Isolation and Identification of antibacterial compound producing Streptomyces parvulus ICN698 from a Wetland Ecosystem of Kanyakumari

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ABSTRACT

Objective: *The alarming threats of drug resistant pathogens have created the search of new antibiotics from different environmental niches. The goal of this study was to identify the antibacterial compound producing actinomycete from Therur pond wetland ecosystem and to isolate the active principle by bioassay-directed isolation.*

Methods: *Actinomycete strain ICN698 was isolated and screened through double layer agar against drug resistant bacterial pathogens. 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.*

Results: *The antibacterial active strain was identified as Streptomyces parvulus ICN698. TLC bioautography revealed the Rf value of the active principle were detected at 0.11 and 0.29. Minimal inhibitory concentration of the partially purified compound showed 16 µg/ml against MRSA, K. pneumonia, A. baumannii and Enterobacter sp. and 64 µg/ml of MIC against P. aeruginosa.*

Conclusion: *The antibacterial active strain ICN698 from Kanyakumari wetland ecosystem proves to be a good source of antimicrobial agent for further studies.*

Key Words: *Wetland ecosystem, TLC bioautography, 16S rDNA sequencing, Enterobacter sp., Streptomyces parvulus.*

INTRODUCTION

Drug resistant pathogens are becoming a serious threat to health care systems globally[1]. Mainly, Methicillin resistant *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumanni* and *Enterobacter sp.* have caused several thousands of deaths and patients have become more complicated to treat these infections worldwide[2]. Many programs aimed at the discovery of new secondary metabolites with interesting biological activities from microbial sources have found impressive number of compounds during the past 30 years[3]. Such potent compounds form an untapped niche is desirable for any drug discovery program. The Indian environment is reported to be rich in biodiversity, especially many novel microorganisms have been discovered[4,5].

Actinomycetes represent one of the most prolific microbial sources for the discovery of bioactive metabolites which primarily inhabit the soil[6]. Searching of novel antimicrobial secondary metabolites from actinomycetes is gaining momentum in recent years especially, their ability to produce antibiotics. These searches have been remarkably successful and approximately two thirds of naturally occurring antibiotics had derived from actinomycetes mostly from the genera *Streptomyce*s and *Micromonospora*[7,8]. The present study is aimed to investigate the antibacterial activity of *Streptomyce*s isolate from a wetland ecosystem of Therur, Kanyakumari.

MATERIALS AND METHODS

Sample Collection and Isolation of actinomycetes

The sediment samples were collected from Therur pond ecosystem of Kanyakumari District, Tamilnadu, India at 8° 07' 14.41" N Latitude 77° 22' 18.48" E Longitude. The central portion of the 6-10 cm sediment sample was taken and transferred to a sterile bag and transported immediately to the laboratory. 1 g of air dried soil sediment sample was serially diluted in sterile distilled water and spread plated over the Actinomycetes Isolation Media (AIM) containing soluble starch 20g, KNO_3 1g, NaCl 0.5g, K_2HPO_4 0.5g, $MgSO_4$ 0.5g, $FeSO_4$ 20µM and agar 15g in 1L distilled water. The medium was supplemented with nalidixic acid 50 µg and nystatin 100µg to inhibit bacterial and fungal contamination respectively. The

plates were incubated at 28°C and colonies were isolated after 7-9 days[5, 9].

Antibacterial screening by double layer agar method
The isolated actinomycete strains were patched on the center of the AIM medium plates and incubated at 28ºC for 5-7 days. After incubation, Luria broth soft agar with fresh inoculums prepared from the overnight culture of bacterial pathogens like Methicillin resistant *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, Enterobacter* sp and *Klebsiella pneumoniae* were prepared and overlaid on the culture medium separately. Plates were then incubated at 37ºC for 24 hours and zone of inhibition was measured. Antagonistic activity of the strain was identified by the observation of inhibition zones.

Morphological characterization
Macroscopically, the actinomycete isolates were differentiated by their colony characters, e.g. size, shape, colour etc. Cultural characteristics of isolated strains were examined by the visible observation of 14-day-old culture grown on AIM medium. Colony morphology was noted with respect to color of aerial and substrate mycelium, branching and the nature of colony under light microscope by cover slip culture method[10] after incubating at 28ºC for 14 days.

