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Antimicrobial and anti-tubercular potential of *Streptomyces lomondensis* SACC 63 isolated from soil samples in Himachal Pradesh, India

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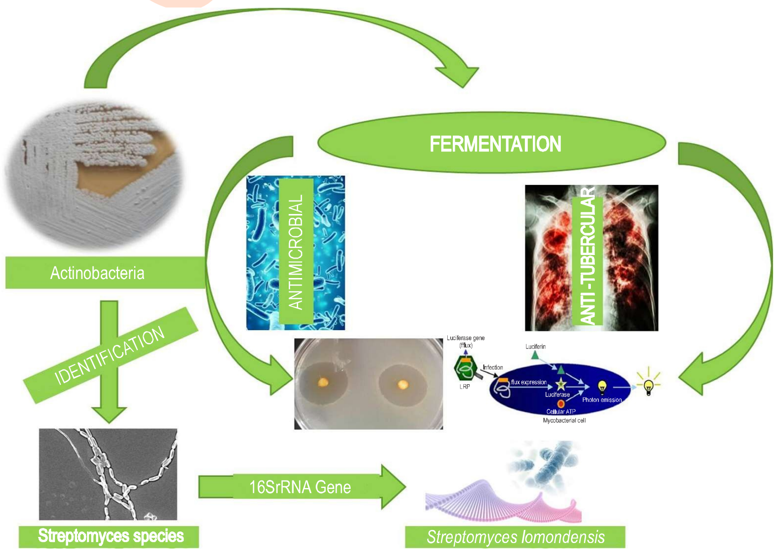
Abstract

Aim : The antimicrobial and anti-tubercular potential of Actinobacteria isolated from soil in Himachal Pradesh, India, was examined in this study.

Methodology: The bioactive metabolites produced from five selected Actinobacterial cultures SACC-63, SACC-75, SACC-76, SACC-77 and SACC-N10 by agar surface fermentation were tested for their antimicrobial and anti-tubercular efficacy in *in-vitro*. The taxonomy of SACC-63 was identified as *Streptomyces lomondensis* using 16S-rRNA sequence analyses.



Results: The ethyl acetate extract of strain SACC-63 showed broad spectrum activity against wide range of bacterial and fungal pathogens at 250 µg per disc concentration. In anti-tubercular screening by luciferase reporter phage (LRP) assay, four actinobacterial strains showed more than 65% inhibition against the standard strain *Mycobacterium tuberculosis* H37Rv and multi drug resistant (MDR) *M. tuberculosis* strain at 100 µg ml^{-1} and 500 µg ml^{-1} concentrations, except the strain SACC-N10. The MIC values of SACC-63 against microbial pathogens ranged between 62.5 µg ml^{-1} and 250 µg ml^{-1}. The strain SACC-63 showed 99% similarity with *Streptomyces* spp. Further, phylogenetic analysis based on 16S rRNA revealed a close relationship between SACC-63 and *S. lomondensis.*

Interpretation: The present study suggested that *S. lomondensis* SACC-63 isolated from Himachal Pradesh region in India could be a promising source for the production of bioactive molecules.

Key words: Antimicrobial, Anti-tuberculosis, Bioprospecting, Luciferase reporter phage assay, *Streptomyces* species

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Introduction

Increased antimicrobial resistance by most pathogens towards the conventional antimicrobial agents have become a great public health concern. Thus, the emergences of antibiotic resistant pathogens demand search for novel and effective drugs from unexplored niches (Jeyanthi and Velusamy, 2016). Natural products harbour unmatched availability of bio-active compounds, and thus provide unlimited opportunities for new drug discovery (Raiz *et al.,* 2016). Microbes, especially have enormous potential to produce bio-active compounds. Among the microbial community, Actinobacteria play an important role and produce nearly 70% of antibiotics existing in the market today, especially by the genus *Streptomyces* (Zothanpuia *et al.,* 2017). Actinobacteria, a group of Gram-positive bacteria with high GC content in their genome, have a widespread diversity. Also, Actinobacteria comprise about ten percent of the microbial inhabitants of extreme habitats (Arocha-Garza *et al.,* 2017; Liu *et al.,* 2016; Qin., 2016).

