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MOLECULAR CHARACTERISATION AND ANTAGONISTIC POTENTIAL OF MANGROVE STREPTOMYCES ISOLATED FROM COASTAL REGIONS OF ANDHRA PRADESH, INDIA

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ABSTRACT: The main aim of the present study is to isolate and identify potent actinobacteria from mangrove sediments of SPSR Nellore district, Andhra Pradesh, India along with their antimicrobial activity. The samples collected were initially pretreated with calcium carbonate followed by dry heating. Dilution plate technique was employed to isolate actinobacteria employing two selective media viz. yeast extract malt extract dextrose agar (YMD) and starch casein agar (SCA) amended with NaCl (4%). The media were supplemented with streptomycin (25µg/mL) and nystatin (25µg/mL) to inhibit the growth of bacteria and fungi. Over all 10 strains (VLKK-1 to VLKK-10) were isolated and screened for antimicrobial activity. The potent strain was identified as *Streptomyces carpaticus* (Gen bank accession number: OR144182) by polyphasic taxonomy including morphological, cultural, physiological and biochemical characters along with 16S rRNA analysis. The growth pattern of the strain was studied by culturing in YMD broth. The culture broth was extracted with ethyl acetate (1:1) and tested for antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. The results of this study suggested that the mangrove actinobacteria from the South Indian coast could provide lead compounds of medicinal value.

INTRODUCTION: The mangrove environment is a potential natural resource worth investigated to search for novel actinobacteria possessing pharmaceutical importance. As a result of the increasing ubiquity of antibiotic resistant pathogens and the pharmacological failure of antibiotics, there is need to investigate new bioactive metabolites from actinobacteria for potential pharmaceutical applications ^{1, 2}. Mangrove actinobacteria have been focussed due to their structural diversity, unique biological activity and functionally active secondary metabolites ³.

They are efficient producers of new secondary metabolites with a broad range of biological activities including antimicrobial, cytotoxic, antiviral, anti-inflammatory, antioxidant, anti-parasitic, anti-malaria, anti-angiogenesis, anti-HIV, neurological, immunosuppressive agents, enzymes and pigments ⁴.

Several novel bioactive compounds were discovered from actinobacteria including marinopyrroles from *Streptomyces*, salinosporamide-A, an anticancer metabolite from *Salinispora*, abyssomicin-C from *Verrucospora* and rifamycin from *Micromonospora* ^{5, 6}. Mangrove actinobacteria are one of the best sources of secondary metabolites and the vast majority of these compounds are derived from the genus *Streptomyces*, which is widely distributed in mangrove as well as terrestrial habitats ⁷. The genus *Streptomyces* is reported to produce more

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than 60% of known industrially important compounds such as β -lactams, tetracyclines, aminoglycosides, macrolides, glycopeptides, lincosamides, aminocyclitols, cyclic peptides, streptogramins, ansamycins and phenylpropanoids^{8, 9}. Screening of different mangrove habitats of SPSR Nellore district, Andhra Pradesh (India) resulted in the isolation of potent strain VLKK-10 with broad spectrum activity against Gram positive and Gram negative bacteria as well as fungi. An attempt is made in the present study to identify the strain with polyphasic taxonomic approach along with its antimicrobial profile.

METHODS AND MATERIALS:

Sampling and Pre-treatment of Soil: The mangrove sediment samples were collected at the depth of 20 cm from the south coastal region of SPSR Nellore, Andhra Pradesh, India. Collected samples were transported to the laboratory under sterile conditions followed by air drying at room temperature for 3 to 5 days. The samples were pretreated with calcium carbonate (10:1 w/w) followed by dry heating in a hot air oven at 45 °C for 1 h and incubated at 37°C for 5 days to reduce the contamination of bacteria and molds.

