Isolation and Identification of antibacterial compound producing Actinomycetes species ICN699 from a terrestrial Ecosystem

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Abstract : The main objective of this study was to screen and isolate novel antibacterial compound producing actinomycete from Therur pond wetland ecosystem and to isolate the active compound by bioassay-medicated isolation. In this study Actinomycete strain ICN699 was isolated and screened using double layer agar method against drug resistant *Enterobacter* sp. Identification of the antagonistic species was carried out through 16S rDNA sequencing analysis. Production, extraction and TLC bioautography based partial purification was carried out and the Minimal inhibitory concentration value was determined using broth dilution method. The potent antibacterial strain was identified *as Streptomyces* sp. ICN699. TLC bioautography revealed the Rf value of the active principle were detected 0.17 to 0.28. Minimal inhibitory concentration of the partially purified compound showed 20 µg/ml against *Enterobacter* sp. The antibacterial active strain ICN699 from Kanyakumari wetland ecosystem proves to be a good source of antimicrobial agent for future studies.

***IndexTerms* – Soil, Actinomycetes, antimicrobial agents, *Enterobacter* sp., drug resistant.**

I. INTRODUCTION

Multi-drug-resistant (MDR) bacteria including *Enterobacter* sp. and *Staphylococcus aureus* constitute a serious problem in hospital environments, which require new active antibiotics of wide spectrum (Doern et al., 1998). Serious infections caused by various bacteria have become resistant to commonly available antibiotics and become a major health problem globally in the 21st century (Alanis, 2005). Methicillin- resistant *Staphylococcus aureus* (MRSA) is a pathogen responsible for a wide range of infections such as pneumonia, boils, osteomyelitis, bacteremia, endocarditis, etc. and has developed resistance to the majority of conventional antibiotics (Enright, 2003). For more than two decades, these strains were effectively controlled with vancomycin. However, there is an increased incidence of emergence of antibiotic-resistant bacterial strains (Bozdogan and Esel, 2003; Chang et al., 2003). In recent years novel therapeutic substances have entered in the clinical area, unfortunately with serious side effects (Wenzel, 2004). Side effects of currently available drugs and drug resistance have become serious public health problems which require the development of new antimicrobial agents (Paterson et al., 2004). Currently, many researchers in the field of drug discovery are working on new antimicrobial drugs, mainly of actinomicetal origin (Oskay et al., 2003). Actinomycetes are producers of various metabolites with antiviral, antitumor activity, antimicrobial, anti-parasite, cytotoxic, etc; whose chemical structures are almost unique. Until recently marine sediments as a source of bioactive actinomycetes have remained as the least explored resources, but now a days it becomes one of the promising sources. *Streptomyces* are a prolific source of secondary metabolites yielded many antibiotics; more than 80% of antibiotics available in the market are from *Streptomyces (*de Lima et al., 2012). The present study is aimed to investigate the antibacterial activity of Actinomycetes species isolated from a terrestrial ecosystem of Thadikkaran konam, Kanyakumari district, Tamilnadu, India

MATERIALS AND METHODS

Isolation of actinomycetes

The soil sediment samples were collected from Thadikkarn konam ecosystem of Kanyakumari District, Tamilnadu, India at 8° 07' 14.41" N Latitude 77° 22' 18.48" E Longitude. It was transferred to a sterile bag and transported to the laboratory. Further, one gram of air dried soil sediment sample was serially diluted in sterile double distilled water and isolated using

Actinomycetes Isolation Media (AIM) (g/L) containing soluble starch - 20, KNO_3 -1, NaCl - 0.5, K_2HPO_4 - 0.5, $MgSO_4$ - 0.5, $FeSO_4$ – 20 µM and agar 15 in double distilled water. The medium was supplemented with nalidixic acid 50 µg and nystatin 100 µg to inhibit bacterial and fungal strains. The plates were incubated at 28 °C and colonies were isolated after 7-9 days of incubation.

Screening of actinomycetes for antibacterial activity by double layer method

The isolated 10 actinomycete strains were patched on the center of the AIM plates and incubated at 28 ºC for 5-7 days. After incubation, Luria broth soft agar with fresh inoculums prepared from the overnight culture of bacterial pathogen *Enterobacter* sp. was prepared and overlaid on the culture medium separately. Plates were then incubated at 37 ºC for 24 h and zone of inhibition was measured. Antagonistic activity of the strain was identified by the observation of zone of inhibition.