Actinobacteria, ubiquitously distributed in terrestrial, freshwater and marine ecosystems, are dominant soil bacterial phylum and are believed to play multiple roles in the environment (Barka *et al.,* 2016). Due to the ease of sampling and their production of novel antibiotics, numerous literatures have previously indicated diversity of terrestrial-derived actinobacteria across the globe (Khieu *et al.,* 2015). Notably, the Actinobacteria from hill stations produce remarkably diverse secondary metabolites which account for roughly two-third of all antibiotics, either as natural products or as derivatives of natural product (Berdy, 2012). Among Actinobacteria, *Streptomyces* species have a complex metabolic mechanism that can produce variety of secondary metabolites which increases the value of streptomyces important in pharmaceutical industries, especially for drug discovery (de Lima Procópio *et al.,* 2012; Barka *et al.,* 2016). Thus, there has been a paradigm shift in drug-discovery research towards natural resources such as those produced by Actinobacteria isolated from undiscovered or poorly explored environments like Himalayan hill stations (Undabarrena *et al.,* 2016).

In India, Actinobacterial diversity has been an important source for natural product discovery. Notably, Northern region of India (Himalayan region) has a huge potential for bio-prospecting, since the region is best known for rich biodiversity and bioactivity (Harish *et al.,* 2016). Himalaya is one of the "hotspots" of biodiversity and is rich in microbial diversity. Its climatic conditions and topological characteristics have served to be a home for thousands of psychrophiles or cold-adapted microbes. The studies of bioactivity of these regions are important because it provides good opportunity to understand the better adaptability of these microorganisms to extreme environmental conditions. Besides having keen interest to understand the biology of these microorganisms, slow growth rate makes the study of these psychrotolerant conditions, but still, the Himalayan region is not extensively explored for the study of microbial biodiversity, bioactivity and economically important microorganisms (Suyal *et al.,* 2014; Kumar *et al.,* 2014). Actinobacteria is one of the major groups dominating in Himalayan regains. However, most parts of the Northern region of India, especially the Himalayan region remain least studied in the context of Actinobacteria. Perhaps, there is no report on anti-tubercular activity of Actinobacteria from Himachal Pradesh. Thus, the current study aims to explore the antimicrobial and anti-tubercular potential of selected Actinobacteria isolated from the soil collected from Himachal Pradesh, India.

Materials and Methods

Sample collection and pretreatment: Soil samples were collected from Himachal Pradesh (Latitude: 32° 29' N; Longitude: 75° 10' E), India and kept for drying at room temperature for two days. Ten gram of soil sample was taken and dried at 55°C for 10 min in order to retard the growth of unwanted bacteria and fungi.

Isolation and characterization of Actinobacteria: The pretreated soil samples were serially diluted up to 10^5 dilutions using sterile distilled water blanks. Hundred microlitre of aliquot from 10^3 to 10^5 dilutions was taken and plated on Starch Casein Agar (SCA) supplemented with nalidixic acid 50 µg ml^{-1} and nystatin 20 µg ml^{-1}. This was performed in triplicate and all the plates were incubated at 28ºC for one month. Microscopic characteristics such as the presence of aerial mycelium, substrate mycelium and mycelial fragmentation were observed under the bright field microscope at 40X magnification. Cultural characteristics such as growth, colony consistency, aerial mass colour, reverse side pigment and soluble pigment on ISP2 agar was recorded by growing the culture on ISP2 agar medium for 7-14 days. Morphologically distinct five Actinobacterial strains *viz.,* SACC-63, SACC-75, SACC-76, SACC-77 and SACC-N10 were selected and purified on ISP2 agar plates. All cultures were preserved on ISP2 agar slants and in 30% glycerol.

Production of bioactive metabolites: Agar surface fermentation method was adopted to produce bioactive metabolites from the selected Actinobacterial strains. Primarily, the spores of Actinobacterial cultures were inoculated in yeast extract malt extract agar media and the plates were incubated at 28°C for 10 days. The growth of Actinobacteria was monitored regularly and at the end of tenth day, the mycelial growth was carefully removed and agar medium which consisted secreted metabolites was cut into pieces. Agar pieces were taken into a conical flask containing ethyl acetate in 1:2 ratio. The mixture was incubated for 24 hrs at room temperature. After incubation, the extract was collected, concentrated and quantified (Eccleston *et al.,* 2008).