Selective Isolation of Actinobacteria: The pretreated soil samples were serially diluted in sterile distilled water and plated on selective media such as YMD agar and SCA amended with 4% NaCl at pH 7.0¹⁸. The media were also supplemented with antimicrobials such as streptomycin (25 μ g/mL) and nystatin (25 μ g/mL) to inhibit bacterial and fungal contamination. Serial dilution plate technique was employed for isolation of actinobacteria. The diluted sample (0.1 mL) was spread over selective media and incubated at 30 \pm 2°C for 14 to 28 days. Actinobacterial colonies were selected based on colony characteristics such as tough, chalky appearance with radiating growth, sub-cultured and preserved on YMD agar slants at 4°C for further studies. In our screening, a total of 10 actinobacteria were isolated and screened for antimicrobial activity against microbial pathogens¹⁰.

Screening of Potent Actinobacterial Strains for Bioactive Metabolites: Over all 10 actinobacterial strains were isolated and screened for bioactive metabolite production. Initially, seed culture was

prepared by culturing the strains in YMD broth for 48 h. 10% of seed culture was inoculated into YMD broth as production medium and incubated at room temperature for 9 days at 120 rpm. The flasks were harvested to separate biomass from broth. The culture filtrate thus obtained was extracted with ethyl acetate and evaporated to dryness in water bath. The residue was used to determine antimicrobial activity by agar well-diffusion method^{11, 12}. Among the ten isolates tested for bioactive compounds, the strain VLKK-10 was found potent as compared to other strains.

Identification of the Potent strain VLKK-10 by Polyphasic Taxonomy:

Morphological, Cultural, Physiological and Biochemical Characteristics of the Strain: The potent actinobacterial strain was characterized by polyphasic taxonomy (morphological, cultural, physiological, biochemical and molecular methods). The morphological characteristics were assessed using scanning electron microscopy (SEM: Model- JOELJSM 5600, Japan)¹³.

To observe the cultural characteristics such as color of aerial and substrate mycelium and pigment production, the strain was grown on seven International Streptomyces Project (ISP) media and three non-ISP media¹⁴. Tyrosine agar (ISP-7) medium was used to study melanin production by the strain¹⁵.

Physiological properties such as influence of pH 5-9, temperature (25-40°C) and effect of NaCl on growth of strain were analyzed. The resistance/susceptibility of the strain to 10 antibiotics was determined by Kirby-Bauer disc diffusion method¹⁶. The strain was screened for production of extracellular enzymes such as amylase, protease, tyrosinase, cellulase, asparaginase and glutaminase.

Various biochemical tests including IMVIC test, production of catalase and urease, nitrate reduction, H₂S production and hydrolysis of starch and gelatin were performed to identify the potent strain VLKK-10¹⁷. Utilization of different carbohydrates such as maltose, cellulose, mannitol, D-glucose, starch, fructose, galactose, lactose, sucrose, arabinose and dulcitol were tested by inoculating the isolate in YMD broth supplemented with

respective carbon sources @ 0.4% and incubated for seven days at 30°C.

Molecular Identification of Potent Strain: The genomic DNA used for the polymerase chain reaction (PCR) was prepared from colonies grown on YMD agar for 3 days. The total genomic DNA extracted from the isolate was isolated by employing DNA purification Kit (Pure Fast® Bacterial Genomic DNA purification kit, Helini Bio molecules, India) as per the manufacturer protocol. Conditions of the PCR were standardized with initial denaturation at 94°C for 3 minutes followed by 30 cycles of amplification (Denaturation at 94°C for 60 seconds, annealing temperature of 55°C for 60 seconds, and extension at 72°C for 60 seconds and an addition of 5 minutes at 72°C as final extension).

The amplification reactions were carried out with a total volume of 50µL in a gradient PCR (Eppendorf, Germany). Each reaction mixture contained 1µL of DNA, 1µL of 10 P mol forward 16S actino specific primer (5'AAATGGAGGAAGGTGGGGAT-3'), 1µL of 10 P mol reverse 16S actino specific primer (5'-AGGAGGTGATCCAACCGCA-3'), 25µL of master mix, and 22µL of molecular grade nuclease free water. Separation was carried out at 90 Volts for 40 minutes in TAE buffer with 5µL of ethidium bromide. PCR product was analyzed using agarose gel (1%) and the fragment was purified (Helini Pure Fast PCR clean up kit, Helini Bio molecules, India) as per the manufacturer's instructions. The bands were analyzed under UV light and documented using Gel Doc. The direct sequencing of PCR products was performed by dideoxy chain termination method using 3100-Avant genetic analyzer (Applied Biosystems, USA).