Morphological characterization

The actinomycete isolates were differentiated by microscopic observation based on their colony characters, such as colour, shape, and size. Cultural characteristics of isolated actinobacterial strains were examined by the visible observation of 14-day-old culture grown on AIM medium. Colony morphology was registered with respect to colouration of aerial and substrate mycelium, branch and the nature of colony under light microscope by cover slip culture method after incubating at 28º C for two weeks.

Taxonomic characterization of Actinomycetes species ICN699

The antibacterial isolate Actinomycetes species ICN699 was grown on Actinomycetes Isolation Medium and incubated at 28 °C for 7 days. The grown biomass was scrapped from the surface of the medium by using plastic loops, carefully transferred to sterile 1.5 ml test tubes and either immediately used for DNA purification or kept at -20 °C until required. Isolation of DNA and identification of the organism was carried out as described previously. The evolutionary history was inferred using the Neighbor-Joining method12 followed by the multiple sequence alignment done through MEGA6 software package13.

Production of secondary metabolites by solid state fermentation and extraction

A loop full of the culture Actinomycetes species ICN699 from a well sporulated mycelium was grown on AIM medium 28°C for 7 days. After 7 days, the mycelium containing agar medium was cut into small pieces in an aseptic condition and equal volume of ethyl acetate was added for cold percolation method extraction. The flask was kept in the shaker at 200 rpm for 18-24 h. The remaining mycelial agar pieces were again extracted using equal volume of methanol. The organic crude extracts were filtered using Whattman No.1 filter paper and the organic solvents were evaporated in vacuum concentrator (Eppendorf 5301) at 30 ºC. After evaporation dry compounds were deposited at ICN Small Molecules Library (ISML) and stored at -20 ºC.

Antibacterial assay and determination of minimum inhibitory concentration (MIC)

In order to evaluate the antibacterial properties of secondary metabolites, the crude extracts were assayed by Kirby-Bauer antibiotic susceptibility test along with Vancomycin (10 µg/disc) as positive control to confirm the antagonistic activity. A disc loaded with methanol alone was used as negative control. Briefly, overnight culture of *Enterobacter* strain was grown to mid-logarithmic phase at 37 ºC in a shaking incubator. These selected pathogenic test strain cultures were swabbed on the Mueller-Hinton agar plate and discs were loaded with the crude extract. Plates were incubated at 37 ºC for 18 h and observed for the presence of zone of inhibition (Bauer et al., 1966). MIC assay of the active TLC fraction was carried out using 0.5 McFarland standards (1 x 108 CFU ml^{-1}) of overnight bacterial culture in a 96 well microtitre plate. Increasing concentrations (0.1, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 µg/ml) of fractioned extract was treated with test pathogens and monitored its inhibitory potential after overnight incubation at 37 ºC.

Purification of secondary metabolities by Thin Layer Chromatography (TLC) and Bioautography analysis

Commercially available TLC silica gel 60 F254 aluminium sheets (Merck) were dried at 80ºC for 15 min. The silica gel sheet was allowed to cool at room temperature and marked about 1 cm from the bottom as the origin. 300 mg of crude extract was dissolved in 1 ml methanol and 5 µl of the dissolved phase was spotted on the TLC sheet on the marked area. The development tank was saturated with suitable mobile phase of chloroform: methanol (9:1) for the purification of crude extract from ICN699. The TLC sheet was kept in the tank without touching baseline by solvent and left for development. The final solvent front was marked and the TLC sheet was dried. The duplicate TLC sheets were visualized in the UV transilluminator. The *Rf* value of the spot was calculated using the following formula, *Rf* = Distance analyte travels / Distance solvent travels. The developed TLC sheet was overlaid with 1 × 106 CFU/mL of *Enterobacter* sp. suspension prepared in a fresh Mueller-Hinton broth in 0.4 % agar. The plate containing sheet was incubated overnight at 37˚C in a humidified environment for 14 h. After incubation, the TLC sheet was sprayed with a 2% solution of 2,3,5-triphenyl-tetrazolium Chloride (TTC) and incubated for further 6 hours. Inhibition zone was observed as clear area against a red-colored background on the TLC sheet.

