

Antimicrobial and Antitubercular Activity of Endophytic Actinobacterium, *Streptomyces* sp. SACC 4 Isolated from the Mangrove Plant *Rhizophora apiculata*

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ABSTRACT

Public health threat due to drug resistant pathogens like *Mycobacterium tuberculosis* and others necessitates the discovery of novel antibiotics. Actinobacteria from understudied sources is emerging as promising source for novel molecules effective against drug resistant pathogens. The present study was undertaken for bioprospecting of endophytic actinobacteria from the mangrove plant *Rhizophora apiculata*. Different actinobacterial cultures isolated from *Rhizophora apiculata* leaves were screened for antimicrobial activity by agar plug method against a wide range of bacterial and fungal pathogens. Antimicrobial metabolites from potential strain SACC4 was produced by agar surface fermentation and extracted using different organic solvents and tested against bacterial pathogens. Antitubercular activity of strain SACC4 was also tested by adopting luciferase reporter phage (LRP) assay. Strain SACC4 was identified based on their phenotypic and molecular characteristics. About 28 distinct actinobacterial strains were isolated from the mangrove leaves in which 50% of the strains showed antimicrobial activity. Notably strain SACC 4 showed broad spectrum activity against bacterial pathogens. Based on the studied phenotypic and molecular characteristics, strain SACC4 was identified as *Streptomyces* species. Among the different solvents tested, methanol extract showed a maximum antimicrobial activity against *S. aureus* in disc diffusion method at 100µg/ml concentration. The methanol extract showed more than 60 percentage inhibition against the standard strain *M. tuberculosis* H37Rv, drug sensitive and MDR *M. tuberculosis* strains at both 100µg/ml and 500µg/ml concentrations. The results of the present investigation revealed that the endophytic *Streptomyces* from mangrove plants are the potent source of novel bioactive metabolites effective against *M. tuberculosis* and other drug resistant pathogens.

KEY WORDS: ACTINOBACTERIA, STREPTOMYCES, ANTIMICROBIAL, ANTI TB, TAXONOMY.

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INTRODUCTION

Antimicrobial resistance (AMR) is a global health crisis. Multi drug resistant pathogens like *Mycobacterium tuberculosis* pose serious threat to public health. Hence, the critical component of AMR solution is the development of truly novel antibiotic. Natural products and their derivatives are mainstays of our antibiotic drugs (Wright, 2017) in which members of the bacterial phylum actinobacteria in particular the genus *Streptomyces* are the known producers of numerous novel secondary metabolites especially antibiotics. However, in recent years, searching of routine sources like terrestrial soil results in the isolation of known actinobacteria which in turn produce known bioactive metabolites (Berdy, 2012). Instead, exploring rare sources like plants may result in the isolation of novel actinobacterial strains with promising bioactive potential. Actinobacteria reside within the healthy plants as endophytes are the promising source for bioactive metabolites to fight against drug resistant pathogens (Golinska et al., 2015; Simpkin et al., 2017). Limited reports on endophytic actinobacteria from plants revealed their enormous bioprospecting potential for clinical, environmental and agricultural applications, (Golinska et al., 2015; Singh et al., 2018).

The present study reports the isolation of endophytic actinobacteria from the mangrove plant *Rhizophora apiculata* and their antimicrobial and antitubercular properties.

MATERIAL AND METHODS

Leaves sample of the mangrove plant *Rhizophora apiculata* was collected from Parangipettai mangrove zone (11.50°N, 79.75°E), Tamil Nadu, India. Endophytic actinobacteria from the leaves were isolated by adopting the method described by Golinska et al., (2015). Morphologically distinct actinobacterial strains were recovered and preserved on ISP2 agar slants at 4°C. All the actinobacterial cultures were screened for antimicrobial activity by agar plug method against clinical strains of *Staphylococcus aureus* MTCC96, clinical strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus fumigates* (Radhakrishnan et al., 2014). Actinobacterial strain SACC 4 which showed promising activity was selected as potential strain.

Phenotypic and molecular characteristics of strain SACC4 was studied by the methods described by Shirling and Gottlieb (1966) and Radhakrishnan et al., (2013). The 16S rRNA gene of SACC4 was amplified by using the primer pairs: 27F 5'AGAGTTTGATCMTGGCTCAG3' (forward) and 1492R 5'TACGGYTACCTTGTTACGACTT3' (reverse) and sequencing was carried out at Eurofins Genomics India Pvt. Ltd., Bangalore. The identification of phylogenetic neighbors and calculation of pair wise 16SrRNA gene sequence similarities were achieved using the MEGA version 6 (Tamura et al., 2013) and BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The

obtained 16SrRNA sequence was submitted to GenBank to get the accession number.

