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Molecular characterization of potent antimicrobial metabolite producing actinomycetes from the soil of Kalaburagi region

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Abstract

This study focuses on isolating and characterizing actinomycetes from soil samples collected in the Kalaburagi region and assessing their antimicrobial activity against *Candida* and various bacterial spp. The isolated actinomycetes were performed by antagonistic activity against *Candida*. The potent isolate VSG-15 ethyl acetate crude extract displayed a significant zone of inhibition against *C. albicans* MTCC1966, *Candida haemulonii* MTCC8303, and *Candida tropicals* MTCC1406 as compared to the Standard drug. The crude extract also showed significant antibacterial activity was recorded against *Brevibacillus brevis* and *Enterococcus faecalis* Based on morphological, biochemical characteristics and 16S rRNA sequencing the isolated strain was identified as *Streptomyces levis* (VSG-15). The present study reveals that *Streptomyces levis* are potential organisms for the production of anticandidal and antibacterial agents against the clinical test organism.

Key words : Actinomycetes, Antimicrobial activity, *Candida* strains, *Streptomyces* sp.

Actinomycetes are filamentous, Gram-positive bacteria widely found in soil and other environmental habitats, contributing substantially to the microbial population. They are distinguished by their DNA, which has a notably high G+C content[3,9]. Among the

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actinobacteria, *Streptomycetes* have been considered as the biggest producer of antibacterial, antifungal, anticancer and immunosuppressive molecules[4]. Majority of commercially available antimicrobial drugs are derived from *Streptomyces* [13]. Majority of invasive fungal infections (IFIs) are caused by *Candida*, *Aspergillus* and *Cryptococcus,* and bacterial infection are caused by *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Escherichia coli.* As per the recent estimate, nearly 7.7 million deaths associated with bacterial related infections and about 1.5 million associated with invasive fungal infections globally[17]. The development of multi-drug resistance among microbial pathogens poses a significant challenge in treating life threatening infectious diseases. It is estimated that over 70% of pathogenic bacteria exhibit resistance to at least one of the currently available antibiotics[7]. Amphotericin B, triazoles, flucytosine antifungal and methicillin, cephalosporin, fluroquinolone antibacterial drugs are now less effective due to increase in drug resistance pathogenic organisms[11]. In order to improve the treatment for infectious diseases caused by drug resistant microbes, the development of new effective and safe drugs is need of the hour. Hence this study aims to explore novel and effective sources for production of antimicrobial agents.

Collection of soil samples :

Soil samples were obtained from various habitats of Kalaburagi region, India. Samples were collected from a depth of 10-15cm, placed in sterilized polythene bag and transported to the laboratory. They were the dried at 45 ^{o}C for 3-4h, crushed, sieved and used for Actinomycetes isolation.

Isolation of Actinomycetes :

One gram of soil sample was suspended in nine ml of sterile distilled water and subjected to serial dilution from 10^{-1} to 10^{-8}. A 0.1ml of aliquot from each dilution was spread onto petriplates containing Starch casein agar medium, followed by incubation at 37 ^{o}C for 7days. Distinct colonies were selected based on morphology, sub cultured and maintained on ISP-4 medium (International Streptomyces project No. 4).

Morphological identification of Actinomycetes :

The morphological identification was carried out by examining the color of aerial and substrate mycelium, spore bearing hyphae, reverse side pigmentation and spore morphology observed under the light microscope using Gram staining technique, following the guidelines in Bergey's manual[14]. Additionally, Scanning Electron Microscopy (SEM) study was performed to reveal the micro-morphology of the active isolate.

Antimicrobial activity of Actinomycetes isolates :

Preliminary screening :

A perpendicular streak plate method was initially screened for antagonistic activity of actinomycetes isolates. Aseptically inoculating single streak of actinomycetes isolate into the center of SCA medium and incubated the petriplates at 37 ^{o}C for 3 days. After observing the distinct growth of

actinomycetes, the test organism was streaked perpendicular to the original streak and incubated at 37 ºC. The zone of inhibition (ZOI) was assessed after 72h, and the isolates showing significant ZOI were subjected for secondary screening[8].

