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RESEARCH ARTICLE

Antimicrobial activity of crude extract obtained from an *Acinetobacter* sp. associated with an entomopathogenic nematode

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Abstract:
The present study was conducted to determine the antimicrobial activity of the crude extract of an *Acinetobacter* sp. associated with an entomopathogenic nematode.The bacterial fermentation was carried out using TSB medium up to 96 hr. After fermentation crude extract was extracted using ethyl acetate. Antimicrobial activity of the crude extract was tested by agar disc diffusion method. Our result clearly indicated that the crude extract recorded significant antimicrobial activity. Purification of this crude extract may result some more pure compounds with antimicrobial property which can be utilized by the pharmaceutical industry.

Keywords: TSB medium ,crude extract, fermentation,antimicrobial activity

Introduction

Xenorhabdus and *Photorhabdus* are Gram-negative bacteria that live in symbiosis with nematodes belonging to the familiessteinernematidae and heterorhabditidae[1]. The bacteria and nematodes share a complex life cycle, which includes symbiotic and pathogenic stages. Throughout their life cycle, the bacteria and the nematodes produce a variety of metabolites to enable them to colonize and reproduce in the insect host. The metabolites produced include molecules to help evade the insect immune system, insect toxins, enzymes such as proteases, lipases and phospholipases to maintain a food supply during reproduction[2] and antifungal and antibacterial agents to prevent degradation or colonization of the insect cadaver while the bacteria and nematodes reproduce[3]. A remarkable feature of *Xenorhabdu*s and *Photorhabdus* is the occurrence of variant cell types that arise during prolonged culture of the bacteria[4]. The primary cell type is carried by the infective juvenile and is characterized by the presence of numerous phenotypic traits. In the variant cell type, referred to as the secondary (or phase 11) form, the primary-specific traits are diminished or altered. Several phenotypic traits are consistently and simultaneously changed in the secondary cells of both *Photorhabdus* and *Xenorhabdus*[4]. These traits include the formation of non-mucoidy colonies, the loss of dye-binding ability and a reduction in the amount of pigments, antibiotics and crystalline inclusion bodies produced. Similarly, the production of fimbriae, the level of lipase, lecithinase and protease activity is reduced in many secondary cells of *Xenorhabdus* and *Photorhabdus*.

The production of secondary metabolites with antimicrobial properties is common to many *Xenorhabdus* species[5]. Although it is widely known that *Xenorhabdus* and *Photorhabdus* species produce chemically different antibacterial compounds[6], there has been no systematic research to find out differences between the antibiotics produced by these strains.

In the present study, cell free culture filtrate of the bacteria were prepared and separated into aqueous and organic fractions. Concentrated the organic fraction using a rotary flash evaporator at 30ºC. The antimicrobial activity of organic fraction was evaluated against pathogenic bacteria, and fungi. The organic fraction had significant antibacterial, antifungal, insecticidal and nematicidal activity. As the bacterial population increased, the antimicrobial activity increased and reached the maximal level at the stationary growth phase of the bacteria.

Materials and methods

Materials

Sterile nuclease-free and protease free glass wares and plastic wares were used for the preparation and storage of reagents and to carry out experimental procedures. The bacterial isolate used in the study is isolate 532. The bacterial and fungal strains used are given in the methods.

Methods

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 stockculture 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 asceptically into 400 ml sterile medium (TSB) and incubated in the gyrorotatory shaker (150 × g) at 30ºC. 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 micro filters to obtain cell free culture filtrate.

Separation of cell free culture filtrates into aqueous and organic fraction

Fifteen litres of TSB culture filtrate were 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 30ºC. The dry residue wa weighed and reconstituted in 6 ml methanol and stored at 20ºC 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

Antimicrobial activity of the crude and purified fractions of TSB medium were measured using agar diffusion assays against the test organism *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*. *Samples* to be tested were filtered through 0.22 µm micro filters. The level of activity was measured by the diameter (mm) of the zone of inhibition.

Agar-well diffusion method:

The assay was conducted followed by Perez et al[7]. 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 (10^6cfu/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 organic fraction was tested using well diffusion method[7]. Wells were made on the agar surface with 6 mm cork borer. The sample of 50 µl was poured into the well using 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

15L TSB was prepared. The cell free culture filtrate of 72 hr showed maximum antimicrobial activity and the cell free culture filtrate was separated into aqueous andorganic fractions.

Table 1: Antibacterial activity of crude extract

Time	Organic fraction	Zone of inhibition (dia in mm)			
		P.aeruginosa	*S. aureus*	*E. coli*	*B. subtilis*
24 hr	50	Nil	Nil	Nil	Nil
48 hr	50	9	10	11	12
72 hr	50	14	18	16	19
96 hr	50	10	11	12	14

Table 2: antifungal activity of crude extract

Time	Concentration (µl)/disc	Zone of inhibition (dia in mm)		
		R.solani	*F.oxysporum*	*A.flavus*
24 hr	50	NIL	NIL	NIL
48 hr	50	19	17	14
72	50	27	16	20
96	50	18	19	15

Organic fractions were concentrated and were used for antimicrobial assay.

Antibacterial activity

Bacillus subtilis which is very sensitive to EPB antibiotics is used to monitor activity during isolation and identification of EPB bioactive compounds. The diameter of zone of inhibition against the test bacteria are shown in Table 1.