Effect of solid-state and submerged fermentation on bioactive metabolite production by the strain SACC4 was investigated against *S. aureus* by agar well diffusion method. Effect of solvents on the extraction of bioactive metabolites from the cell free supernatant of strain SACC4 was studied using different solvents such as n-hexane, chloroform and ethyl acetate at 1:2 ratio for 24 hours. The antibacterial activity of different solvent extracts was tested against *S. aureus* MTCC96 by disc diffusion method (Bavya et al., 2011). The antimicrobial activity of methanol extract of strain SACC4 was evaluated against wide range of bacterial pathogens by disc diffusion method at 100 µg/disc concentration. Zone of inhibition was measured after 24 hours of incubation at 37°C and expressed in millimetre in diameter. Effect of different concentration of YEME medium (2X, 1X, 1/2X, 1/4X and 1/10X) on antimicrobial activity of strain SACC 4 was also tested (Yilmaz et al., 2008).

Antitubercular activity of potential strain of SACC4 was studied against standard laboratory strain *Mycobacterium tuberculosis* H37Rv, drug sensitive and multi drug resistant (MDR) clinical isolates of *M. tuberculosis* by adopting LRP assay. The relative light unit (RLU) was measured in a luminometer (Radhakrishnan et al., 2014).

Percentage RLU reduction = $\frac{\text{Control RLU} - \text{Test RLU}}{\text{Control RLU}} \times 100$

Extract showing RLU reduction by 50% or more when compared to control were considered as having anti-tubercular activity.

RESULTS AND DISCUSSION

Totally 28 endophytic actinobacterial cultures were isolated from the *Rhizophora apiculata* leave. Based on their morphology, about 75 % of the cultures were tentatively identified as *Streptomyces* and the remaining 25% are rare actinobacterial strains. Actinobacteria from understudied ecosystems are the promising source for novel bioactive natural products. Limited reports on endophytic actinobacteria from plants revealed their enormous bioprospecting potential for clinical, environmental and agricultural applications (Golinska et al., 2015; Singh et al., 2018). In India, reports on endophytic actinobacteria are very few. In a previous study, Du et al. (2013) analyzed the reported 600 actinobacteria belonging to 34 genera and 7 unknown taxa from 37 medicinal plants in which maximum from root followed by stem and least from leaves. However in the present study 25% of the actinobacterial strains recovered from leaves were found to be rare actinobacterial strains.

In preliminary screening, 18 actinobacterial cultures (64%) showed inhibition against any one of the bacterial pathogens tested. Notably, strain SACC 4

showed promising inhibition against *S. aureus* with 15-18 mm zone of inhibition against *S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and the fungi *C. neoformans*.

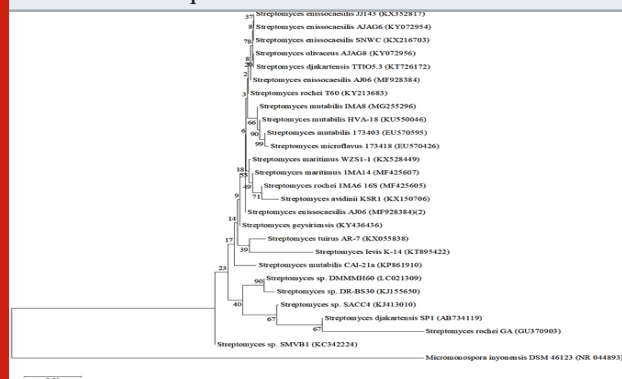
Microscopic, cultural and physiological characteristics of strain SACC4 was given in table 1. PCR amplification and BLAST analysis revealed that the SACC 4 sequence showed 99.9% similarity with the genus *Streptomyces*. Phylogenetic tree constructed based on neighbor-joining method also indicated its closest similarity with *Streptomyces djakartensis* (99%) and other species (Figure 1). The 16s rRNA gene sequence of strain SACC

4 was published in GenBank with the accession number KJ413010. Strain SACC4 was showed 99.8% close similarity with the 16SrRNA sequence of *Streptomyces djakartensis*. However, certain differences were noted when comparing the physiological characteristics of strain SACC 4 with the characteristics of *S. djakartensis* (Kounouz et al., 2017). Hence it was assumed that the strain SACC 4 is found to be novel at strain level. In another previous study, it was reported that 85 % of the total 123 endophytic actinobacterial isolates studied were determined to be unique at the strain level (Janso et al., 2010). Further DNA-DNA hybridization and G+C content analysis is needed to confirm its novelty.

Table 1: Characteristics of endophytic actinobacterial strain SACC 4

	Characteristics	SACC4
Micromorphology	Aerial mycelium	+
	Substrate mycelium	+
Cultural characteristics	Fragmentation	-
	Colony consistency	Powdery
	Aerial mass colour	Gray
	Reverse side pigment	-
Physiological Characteristics	Soluble pigment	-
	ISP1 (Tryptone Yeast Extract Agar)	-
	ISP2 (Yeast extract malt extract agar)	+
	ISP3 (Oat meal agar)	-
	ISP4 (Inorganic salt starch agar)	+
	ISP5 (Glycerol asparagine agar)	-
	ISP6 (Peptone yeast Iron agar)	+
Carbon utilization	ISP7 (Tyrosine agar)	+
	Arabinose	+
	Xylose	+
	Inositol	+
	Mannitol	+
	Fructose	+
	Rhamnose	+
Enzyme production	Asparagine	+
	Glutamine	+
PH tolerance	5	-
	6	-
	7	+
	8	+
	9	+
	10	+
	11	+
Temperature tolerance (° C)	20	+
	30	+
	40	+
NaCl tolerance (%)	0	+
	1	+
	2	+
	3	+
	5	-
“+” grown, “-” no growth		

Figure 1. The phylogenetic relationship of the potential actinobacterial strain SACC4 based on 16S rRNA gene homology. The tree was constructed using the neighbor-joining method with pairwise-deletion model analyses, which were implemented in the Molecular Evolutionary Genetics Analysis (MEGA), version 6.0 program. The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates. *Micromonosporainyonens* is used as out group. Scale bar indicates the number of substitutions per site.