Fermentation and extraction of secondary metabolites :

Secondary metabolites were extracted through submerged fermentation using an optimized production medium. A loopful of culture was inoculated into a 250ml of Erlenmeyer flask containing ISP4 production medium and incubated at 37 ºC on a rotary shaker (150 rpm) for 15 days. The cell free culture broth was obtained by centrifugation (REMI R-24) at 10,000rpm for 15 min. The supernatant was transferred aseptically into a conical flask, mixed with an equal volume of ethyl acetate, and kept on orbital shaker for 1h. The organic layer was then collected, and the solvent was evaporated to dryness. The resulting dried residue was used as crude extract. About 10mg/ml of crude extract was dissolved in dimethyl sulfoxide (DMSO) for antimicrobial testing against test organisms.[23]

Secondary screening by agar well diffusion assay :

The ethyl acetate crude extract was used to perform the antimicrobial activity by using agar-well diffusion assay to determine the antifungal and antibacterial efficacy against test organisms[1]. The petriplates were prepared by pouring 20ml of sabouraud dextrose agar (SDA) and Muller Hinton agar (MHA, Himedia) media to perform the anticandial and antibacterial activity, respectively. Twenty-four hours freshly sub-cultured *Candida* and bacterial strains were streaked over the respective medium. The agar wells were made by using sterile cork borer (5mm in diameter) over the lawn cultures of *Candida* and bacteria. 100µl of each crude extract, standard drugs (fluconazole for *Candida* and streptomycin for bacteria) as positive control and dimethyl sulfoxide as negative control were added into separate well. The plates incubated with *Candida* strains were incubated at 28 ºC and the bacterial plates were incubated at 37 ºC for 24h. The antimicrobial activity was evaluate by measuring the ZOI and performed the experiment in triplicate.

Biochemical characterization :

The potent isolates were screened for salt tolerance, urease, citrate, starch hydrolysis tests, lipase, oxidase, catalase, methyl red, indole and voges-proskauer tests by using conventional methods. Similarly, the presence of glucose, sucrose, fructose, mannitol, sorbitol, rhamnose, arbinose, xylose, salicin, maltose, methionine, phenylalanine and proline were analyzed in culture broth[20,21].

Molecular characterization and Phylogenetic analysis :

Chromosomal DNA was extracted using a spin column kit (HiMedia,India). The 16S rRNA gene (1500bp) was amplified through Polymerase chain reaction (PCR) in a thermal cycler and purified with Exonuclease I-shrimp Alkaline Phosphatase (EXO-SAP)[5,6]. The purified PCR products were sequenced using Sanger method with ABI 3500XL genetic

analyzer (Lite Technologies, USA). The sequences were edited with Chromas Lite (version 1.5) software and aligned with multiple sequence alignment. The edited sequences were analyzed with BLAST search tool to compare nucleotide sequences, evaluate statistical matches, and identify related organisms[10]. The phylogenetic analysis was performed to predict species accurately and assess evolutionary relationships, identifying the closest match from the National Centre for Biotechnology Information (NCBI) database[2].

Isolation of Actinomycetes :

Nine soil samples were collected from various habitats in and around Kalaburagi region in order to isolate actinomycetes. A total of 48 actinomycetes isolates were isolated based on their morphological characteristics.

Antimicrobial activity of Actinomycetes isolates :

Among the actinomycetes, 16 isolates showed significant antagonistic activity against *Candida* strains. In the secondary screening, the isolate VSG-15 exhibited the highest ZOI against all tested *Candida* strains and bacterial spp. VSG-15 crude extract showed maximum of 15±0.96mm ZOI against *C. albicans* MTCC1966 and minimum of 8±0.33mm ZOI against *C.albicans MTCC3017* as compared to 16±0.33 and 12±0.26mm in fluconazole. (Table-1). The antibacterial activity of VSG-15 showed maximum of 16±0.66 mm ZOI against *Brevicillus brevis* and minimum of 6±0.83mm ZOI against *Staphylococcus epidermidis* as compared to 24±0.33mm and 12±0.66mm in streptomycin (Table-2).

Table-1. Anticandidal activity of Actinomycetes isolate VSG-15 against *Candida* strains

Candida strains (MTCC)	Ethyl acetate extract of VSG-15 (100µl)	Fluconazole (Standard)
C. albicans 183	11±0.63	15±0.66
C. albicans 1637	11 ± 0.5	12±0.33
C. albicans 3017	8 ±0.33	12±0.26
C. glabrata 3814	11±0.03	12±0.33
C. glabrata 3019	9±0.6	11±0.33
C. glabrata 3981	9±0.5	11±0.83
C. tropicalis 230	11±0.66	11±0.33
C. tropicalis 1406	13± 0.33	12±0.33
C. haemulonii 2766	11±0.33	18±0.66
C. albicans 1966	15±0.96	16±0.33
C. albicans 2795	11±0.33	14±0.26
C. haemulonii 8303	15±0.93	22±0.33

Note: Each experiment was carried out in triplicate and represented as means ±SD of three independent experiments

Table-2. Antibacterial activity of actinomycetes isolate VSG-15 against bacterial sp.