Taxonomic characterization by 16S rDNA sequencing and Phylogenetic analysis
The antibacterial strain *Streptomyces parvulus* ICN698 was swabbed on Actinomycetes Isolation Medium and grown 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. It was suspended in 5 ml sucrose TE buffer containing 2 mg/ml lysozyme. The suspension was incubated at 37 °C and titrated every 15 min continuously till completely lysed. Lysis was checked by placing a drop of 10% SDS on a drop of suspension on a slide. Clear solution indicated complete lysis of colonies. 1.2 ml of 0.5 M EDTA and 0.13 ml of pronase were added mixed gently and incubated at 30 °C for 5 minutes. 0.7 ml of 10% SDS was added and mixed, incubated at 37°C for 2 hrs. 6 ml buffered phenol was added and mixed for 5 minutes at room temperature. Spun at 6,000 rpm for 20 min. Aqueous phase was carefully transferred to another tube with autoclaved cut tips. One phenol: chloroform (1:1) wash with equal volume, two chloroform washes with equal volume were given at 10,000 rpm for 10 minutes. RNaseA final concentration 40 µg/ml was added and incubated at 37°C for 30 minutes. DNA was precipitated with two volume of cold ethanol (100%). Spooled DNA was taken in screw tight cap with 70% ethanol wash and air dried. The 16S rRNA universal primer (27f and 1525r) and genomic DNA from ICN698 as template were used and PCR was carried out. DNA sequencing and sequence assembly were carried out and the merged sequences were blasted with the NCBI database and analyzed for homology. The 16S rRNA similarity search was carried out using NCBI nucleotide database and through EZtaxon database[11]. The evolutionary history was inferred using the Neighbor-Joining method[12] followed by the multiple sequence alignment done through MEGA6 software package[13].

Secondary Metabolite Production by solid state fermentation and extraction
A loop full of the culture *Streptomyces parvulus* ICN698 from a well sporulated mycelium was streaked in AIM medium and grown at 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 for 18-24 hours at 220 rpm. 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 30ºC. After evaporation dry compounds were deposited at ICN Small Molecules Library (ISML) and stored at -20ºC for further use.

Antibacterial assay and MIC determination
In order to find the presence of antibacterial active 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 Methicillin Resistant *S. aureus, P. aeruginosa, A. baumannii*, *Enterobacter* sp. and *K. pneumoniae* strains were grown to mid-logarithmic phase at 37ºC 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 37ºC for 18 hours and observed for the presence of inhibition zones[14]. Minimal Inhibitory Concentration (MIC) assays of the active TLC fraction was carried out using 0.5 McFarland standards (1×10^8 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 37ºC.

Purification by Thin Layer Chromatography (TLC) and Bioautography analysis
Commercially available TLC silica gel 60 F_{254} 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 ICN698. 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 R*f* value of the spot was calculated using the following formula, R*f* = Distance analyte travels / Distance solvent travels. The developed TLC sheet was overlaid with 1×10^6 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 hours. 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

Isolation and Antibacterial screening

During our continuous search for novel antibacterial metabolites of Kanyakumari wetland ecosystem, *Streptomyces parvulus* ICN698 had isolated and characterized. The morphological characters based on the color of the colony, pigmentation in the mycelium, aerial mycelium, substrate mycelium, melanin pigment, metabolic exudation and spore chain are tabularized (Table 1). The antibacterial activity of the strain ICN698 showed considerable inhibitory effect against the tested bacterial pathogens. ICN698 had shown 58 mm zone of inhibition against MRSA, 10 mm against *P. aeruginosa,* 28 mm against *A. baumannii and Enterobacter sp*. and 56 mm against *K. pneumoniae* through double layer agar method (Table 2).

Identification of the producer strain

Sequence homology through similarity searches on BLAST at NCBI database and EZtaxon analysis shows maximum similarity (99.9%) with *Streptomyces parvulus*. Both the BLAST and EZtaxon results show that the Strain ICN698 is 99.9% identical to *Streptomyces parvulus*. A phylogenetic tree (Fig. 2) of similar sequences with bootstrap replication values of 500 was constructed. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood is shown in Fig. 2. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated.

Production and Bioassay guided detection 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 ICN698 extract revealed the active compounds with relatively lower Rf values of 0.11 and 0.29 (Fig. 1). Further the active components were scrapped and pooled for its MIC determination. The fractioned substance from ICN698 was inhibited the bacterial growth at 16 µg/ml against MRSA, *K. pneumonia, A. baumannii* and *Enterobacter sp.* It showed 64 µg/ml of MIC against *P. aeruginosa.*

Table 1: Morphological characteristics of active strain *Streptomyces parvulus* ICN698

Morphological characteristics	ICN 698
Color of the colony	White to Pale green
Pigmentation in the medium	No pigmentation
Aerial mycelium	Present, White
Substrate mycelium	Present, Brown, lightly branched
Melanin pigment	Dark brown
Metabolite Exudation	Absent
Spore chain	Rectus flexibilis