In fermentation study, even until 20 days of fermentation, strain SACC 4 was failed to produce bioactive compounds in submerged fermentation whereas it produced bioactive compounds in agar surface fermentation. The agar plug from ISP2 agar showed activity against *S. aureus* even from 2nd day (11.2 ± 0.72 mm in dm) of fermentation. The zone of inhibition eventually increased from 2nd day to 8th day (17.1 ± 1.09 mm in dm) of fermentation. This is due to the antibiotics production through solid state fermentation are higher quantities and more stable than liquid state fermentation (Subramanian et al., 2012). This finding is in agreement with the antibiotic production by desert soil, *Streptomyces* strain D25 which showed antibacterial activity and also produced antitubercular pigment only in solid culture (Radhakrishnan et al., 2015).

In solid state fermentation, strain SACC4 showed good growth and the methanol extract obtained from the fermented medium showed 18mm inhibition against *S. aureus* but failed to show activity in liquid state fermentation. Isolation of crude extract by solvent extraction is an important phenomenon to find a good solvent that has the potential to extract high yield and most potent bioactive compounds. Our research findings demonstrated that the extract of methanol have significant antimicrobial and antitubercular activity. These results were clearly comparable to that, who evaluated *Streptomyces mutabilis* which produced an antitubercular activity and antimicrobial activity against gram positive and gram negative pathogens (Mahmoud et al., 2015). Among the different concentrations of YEME agar, 1X concentration of YEME agar was found to support good growth and antimicrobial compound production which was expressed by its activity against *S. aureus* (10.2 ± 0.12), *B. subtilis* (15.0 ± 0.64) and *E. coli*

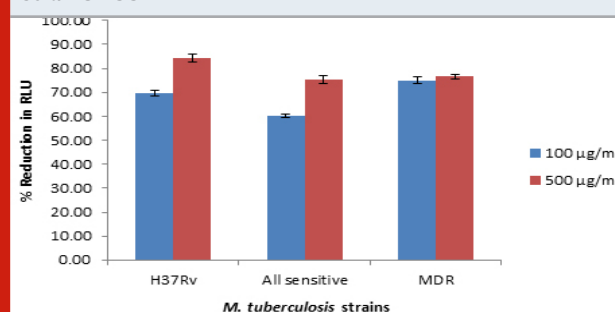
(9.8 ± 0.44). The methanol extract of strain SACC 4 was found to be active against *S. aureus* and wide range of Gram negative bacterial pathogens (Table 2).

Table 2. Antimicrobial activity of methanol extract of endophytic actinobacterial strain SACC 4.

Bacterial pathogens	Zone of inhibition (mm in diameter)*
<i>Staphylococcus aureus</i>	18.2 ± 0.15
<i>Klebsiella pneumonia</i>	17.0
<i>Pseudomonas aeruginosa</i>	18.0
<i>Vibrio cholerae</i> 01 ogawa	8.1 ± 0.17
<i>Vibrio cholerae</i> 0139	10.2 ± 0.15
<i>Vibrio parahaemolyticus</i> 03:K6	12.8 ± 0.47
<i>Aeromonassorbia</i>	8.3 ± 0.17
<i>Aeromonashydrophilla</i>	10.2 ± 0.15
Enteropathogenic <i>E.coli</i> 0115	10.2 ± 0.37
Enteropathogenic <i>E.coli</i> 015	10.9 ± 0.35
Enteropathogenic <i>E.coli</i> 0114	10.9 ± 0.21
<i>Salmonella typhimurium</i>	12.0 ± 0.32
<i>S. worthington</i>	12.9 ± 0.38
<i>S. infantis</i>	11.4 ± 0.31
<i>Shigella sonnei</i>	0
<i>S. dysenteriae</i> sero 5	0
<i>S. boydii</i> sero 1	0
<i>S. flexneri</i> type 2a	0

In LRP assay, the methanol extract of strain SACC 4 was showed more than 60% inhibition against standard laboratory strain *Mycobacterium tuberculosis* H37Rv, drug sensitive and MDR *M. tuberculosis* strains (Figure 2).

Figure 2: Antitubercular activity of potential actinobacterial strain SACC4



Findings of the present study evidenced that mangroves are the promising source for the isolation of bioactive endophytic actinobacteria.

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Conflict of Interest: Authors have no conflict of interest.

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