***In vitro* screening for antimicrobial activity:** Antimicrobial activity was evaluated against the selected pathogens (Table 2), which were obtained from Medical Microbiology Department, Periyar University, Salem district, Tamilnadu, India. Bacterial and fungal inoculum with 0.5 McFarlands standard was prepared using sterile nutrient and Sabouraud broth and inoculated onto Muller Hinton Agar (MHA). About 10 mg ml^{-1} concentration of

Actinobacterial extracts were prepared using ethyl acetate and filtered using sterile 0.45 µm syringe filter. One mg per ml of working stock solution was prepared. Antibiotic discs were prepared at 250 µg per disc concentration from working extract on sterile Whatman No. 1 filter paper disc (5 mm diameter) and allowed to dry. The extract impregnated discs were placed over pathogens overlaid MHA plates. All plates were incubated for 24 hrs at 37°C and after incubation period, the zone of inhibition was measured and expressed in millimetre in diameter. Disc impregnated with only ethyl acetate served as negative control.

***In-vitro* screening for anti-tubercular activity:** The ethyl acetate extract of actinobacterial strains were screened for anti-tubercular activity by Luciferase Reporter Phage (LRP) assay (Radhakrishnan *et al.,* 2010). A 50% reduction in relative light units (RLU) as measured by luminometer was considered sensitive. Standard laboratory strain *M. tuberculosis* H37Rv, SHRE sensitive and SHRE resistant clinical strains of *M. tuberculosis* were obtained from the Department of Bacteriology, National Institute for Research in Tuberculosis.

The viability of all isolates was maintained on LJ slopes. High titer of mycobacteriophage phAE129 used in this study was prepared using *M. smegmatis* mc2155 in Middlebrook 7H9 complete medium (Vanaja *et al.,* 2007). About 350 µl of G7H9 broth supplemented with 10% albumin dextrose complex and 0.5% glycerol was taken in cryovials and added to 50 *µ*l of crude extract in order to get the final concentration of 100 µg ml^{-1}. A 100 *µ*l of *M. tuberculosis* cell suspension was added to all vials. The above procedure was followed for all three *M. tuberculosis* isolates. DMSO (1%) was also included in the assay as a solvent control. All vials were incubated at 37°C for 72 hrs. After incubation, 50 *µ*l of high titer phage phAE129 and 40 *µ*l of 0.1M $CaCl_2$ solution was added to test and control vials. All vials were incubated at 37°C for 4 hrs. After incubation, 100 *µ*l from each vial was transferred to luminometer cuvette. About 100 µl of D-Luciferin was added and relative light unit (RLU) was measured with a luminometer.

Effect of fermentation method on bioactive metabolites production: To study the effect of solid-state and submerged fermentation on the production of bioactive metabolites, the strain SACC-63 was inoculated into both YEME broth and YEME agar. YEME agar plates and YEME broth were incubated at 28°C for 12 days. Actinobacteria from YEME agar and YEME broth was tested for antimicrobial activity against *S. aureus* by disc diffusion method (Hudzicki, 2009).

Production of bioactive metabolites by submerged fermentation: Since the strain SACC-63 was found to produce bioactive metabolites in submerged culture, further large scale production was carried out by using YEME broth. To study the effect of solvents on extraction of bioactive metabolites from cell free supernatant, different solvents such as n-hexane, chloroform and ethyl acetate were tested. The extracts were concentrated appropriately and tested against *S. aureus* (Hudzicki, 2009).

Determination of Minimal Inhibitory Concentration (MIC): Minimal Inhibitory Concentration (MIC) of crude ethyl acetate extract was determined by adopting broth dilution method (Zothanpuia *et al.,* 2017) at concentration ranging from 12.5 µg ml^{-1} to 250 µg ml^{-1} against the pathogens *viz., Staphylococcus aureus*- ATCC 29213, *Escherichia coli*- ATCC-25922, *Pseudomonas aeruginosa*- ATCC-27853, *Klebsiella pneumonia*- ATCC-13882, and the clinical pathogens *Bacillus cereus, Enterobacter aerogenes, Vibrio parahaemolyticus, Salmonella typhi, Shigella sonnei, Salmonella enteritidis, Aeromonas hydrophila, Providencia species* and *Cryptococcus neoformans* which were obtained from Medical Microbiology Department, Periyar University, Salem district, Tamilnadu, India.

