mm zone diameter) in case of *A. flavus* and lower in case of *F. oxysporum* (18 mm zone diameter). Antimycotic and antibacterial activity was not observed in 24 hr organic fraction. From the study, it can be concluded that the organic fraction has significant antibacterial and antifungal activity. The present study also reveals that this entomopathogenic bacteria will be useful for the production of bioactive metabolites effective against bacterial and fungal diseases of plants and animals.

INTRODUCTION

Entomopathogenic nematodes (EPNs) lead a symbiotic association with specific enterobacteria. *Xenorhabdus* and *Photorhabdus* are two genera of bacteria that are symbiotically associated with specific nematodes belonging to the families Steinernematidae and Heterorhabditidae, respectively (Poinar, 1990).

Rhabditis (*Oscheius*) sp. isolated from different agro-climatic zones of Kerala resembles EPN and was found to be effective for the control of areca nut spindle bug in the field (Mohandas et al., 2004). These were found to kill a number of important insect pests within 24–72 hr in laboratory conditions. *Rhabditis* (*Oscheius*) sp. was also reported as a biological control agent against rice yellow stem borer, *Scirpophaga incertulas* (Walker) (Padmakumari et al., 2007).

A striking feature of Xenorhabdus and Photorhabdus is phase variation, which affects a large number of membrane-bound, intra and extracellular proteins and secondary metabolites (Akhurst 1996; Forst et al., 1997).

Phase I variants are involved in the symbiotic relationship with EPN and are isolated from the non-feeding infective stage nematodes and from the body cavities of insects killed by these nematodes. No role in symbiosis has yet been determined for phase II, which is associated only with entomopathogenic nematodes under laboratory conditions. They represent one important part of the spectrum of biocontrol agents that are used to control insect pests of economically important crops.

The importance of entomopathogenic bacteria (EPB) as a source for the discovery of antibacterial and antifungal molecules has been studied in depth, as highlighted in various reviews published in different time periods (Paul et al., 1981; Webster et al., 2002; Bode, 2009).

MATERIALS AND METHODS

Culture of insect and nematode

Greater wax moth (*Galleria mellonella*) was reared continuously in the laboratory using artificial diet following Woodring and Kaya (1988). EPN isolates were raised continuously on the laboratory-reared *Galleria mellonella* larvae (Figure 1).

Fig. 1. EPN infected with *Galleria mellonella* larvae

Bacterial strains

The bacteria were isolated from infective third stage dauer juveniles (Figure 2) of the nematode isolate (532) collected from the soil of Vellayani, Thiruvananthapuram, Kerala.

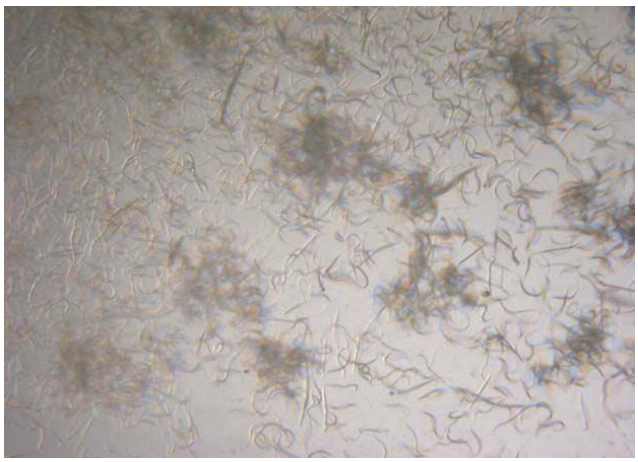

Fig. 2. Infective juveniles of EPN

Isolation of bacteria from infective juveniles

Infective juveniles (IJs) of nematodes (30 nos.) were transferred to 2 ml distilled water, treated with streptomycin (5000 units/ml) solution for one hour for surface sterilization. The nematodes were triple rinsed in sterile distilled water (Akhurst, 1980) and transferred into a microtube having 2 ml nutrient broth. It was then kept in a vortex shaker for 24 hr. The solution was then streaked on to nutrient agar plates (Woodring and Kaya, 1988) and kept at room temperature for 24 hr.

Isolation of primary colonies of bacteria

Nutrient agar (NA) of 100 ml was prepared and 0.0025 gm Bromo Thymol blue (BTB) was added and autoclaved at 121°C for 15 min. Just before pouring the medium Triphenyl Tetrazolium Chloride (TTC) (0.04 gm) was added.