Antifungal activity

The antifungal activity of organic fraction was tested against *A. flavus*, *F. oxysporum* and *R. solani*. It is displayed in Fig.6.7-6.9 and diameter of zones of inhib ition is given in Table2.

Discussion

The organic fraction of 72 hr exhibited significant antibacterial activity against both Gram- positive and Gram-negative bacteria and antifungal activity. The increase in antibiotic activity can be explained by the accumulation of the antibiotics synthesized and released by the bacterial isolate. The present study shows that organic fraction have highest antibacterial activity against *B. subtilis* with a zone diameter of 19 mm and lowest antibacterial activity with a zone diameter of 14 mm against *P. aeruginosa*. 24 hr organic fraction lacks anti bacterial activity. Akhurst[8] demonstrated the antibiotic activity of cultures of *Xenorhabdus* sp. against a wide variety of microorganisms, including the Gram-positive *Micrococcus*, *Staphylococcus* and *Bacillus*, the Gram-negative *Escherichia*, *Shigella*, *Enterobacter*, *Serratia*, *Proteus*, *Erwinia*, *Flavobacterium* and *Pseudomonas*. Maxwell et al.[9] recorded that antibacterial activity of some *X. nematophila* isolates was greatest against bacteria of the family *bacillaceae*, moderate against those of the family *pseudomonadaceae*and least against the family *enterobacteriaceae*.Organic fraction of 72 hr has highest antifungal activity (27 mm zone diameter) in case of *A. flavus* and low in case of *F. oxysporum* (16 mm). Antimycotic activity was not observed in 24 hr organic fraction. In this study the metabolites of the bacterial strain have effective fungicidal activity against*A. flavus*, *F. oxysporum* and *R. solani*. Through improving the efficiency of fermentation techniques and also by purification, it is possible that the activity may increase manifold.

Sustainable agriculture will rely increasingly on alternatives to conventional chemical insecticides for pest management that are environmentally friendly and reduce the amount of human contact with hazardous pesticides. Microbial control in conjunction with other IPM components can provide effective control. Aspects that warrant further study and attention are storage, marketing and transfer of technology to growers. In all,

281 biopesticides were available in the market in 1993, with active ingredients of bacteria, EPN, fungi and viruses[10].

Screening microbial secondary metabolites is an established method to identify novel biologically active molecules. Preparation of biological screening samples from microbial fermentation extracts requires growth conditions that promote synthesis of secondary metabolites and extraction procedures that capture the secondary metabolites produced. Microbial extracts have been and continue to be a productive source of new biologically active molecules for drug discovery. It is estimated that more than 30% of worldwide human pharmaceutical sales have compounds from natural sources as their origin.

Conclusion

From the study it can be concluded that the cell free culture filtrates of the bacteria have significant antibacterial, antifungal, nematicidal and insecticidal activity. Although in the present study, the broth medium used was TSB, further experimentation is required to isolate and characterize the bacterial metabolites from different media and compare with that in insect haemolymph as suggested by earlier workers.

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References

1. Forst, S., Dowds, B., Boemare, N. and Stackebrandt, E. 1997. *Xenorhabdus* and *Photorhabdus*spp. : bugs that kill bugs.*Ann. Rev. Microbiol.*, 51: 47-72.
2. Thaler, J. O., Duvic, B., Givaudan, A. and Boemare, N. E. 1998. Isolation and entomotoxic properties of the *Xenorhabdus nematophilus* F1 lecithinase. *Appl. Environ. Microbiol*., 64: 2367-2373.
3. Akhurst, R. J. 1993. Bacterial symbionts of entomopathogenic nematodes— the power behind the throne. In: Bedding, R., Akhurst, R. and Kaya, H. (Eds). *Nematodes and the biological control of insect pests*. Melbourne, Australia, CSIRO Press, 127-135 pp.
4. Boemare, N. E., Thaler, J.O. and Lanois, A. **1997.** Simple bacteriological tests for phenotypic characterization of *Xenorhabdus*and *Photorhabdus* phase variants. *Symbiosis*, 22:167-175.
5. Webster, J. M., Chen, G. and Li, J. 1998. Parasitic worms: An ally in the war against the superbugs.*Parasitol. Today*, 14: 161-163.
6. Webster, J. M., Chen, G., Hu, K. and Li, J. 2002. Bacterial metabolites. In: Gaugler, R (ed.), *Entomopathogenic nematology*. CABI Publishing, New York. 99-114.
7. Perez, C., Paul, M. and Bazerque, P. 1990. Antibiotic assay by agar-well diffusion method. *Acta. Biol. Med. Exp*, 15: 113-115.
8. Akhurst, R. J. 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. *J. Gen. Microbiol.*, 128: 3061-3065.
9. Maxwell, P. W., Chen, G., Webster, J. M. and Dunphy, G. B. 1994. Stability and Activities of Antibiotics produced during Infection of the Insect *Galleria mellonella* by Two Isolates of *Xenorhabdus nematophilus*. *Appl. Environ. Microbiol.,*Vol. 60, No.2. p715-721.
10. Lisansky, S. G. and Coombs, J. 1994. Developments in the market for biopesticides. Brighton Crop Protection Conference - Pests and Diseases, 1049-1054.