Characterization and taxonomy of potential strain SACC-63: Strain SACC-63 was identified through phenotypic, chemotaxonomic and genetic characterization. The micromorphology of strain SACC-63 was examined using bright field microscope and scanning electron microscopy (JEOL model JSM5600LV). Cultural features of SACC-63 were determined using 14 day culture incubated at 28ºC on different ISP agar medium. The cultural characteristics such as nature of growth, aerial mass colour, consistency, pigmentation were recorded. Basal agar medium was used for utilization of carbon and nitrogen. The tolerance of NaCl, pH and the effect of temperature of the strain SACC-63 was determined on ISP2 medium (Shirling and Gottileb, 1966).

The total genomic DNA was extracted from the strain SACC-63 using solute ready genomic DNA kit. The 16S rRNA of SACC-63 was amplified by using the primer pairs: 27F 5´AGAGTTTGATCMTGGCTCAG3´ (forward) and 1492R 5´TACGGYTACCTTGTTACGACTT3´ (reverse) (Gothwal *et al.,* 2007). The PCR product of SACC-63 was sequenced and aligned with similar sequences available in GenBank using MEGA 6 program (Saitou and Nei, 1987). The aligned sequences were used to construct the phylogenetic tree by following neighbor joining algorithm (Saitou and Nei, 1987) in MEGA 6 software. The bootstrap estimation (Felsenstein, 1985) was used to determine the confidence of branches of phylogenetic tree. The partial 16S rRNA nucleotide sequence of potential Actinobacteria strain SACC-63 was deposited in GenBank database.

Statistical analysis: The antimicrobial and anti-tubercular activity was performed in triplicate process and repeated three times. Readings were taken as mean ± standard deviation of three replicates calculated using Microsoft Excel XP 2010.

Results and Discussion

Actinobacteria from rare ecosystems are known to produce bioactive compounds (Barka *et al.,* 2016; Nachtigall *et al.,* 2011). All strains showed good growth on ISP2 agar. All the strains formed powdery type of colonies, except SACC-75, SACC-76, SACC-77 and SACC-N10 produced both reverse side and soluble pigment; however, SACC-63 was unable to produce

Table 1: Antimicrobial activity of ethyl acetate extract of Actinobacteria isolated from soil samples of Himachal Pradesh against bacterial and fungal pathogens

Test pathogens	Zone of inhibition (mm)				
	SACC-63	SACC-75	SACC-76	SACC-77	SACC-N10
Staphylococcus aureus- ATCC 29213	15.3±0.8	9.8±0.8	0.0	0.0	0.0
Bacillus cereus	11.0±0.5	11.5±1.2	0.0	14.1±1.1	0.0
Escherichia coli- ATCC 25922	19.0±1.1	0.0	0.0	0.0	0.0
Salmonella abony	0.0	0.0	0.0	0.0	0.0
Enterobacter aerogens	15.8±0.6	0.0	0.0	0.0	0.0
Pseudomonas aeruginosa¬- ATCC 27853	14.5±0.7	0.0	0.0	0.0	0.0
Vibrio cholera	0.0	0.0	0.0	0.0	0.0
Vibrio parahaemolyticus	13.7±1.2	0.0	0.0	0.0	0.0
Salmonella typhi	15.7±0.6	0.0	0.0	0.0	0.0
Shigella sonnei	18.8±0.7	0.0	0.0	0.0	0.0
Shigella boydii	0.0	0.0	0.0	0.0	0.0
Klebsiella pneumonia- ATCC 13882	17.3±1.4	0.0	0.0	0.0	0.0
Salmonella enteritidis	15.2±0.6	0.0	0.0	0.0	0.0
Aeromonas hydrophila	10.5±0.4	0.0	0.0	0.0	0.0
Providencia species	12.5±0.7	0.0	0.0	0.0	0.0
Chromobacterium violaceum- ATCC 6258	16.5±0.4	0.0	0.0	0.0	0.0
Candida albicans	0.0	0.0	0.0	0.0	0.0
Cryptococcus neoformans	16.2±1.0	0.0	0.0	0.0	0.0

both. Also, aerial and substrate mycelium were observed in all the strains under bright field microscope. Based on cultural and micromorphology, all cultures were suspected as *Streptomyces* (van Dissel *et al.,* 2014).