Standardization of incubation time and preparation of cell-free culture filtrate

A pure culture of the bacterium was obtained from the third stage infective juveniles of the nematode isolate 532 and bacterial fermentation was carried out using Tryptic Soya Broth (TSB). Aliquots of the stock culture were added separately into 100 ml sterile medium. The flasks were incubated in a gyrorotatory shaker (150 × g) at 30°C for 24 hr. When the optical density of the culture at 600 nm was approx 1.7, the bacterial cultures were transferred aseptically into 400 ml sterile medium (TSB) and incubated in the gyrorotatory shaker (150 × g) at 30°C. Fermentation was carried out for 4 days during which samples (100 ml) were withdrawn at regular intervals (24 hr, 48 hr, 72 hr and 96 hr). The culture media were then centrifuged (10,000 × g, 20 min, 4°C) followed by filtration through a 0.45 µm microfilters to obtain cell-free culture filtrate.

Separation of cell-free culture filtrates into aqueous and organic fraction

Fifteen litres of TSB culture filtrate was separated into aqueous and organic fractions. For this, the filtrate was neutralized with concentrated hydrochloric acid and extracted with an equal volume of ethyl acetate thrice. The ethyl acetate layers were combined, dried over anhydrous sodium sulphate, and concentrated using a rotary flash evaporator at 30°C. The dry residue was weighed and reconstituted in 6 ml methanol and stored at -20°C for further studies.

Test bacteria, their source and maintenance

The following four bacteria *Bacillus subtilis* MTCC 2756, *Escherichia coli* MTCC 2622, *Staphylococcus aureus* MTCC 902 and *Pseudomonas aeruginosa* MTCC 2642 were purchased from IMTECH, Chandigarh and are maintained on Nutrient agar (NA) slants and sub cultured using standard aseptic laboratory techniques, every 2 weeks.

Test fungi, their source and maintenance

Aspergillus flavus MTCC 183, *Fusarium oxysporum* MTCC 284 and *Rhizoctonia solani* MTCC 2644 were purchased from IMTECH, Chandigarh. All fungi were maintained and sub cultured biweekly on potato dextrose agar (PDA) slants.

Antibacterial activity

Antibacterial activity of the crude and organic fractions of culture filtrate was measured using agar diffusion assays against the test organism *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*.

Agar-well diffusion method

The assay was conducted followed by (Perez *et al*., 1990). The test bacteria cultured on nutrient agar and incubated at 37°C for 18 hr were suspended in saline solution (0.85 % NaCl) and adjusted to a turbidity of 0.5 Macfarland standards (10^6 cfu/ml). The suspension was used to inoculate on Muller Hinton Agar (MHA) plates with a sterile non-toxic cotton swab. Wells were punched (6 mm dia) in the agar and filled with 50 µl of samples. Plates were incubated at 37°C for 24 hr. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone.

Antifungal activity

The antifungal activity of crude and the organic fraction was tested using the agar-well diffusion method (Perez *et al*., 1990). Wells were made on the agar surface with 6 mm cork borer. The sample of 50 µl was poured into the well using a sterile syringe. The plates were incubated at 37°C for 48 hr. The plates were observed for the zone formation around the wells.

RESULTS

Preparation of cell-free culture filtrate and its separation

The cell-free culture filtrate of 72 hr showed maximum antimicrobial activity.

Antibacterial activity

The diameter of zone of inhibition against the test bacteria is shown in Table 1.

Antifungal activity

The antifungal activity of organic fraction was tested against *A. flavus*, *F. oxysporum* and *R. solani*. The diameter of the zones of inhibition is given in Table 2.

Table 1. Antibacterial activity against *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*

Organic fraction	Concentration (µl)/disc	Zone of inhibition (dia in mm)			
		B. subtilis	***E. coli***	***S. aureus***	***P. aeruginosa***
24 hr	50	Nil	Nil	Nil	Nil
48 hr	50	10	09	07	05
72 hr	50	20	16	15	13
96 hr	50	13	11	12	10

Table 2. Antifungal activity against *A. flavus*, *F. oxysporum* and *R. solani*

Organic fraction	Concentration (µl)/disc	Zone of inhibition (dia in mm)		
		A. flavus	***F. oxysporum***	***R. solani***
24 hr	50	NIL	NIL	NIL
48 hr	50	19	17	14
72 hr	50	29	18	22
96 hr	50	18	20	13

DISCUSSION

The organic fraction of 72 hr exhibited significant antibacterial activity against both Gram-positive and Gram-negative bacteria and antifungal activity. The organic fraction of 72 hr has highest antibacterial activity (20 mm zone diameter) against *B. subtilis* and lower (13 mm zone diameter) against *P. aeruginosa* and antifungal activity (29 mm zone diameter) in case of *A. flavus* and lower in case of *F. oxysporum* (18 mm zone diameter). Antimycotic and antibacterial activity was not observed in 24 hr for an organic fraction.

CONCLUSION

From the results, it can be concluded that the crude organic extract of the entomopathogenic bacteria *Acinetobacter* spp. isolated from entomopathogenic nematode and *Rhabditis* (*Oscheius*) spp. have strong antimicrobial activity. The present study also reveals that this entomopathogenic bacteria will be useful for the production of bioactive metabolites effective against bacterial and fungal diseases of plants and animals.

CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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