Name of the Pathogen	VSG-15 (100µl) Ex	Strepto-mycin
Gram-positive bacteria		
Staphylococcus aureus	10±0.66	12±0.33
Enterococcus faecalis	16±0.33	08±0.57
Staphylococcus epidermidis	6±0.83	12±0.66
Brevicillus brevis	16±0.66	24±0.33
Gram-negative bacteria		
Escherichia coli	12±0.57	15±0.57
Pseudomonas aeruginosa	11±0.83	22±0.33
Salmonella typhi	12±0.57	18±0.66

Identification of Actinomycetes :

The VSG-15 revealed extensive branched substrate and aerial hyphae bearing

chains of whitish aerial spores, which releases brown-coloured diffusible pigment (Fig. 1). Whereas, the scanning electron microscopic images revealed that, the VSG-15 produced rectiflexibile (RF) long chain forming 40-45 spores per chain and spore surface was smooth (Fig. 2). The potent isolate VSG-15 has utilized glucose, sucrose, fructose, mannitol, sorbitol, rhamnose, arbinose, xylose, salicin, maltose as carbon sources and methionine, phenylalanine and proline as nitrogen sources. Salt tolerance, urease, citrate, starch hydrolysis tests and lipase were found positive. Catalase, oxidase, methyl red, indole and voges-proskauer activities were found negative (Table-3). Based on morphological and biochemical observations, the isolates were related to a group of *Streptomyces*[18,24].

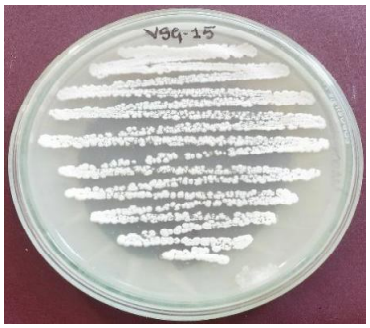

Fig. 1. Pure culture plate of actinomycetes VSG-15

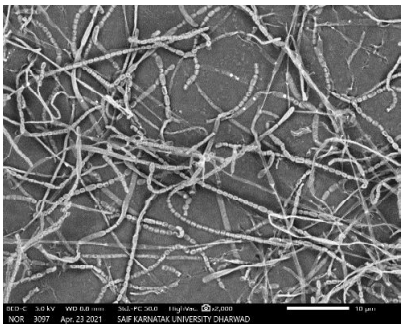

Fig 2. Scanning electron microscopic image of *Streptomyces levis* VSG-15

Table-3. Biochemical characterization of active isolate VSG-15

Biochemical assay	VSG-15
Starch hydrolysis	+
Indole production	–
Methyl red test	–
Voges Proskauer	–
Citrate agar	+
Lipase	+
Oxidase	–
Salt tolerance	+
Urease	+
Catalase	–
Carbon sources	
Glucose	+
Sucrose	+
Fructose	+
Mannitol	+
Sorbitol	+
Rhamnose	+
Arbinose	+
Xylose	+
Salicin	+
Maltose	+
Nitrogen sources	
Methionine	+
Phenylalanine	+
Proline	+

+ : Positive: - : negative.

Molecular characterization :

Genomic DNA was extracted using the Hipur Bacterial Purification Kit (HiMedia MB505) and confirmed in 1% agarose gel stained with EtBr. The DNA was visualized under a UV transilluminator, showed a high

yield. The PCR products were analysed through agarose gel electrophoresis and the DNA of the expected size was purified and sequenced (Fig. 3). Molecular characterization of isolate VSG-15 was performed using 16S rRNA gene sequencing and the sequence was submitted to Gene Bank (Accession number VSG15- ON982539) (Fig. 4). NCBI BLAST search result reveled 99% sequence similarity between VSG-15 and *Streptomyces levis* NR_115778.1 (Accession no NRRL B-16370). Phylogenetic tree was constructed using the maximum likelihood methods with 1000 bootstrap replications, illustrating the evolutionary relationship among the isolates. Based on the molecular studies, the isolate VSG-15 was identified as *Streptomyces levis* respectively.