Fermentation is a cost-effective way of mass production of bioactive molecules. There are multitude antibiotics that are produced by fermentation process. Therefore, fermentation technique must be decided based on the strain that is being used for production. Recent advances have suggested that antibiotics produced through solid surface fermentation are more stable and produced in higher quantities than submerged fermentation (Kagliwal *et al.,* 2009; Bussari *et al.,* 2009; Subramaniyam and Vimala, 2012). In the present study, crude bioactive metabolite from five Actinobacterial cultures were obtained by simple agar surface fermentation, a variant of solid state fermentation which results in good extraction of bioactive metabolites, *i.e.,* around 30 – 40 mg 100 ml^{-1} of ISP2 medium when extracted with ethyl acetate. In a previous study, agar surface fermentation technique was followed to produce bioactive metabolites from 54 Actinobacterial strains (Radhakrishnan *et al.,* 2014).

Actinobacterial strains were screened for their antimicrobial activity against test pathogens using disc diffusion method. Out of five Actinobacterial strains, SACC-63 showed broad spectrum of antimicrobial activity against all the clinical pathogens, except *Vibrio cholerae, Shigella boydii, Salmonella abony* and *Candida albicans.* It showed the maximum inhibition against *E. coli*- ATCC-25922 followed by *Shigella sonnei,* K. *pneumonia*- ATCC-29212 and *Chromobacterium violaceum*- ATCC 6258 (Table 1). Similarly, Abid *et al.* (2016) reported that *Streptomyces* strains isolated from soil samples, collected from Jazan in Saudi Arabia showed antibacterial activity against *B. subtilus, S. aureus, P. aeruginosa, E. coli and S. sonnei.* Certain issues are known to be associated with primary screening in solid medium and liquid culture based secondary screening. There can be loss of bioactive metabolite production in secondary screening when the medium used for secondary metabolite production is different from the one used for primary screening. Certain Actinobacteria strains produce antibiotic compounds only in solid medium but fail to produce the same in liquid medium (Ilic *et al.,* 2007). The crude extract from the same medium was tested for *M. tuberculosis.* Most of the secondary metabolites including antibiotics are extracellular in nature and those extracellular products of Actinobacteria exhibit potent antimicrobial activities (Radhakrishnan *et al.,* 2011).

Table 2: Anti-tubercular activity of ethyl acetate extract of ctinobacteria isolated from soil samples of Himachal Pradesh against H37Rv and MDR *M. tuberculosis*

SACC No.	Percentage of reduction in RLU			
	M. tuberculosis H37Rv		MDR *M. tuberculosis*	
	100 µg ml^{-1}	500 µg ml^{-1}	100 µg ml^{-1}	500 µg ml^{-1}
SACC-63	29.2±0.4	73.5±1.0	0.0	77.5±0.8
SACC-75	35.5±0.3	61.8±0.8	0.0	41.8±0.8
SACC-76	54.2±0.9	84.2±1.3	21.0±0.0	99.0±0.0
SACC-77	55.5±1.3	99.0±0.0	23.2±1.0	99.0±0.3
SACC-N10	34.2±1.0	45.2±0.7	25.5±0.5	39.2±1.0

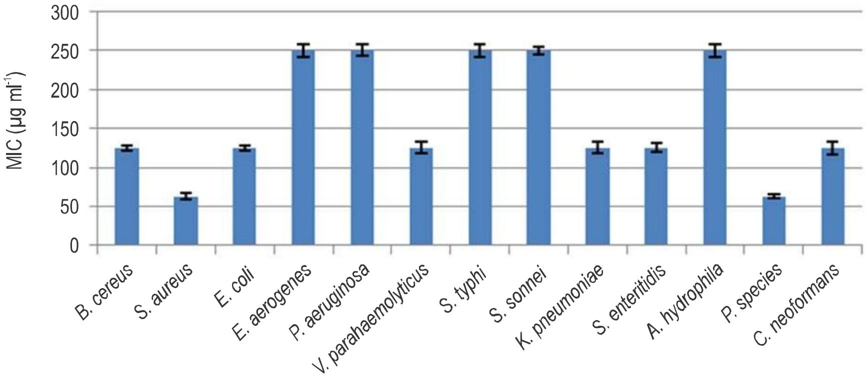

Fig. 1: MIC of ethyl acetate extract of Actinobacterial strain SACC-63 isolated from soil samples of Himachal Pradesh.