RESULTS AND DISCUSSION

Isolation and screening of antibacterial compound from Actinomycetes sp. ICN699

In the present study the potent antibacterial secondary metabolite producing Actinomycetes species ICN699 was isolated and characterized. The isolate was white to pale green in colour, without any pigmentation, aerial mycelium was observed with white in colour, substrate mycelium is brown in colour and lightly branched, melanin pigment is dark brown and metabolite

exudation is absent. The antibacterial activity of the strain ICN699 showed considerable inhibitory effect against the tested bacterial pathogens. The present study highlighted the antibacterial potential of Actinomycetes species 699 isolated from a terrestrial ecosystem of Thadikkaran konam, Kanyakumari district. Among all the actinomycete species, Actinomycetes species are the biggest contributors of antibiotics (Pandey et al., 2004). Most of these antimicrobial compound producing strains are reported to be terrestrial and are still to be very much explored, prove to be more novel product providers. Our study area, Thadikkarankonam terrestrial ecosystem is rubber plant vegetation from which the strain ICN699 was isolated. This protected ecosystem could provide a diverse variety of microbes with novel applications. Remarkably, 16S rDNA sequence similarity searches in EZtaxon database analysis15 revealed the similarity of ICN 699 to Actinomycetes species (Fig. 1). The BLAST results show higher similarity to *Streptomyces parvulus* and the phylogenetic analysis shows close relation of ICN699 with *Streptomyces bellus*. The *Streptomyces* species relating to ICN699 are previously reported to produce antibiotics like althiomycin16 and Actinomycin D17. Similar to these phylogenetic neighbor strains ICN699 show activity against, *Enterobacter* sp.

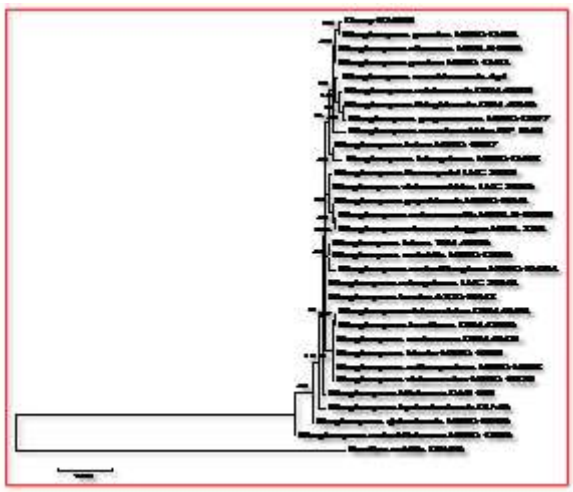

Fig. 1. Phylogenic relationship of ICN699 and closely related sequences within the genus based on the 16S rRNA gene sequence

Production and Bioassay guided detection of antibacterial substance

Purification through TLC on chloroform: methanol (9:1) mobile phase revealed many bands in UV transilluminator and the TLC autobiography with TTC of ICN699 extract revealed the active compounds with relatively lower Rf values of 0.11 and 0.29 (Fig. 2). Further the active components were isolated and pooled for its MIC determination. The fractioned substance from ICN699 was inhibited the bacterial growth at 20 µg/ml against *Enterobacter* sp. The production of secondary metabolites is more strain specific rather than species specific, which implies the possibility of a same species of different strains producing same compound less frequent. Our search for novel strains on relatively less exploited terrestrial wetlands provided an active compound with a wide activity ranges against the tested pathogens. Minimal Inhibitory Concentration (MIC) results of ICN699 extract proves to be promising source of antimicrobial compounds. A previous study reported the TLC autobiography guided detection of compounds against *Enterococcus* sp. from Actinomycetes species and reported that the R*f* value of the compounds from 0.17 to 0.28. However, TLC autobiography of ICN699 extract reveals the active compounds being less mobile on chloroform: methanol (9:1) mobile phase with relatively lower R*f* values. A similar study on the extracts obtained from *Streptomyces* isolated from soil sediment showed various bioactive regions with Rf values ranges from 0.21 to 0.96 (Ilic et al., 2007). Similar result was reported from the cultural extracts of marine sponges associated *Streptomyces* (Dharmaraj and Sumantha, 2009).

Fig. 2: TLC bio autography analysis of crude extract from ICN 699 against *Enterobacter* sp. Zones of inhibition shows the active spots with Rf values 0.23 and 0.69.

CONCLUSION

Soils of different ecological niches from Tamilnadu, India were processed to obtain various Actinomycetes isolates. Secondary metabolites were extracted from the organism and showed potent activity against *Enterobacter* sp. The current stud has enlightened the functional characterization of novel secondary metabolities from soil Actinomycetes.

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