Pair Wise Sequence Alignment: The gene sequence of the isolate was aligned using BLAST against the gene library available for *Streptomyces* species in the NCBI and the GenBank. Pairwise evolutionary distances were computed by MEGA-6 software.

Multiple Sequence Alignment: The phylogenetic analysis was conducted with maximum parsimony method using BLAST and CLUSTAL W. The closely related homologous isolates were identified,

retrieved and compared to the sequence of the strain using CLUSTAL W available with the MEGA 6 Version¹⁸.

Nucleotide Sequence Accession Numbers: The 16S rRNA gene sequence of the isolate VLKK-10 was registered in the GenBank database.

Growth Pattern of the Strain VLKK-10: The strain was inoculated into 250 ml flasks containing 100 ml YMD broth with 4% NaCl and incubated at 30±2°C on a rotary shaker at 120 rpm. The flasks were harvested at 24 h interval up to 10 days and growth of strain was determined by taking dry weight of biomass. The culture filtrate obtained after separating biomass was extracted with equal volume of ethyl acetate and antimicrobial activity of the solvent extract was determined by agar well diffusion method¹⁹.

Extraction of Metabolites and Antimicrobial Assay: Agar well diffusion assay was used to determine antimicrobial activity of strain VLKK-10. The homogenous culture suspension prepared by suspending 3-day-old culture in sterile saline was used to inoculate YMD broth (seed medium) and the culture was incubated at 30°C for 48h on a rotary shaker at 120 rpm. Seed culture @ 10% was transferred to YMD broth (fermentation medium). The fermentation was carried out at 30±2°C for 120 h under agitation at 120 rpm. The secondary metabolites were recovered from filtrate by extraction method. Equal volume of ethyl acetate (1:1) was added to the filtrate and shaken vigorously. The ethyl acetate extract was evaporated to dryness and the residue was used to determine antimicrobial activity. The ethyl acetate solvent was used as negative control. About 80 µl of crude extract and 80 µl of negative control were poured into separate wells. Plates were incubated at 37°C for 48 h and inhibition zones (mm) were measured²⁰. The experiment was carried out in triplicates for each test organism and the mean values were tabulated.

Test Microorganisms:

Bacteria: *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 9027), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (MTCC 3160), *Streptococcus mutans* and *Xanthomonas campestris* (MTCC 2286).

Fungi: *Aspergillus niger* (ATCC 16404), *Penicillium citrinum* (MTCC 6849) and *Candida albicans* (MTCC 183).

RESULTS AND DISCUSSION:

Selective Isolation of Actinobacteria: In the present study, a total of ten actinobacterial strains were isolated from mangrove ecosystem of SPSR Nellore district on different media and designated as VLKK-1 to VLKK-10. Combined pre-treatment of the mangrove soils with calcium carbonate (10:1 w/w) and dry heat (45°C for 1 h) reduced the growth of microbial contaminants and facilitated the isolation of actinobacteria. The actinobacteria thus isolated were preserved on YMD slants at 4°C. The strain VLKK-10 exhibited high bioactivity among the isolates tested, thus selected for in-depth taxonomic studies.

Identification of the Potent strain VLKK-10 by Polyphasic Taxonomy:

Cultural Characteristics of the Strain VLKK-10: The cultural characteristics of the strain are represented in **Table 1**. The strain exhibited good growth on tryptone yeast extract-agar, YMD agar, starch casein agar and humic acid vitamin-B agar while the growth was moderate on tyrosine agar, nutrient agar and oat meal agar. The growth was not found on inorganic salts starch agar, glycerol asparagine agar and Czapek–Dox agar media. The color of aerial and substrate mycelia was gray. Soluble black colored pigment production was found on all the media tested except ISP-4, ISP-5, CDA and nutrient agar.

Morphological Characteristics of the Strain VLKK-10: Micro-morphological features of the strain were examined under SEM. Branched substrate mycelium produced abundant aerial hyphae with massive sporulation. Spores are

globose with smooth-surface. Paired spores on hyphae were also observed **Fig. 1**.