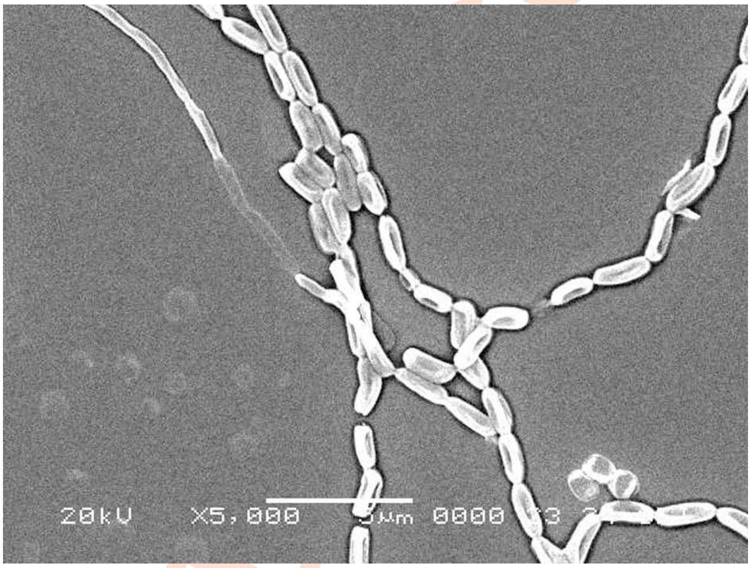

Fig. 2: Scanning electron micrograph showing spore chain morphology of *Streptomyces lomondensis* SACC-63, prepared without using any chemical fixative and dehydrating agent.

Actinobacteria from less or under explored ecosystems are the promising source for anti-tubercular metabolites. It has been previously reported that the anti-tubercular activity of Actinobacteria isolated from marine, forest, desert and cave ecosystems (Radhakrishnan *et al.,* 2011; Radhakrishnan *et al.,* 2014). Furthermore, discovery of streptomycin (first discovered anti-tuberculosis drug from *Streptomyces griseus*), the drug discovery and development programmes have inclined toward the anti-tubercular agents than chemical compounds (Shivlata and Satyanarayana, 2015). Raja *et al.* (2011) also reported the anti-TB activity of psychrophilic Actinobacteria from Manali Ice point, Himachal Pradesh. Similar, anti-tubercular activity of ethyl acetate extract of five Actinobacterial cultures is given in Table 2. Among the five cultures, the ethyl acetate extract of four cultures, SACC-63, SACC-75, SACC-76 and SACC-77 revealed >50% reduction in RLU against *M. tuberculosis* H37Rv whereas the ethyl acetate extract of SACC-63, SACC-76 and SACC-77 were active against MDR *M. tuberculosis* at 500 µg ml^{-1}. The ethyl acetate extract of SACC-76 and SACC-77 exhibited >50% reduction against *M. tuberculosis* H37Rv at 100 µg ml^{-1} concentration whereas none of the extracts were active against MDR *M. tuberculosis* at 100 µg ml^{-1} concentration (Table 2).