Table 2: Antimicrobial activity of *Streptomyces parvulus* ICN 698 using double layer agar method

Bacterial pathogens	Zone of inhibition (mm)	MIC value (µg/ml)
Methicillin resistant *Staphylococcus aureus*	58	16
Pseudomonas aeruginosa	10	64
Actinetobacter baumannii	28	16
Enterobacter Species	28	16
Klebsiella species	56	16

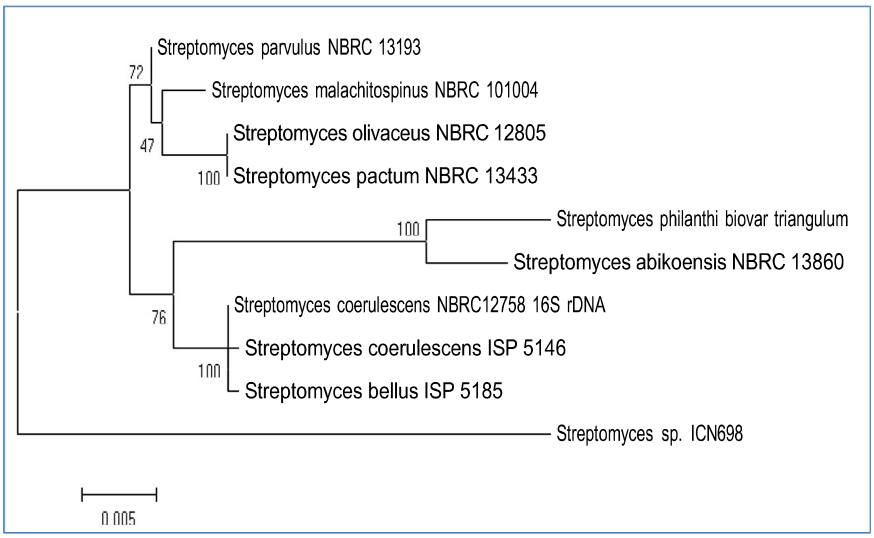

Fig. 1: Molecular phylogenetic analysis of ICN698 by maximum likelihood method based on the Tamura-Nei model

Fig. 2: TLC bio autography analysis of crude extract from ICN 698 against *Enterobacter sp*. Zones of inhibition shows the active spots with R*f* values 0.11 and 0.29 respectively

DISCUSSION

The present study highlighted the antibacterial potential of *Streptomyces parvulus* isolated from a wetland ecosystem of Therur, Kanyakumari district. Among all the actinomycete species, *Streptomyces* species are the biggest contributors of antibiotics[6]. 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, Therur pond wetland ecosystem is a designated bird sanctuary from which the strain ICN698 was isolated. This protected ecosystem could provide a diverse variety of microbes with potent applications. Remarkably, 16S rDNA sequence similarity searches in EZtaxon database analysis[15] revealed the similarity of ICN 698 to *Streptomyces parvulus*. The BLAST results show higher similarity to *Streptomyces parvulus* and the phylogenetic analysis shows close relation of ICN698 with *Streptomyces bellus*. The *Streptomyces* species relating to ICN698 are previously reported to produce antibiotics like althiomycin[16] and Actinomycin D[17]. Similar to these phylogenetic neighbor strains ICN698 show activity against Methicillin resistant *S. aureus, P. aeruginosa, A. baumannii, Enterobacter* sp. and *K. pneumoniae.*

The production of secondary metabolites is more strain specific[18] rather than species specific, which implies the possibility of a same species of different strains producing same compound less frequent. Thus, novelty of a compound among different strains is theoretically more possible. Practically, most hunts for novel compound discovery ends to unfruitful repetition of

previously reported compounds. 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 ICN698 extract proves to be promising source of antimicrobial compounds. A previous study[5] reported the TLC autobiography guided detection of anti-MRSA compounds from *Streptomyces* sp. and reported that the R*f* value of the compounds from 0.65 to 0.86. However, TLC autobiography of ICN698 extract reveals the active compounds being less mobile on chloroform: methanol (9:1) mobile phase with relatively lower R*f* values.

CONCLUSION

The present study deals the isolation and identification of actinomycetes based on the cultural, morphological, molecular sequence and its potential study by antibacterial and TLC characteristics. Further studies on production optimization, purification and structural characterization of active compound are currently in progress. It is expected that the current attempt for the isolation and antibacterial activity of *Streptomyces parvulus* ICN698 from Kanyakumari wetland ecosystem will be helpful for the identification of effective antibiotics effective against bacterial pathogens.

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