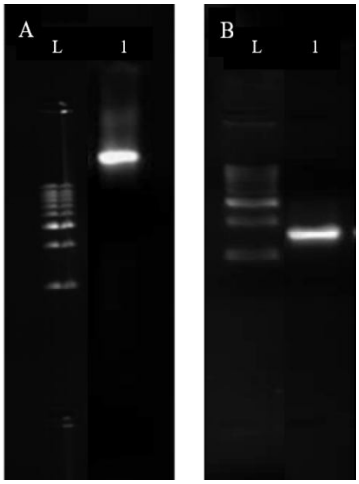

Fig 3. **A.**: Isolation of genomic DNA; **B**: 16S rRNA PCR of *Streptomyces levis* VSG-15

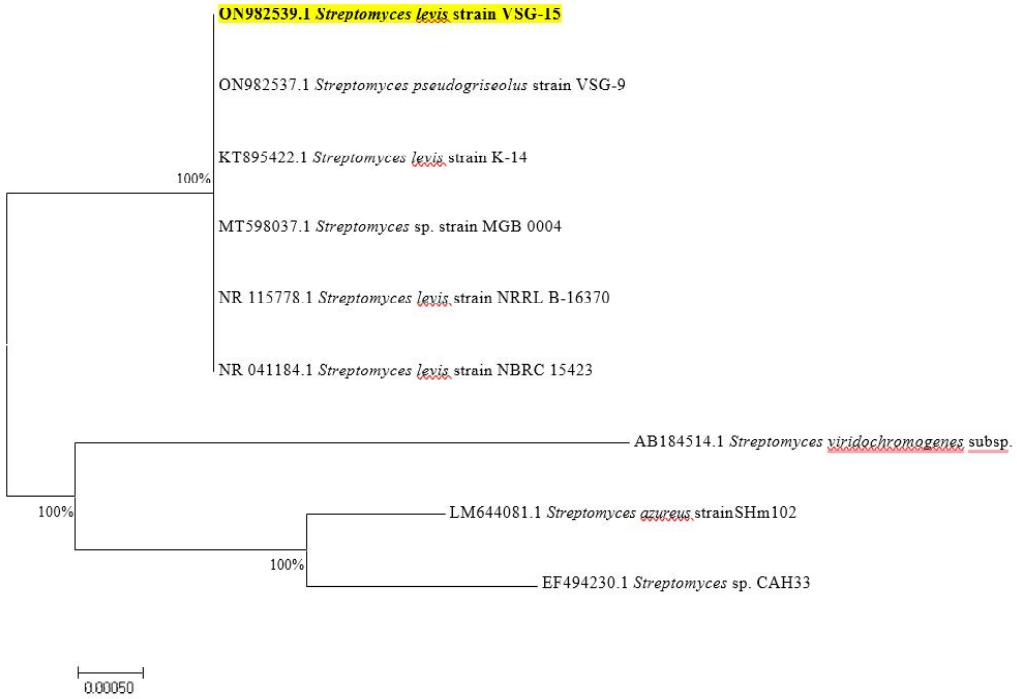

Fig 4. Neighbour-joining phylogenetic tree indicating the taxonomic position of *Streptomyces levis* VSG-15

Streptomyces species were the most dominant among the isolates in the majority of soil samples, consistent with findings reported by earlier researchers[15]. In the present investigation, a total of 48 isolates were isolated from various soil samples. Among them VSG-15 showed the highest antimicrobial activity in preliminary screening and was selected for further studies. Oskay[16], isolated 50 actinomycetes strains from north Cyprus soil and were screened against several human pathogens. Khattab *et al.*,[12] isolated 50 actinomycetes isolates from the soil sediment samples of Red sea, Sudan and determined antibacterial activity in twenty-one isolates. The potent isolate VSG-15 exhibited significant ZOI against *C. tropicalis* MTCC 1406 (13±0.33mm) as compared to standard drug (12±0.33mm). The ethyl acetate extract also showed highest of 16±0.33mm antibacterial activity in VSG-15 against *Enterococcus faecalis,* respectively as compared to Streptomycin (8±0.57mm). In conclusion, the antimicrobial activity reported by VSG-15 were found better than the fluconazole and streptomycin. These results were compared with the previous studies reported by Singh *et al.*,[19] where the ethyl acetate extract of *Streptomyces tanashiensis* strain A2D showed antimicrobial activity against *B. substilis*, *S. aureus*, *E.coli*, *K. pneumonia*, *Micrococcus luteus*, *Mycobacterium phlei* and *C. albicans*. In another study Vijayakumar *et al.*,[23] reported antimicrobial studies of ethyl acetate extract of *Streptomyces* sp. VPTSA18 against *B. substilis*, *E. coli*, *P. mirabilis*, *S. typhi*, *Staphylococcus aureus*, *S. epidermis* and *C. albicans*. The morphological, biochemical and molecular characteristics supports for the identification of isolate VSG-15 as *Streptomyces levis,* respectively.

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Declarations

The authors declare that they have no potential competing financial interests

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