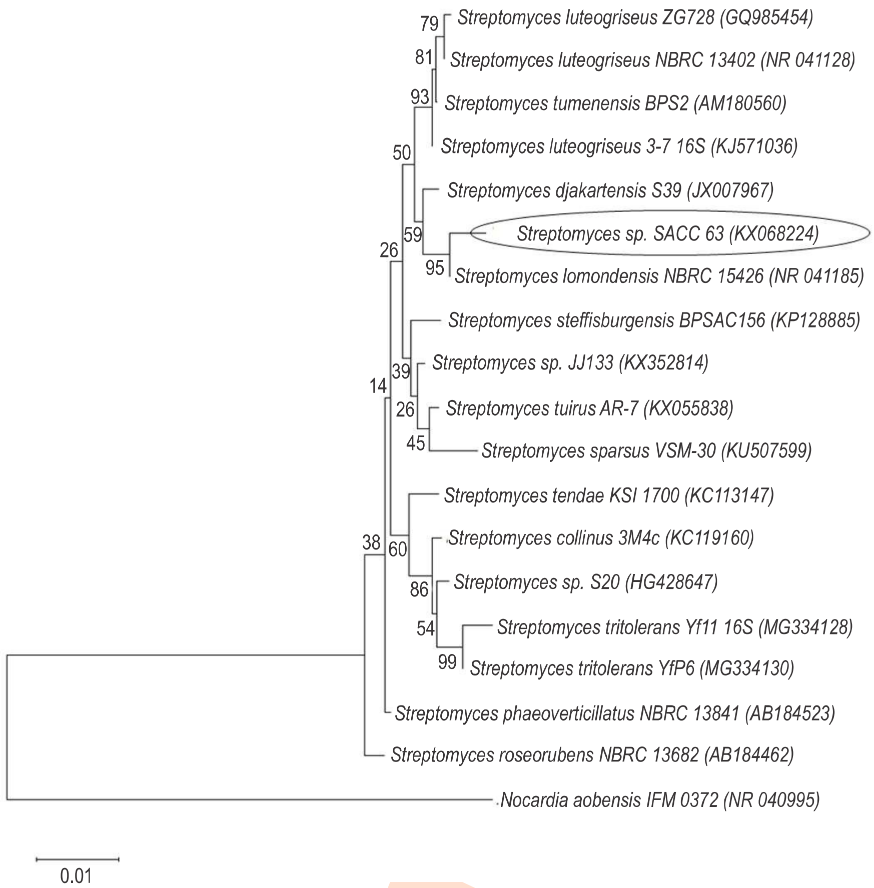

Fig. 3: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences shows the strain SACC-63 to be a sister taxa of *Streptomyces lomondensis* within the family Streptomycetaceae. *Nocardia beijingensis* IFM 0372 was used as out-group.

Among the five Actinobacterial strains, SACC-63 showed an enhanced antimicrobial and anti-tuberculosis activity, and hence the strain SACC-63 was selected for further studies. Further, the cell free supernatant collected from 4th day of fermentation showed antibacterial activity whereas agar plug from ISP2 agar showed antibacterial activity against *S. aureus* from 2nd day of fermentation onwards. n-butanol, ethyl acetate and chloroform were used as solvents for the extraction of antibacterial compound and in each case, some antibacterial activity was observed, however, ethyl acetate extract showed the maximum antibacterial activity against *S. aureus* (24mm), followed by chloroform extract (16 mm). The MIC results of ethyl acetate extract of strain SACC-63 is given in Fig. 1. The MIC of ethyl acetate extract ranged between 62.5 µg ml^{-1} and 250 µg ml^{-1}.

The taxonomical identification of a potential strain is important in systematic of bacteriology. In Actinobacterial taxonomical identification, the morphological features and physiological characteristics are largely used (Raja *et al.,* 2011; Nonomura *et al.,* 1974). In addition, the usage of chemotaxonomic criteria has been used as a reliable tool to identify Actinobacteria at genus level. In the current study, the microscopic observations revealed the substrate mycelium to be lengthy while aerial mycelium was dark with recti flexible (RF) arrangement. The aerial and substrate mycelia did not exhibit fragmentation. Smooth, oval shaped spores were arranged in hyphae as long straight chains to spirals with 30-40 spores. SEM images of this strain showed substrate mycelia and extensively branched aerial hyphae that further differentiated into smoothly surfaced spores (Fig. 2). Strain SACC-63 showed good growth on ISP2, ISP3, ISP4, ISP5 and ISP7 medium while moderate growth was observed on ISP1 and ISP6 medium. It produced powdery colonies on all ISP media. The strain SACC-63 was found to utilize a wide range of carbon and nitrogen sources on basal medium supplemented with glucose, fructose, sucrose, rhamnose, mannitol and asparagine. The growth of strain SACC-63 was observed at a wide range of temperature (20°C – 40°C) at pH 7 and 9, in the presence of 0-5.0% NaCl. The 16S rRNA sequence analysis confirmed that the strain SACC-63 belonged to a species of genus *Streptomyces.* It showed 99% similarity with

16S rRNA gene sequence of its closely related *Streptomyces* species and deposited in the GenBank database with accession number KX068224. The neighbor-joining phylogenetic tree indicated the strain SACC-63 to be a sister taxa to *S. lomondensis* within the genus *Streptomyces* (Fig. 3). Based on the results of sequence analyses, the potential Actinobacterial strain SACC-63 isolated from Himachal Pradesh ecosystem is putatively a novel species of genus *Streptomyces* and phylogenetically close neighbour of *Streptomyces lomondensis*. However, the taxonomic identification needs to be evaluated further using whole genome sequence data. The findings of the present study showed that Actinobacteria from Himachal Pradesh region are promising source for bioprospecting. *Streptomyces* sp. SACC-63 isolated from Himachal region is a potential source for the isolation of antimicrobial and anti-tubercular metabolites. However, further studies will be necessary to determine the structure of active compounds.

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