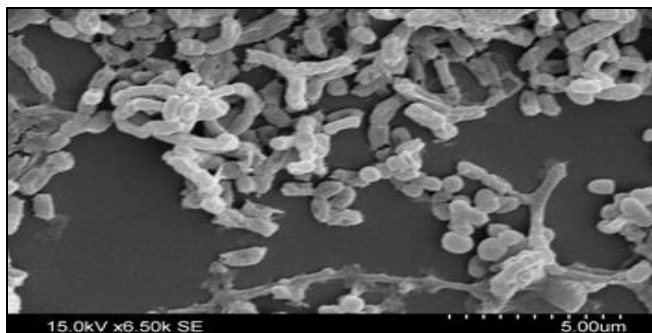


FIG. 1: SCANNING ELECTRON MICROSCOPIC PHOTO-GRAPH OF *STREPTOMYCES CARPATICUS* VLKK-10

Biochemical and Physiological characteristics of the strain VLKK-10: The physiological and biochemical characteristics are significant tools for identification of actinobacteria. The strain exhibited positive response to indole production, urease activity, gelatin liquefaction, methyl red and catalase activity but negative to H₂S production, Voges-Proskauer and citrate utilization. It could also produce enzymes like L-asparaginase, amylase and glutaminase **Table 2**. The strain showed good growth at pH range between 5 and 9 with the optimum being 7 and the range of temperature for growth was 30-40°C with the optimum being 35°C. Sodium chloride tolerance of the strain was also studied as the salt concentration has a profound effect on the production of antibiotics. The strain could grow well in the medium supplemented with 4% sodium chloride and showed tolerance up to 12%. Though the strain utilized a wide range of carbon sources, maltose, D-glucose and arabinose supported good growth of the strain (table 3). The strain showed sensitivity to gentamicin, imipenem, vancomycin, ampicillin, chloramphenicol and tetracycline **Table 4**.

TABLE 1: CULTURAL CHARACTERISTICS OF THE STRAIN VLKK-10

Culture Media	Growth	Aerial and substrate mycelium	Pigmentation
Tryptone yeast extract agar (ISP-1)	Good	Gray	Black
Yeast extract malt extract dextrose agar (ISP-2)	Good	Gray	Black
Oat-meal agar (ISP-3)	Moderate	Gray	Black
Inorganic salts starch agar (ISP-4)	-	-	-
Glycerol asparagine agar (ISP-5)	-	-	-
Tyrosine agar (ISP-7)	Moderate	Gray	Black
Starch-casein agar	Good	Gray	Black
Humic acid vitamin-B agar	Good	Gray	Black
Czapek-Dox agar	-	-	-
Nutrient agar	Moderate	Gray	Nil

‘-’: No growth; ‘ISP’: International *Streptomyces* Project

TABLE 2: MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF VLKK-10

Character	Response
Morphological characters	
Color of aerial and substrate mycelium	Gray
Physiological characters	
Grams reaction	Gram-positive
Range of temperature for growth	30 – 40 °C
Optimum temperature for growth	35°C
Range of pH for growth	5 – 9
Optimum pH for growth	7
NaCl tolerance	Up to 12%
Optimum NaCl Concentration	4%
Biochemical characters	
Catalase production	+
Urease production	+
H ₂ S production test	-
Nitrate reduction	+
Starch hydrolysis	-
Gelatin liquefaction	+
Methyl red test	+
Voges proskauer test	-
Indole production	+
Citrate utilization	-
Enzymatic activity	
Amylase	P
Asparaginase	P
Glutaminase	P
Protease	N
Tyrosinase	P
Cellulase	N

‘P’: Positive; ‘N’: Negative; ‘+’: Positive; ‘-’: Negative

TABLE 3: UTILIZATION OF THE CARBON SOURCES BY THE STRAIN VLKK-10 (W/V)

Carbon Sources	Response
Maltose	+++
Cellulose	+
Mannitol	+
D-glucose	+++
Starch	+
Fructose	-
Galactose	++
Sucrose	+
Lactose	+
Arabinose	+++
Dulcitol	-

‘+++’: Good growth; ‘++’: Moderate growth; ‘+’: Weak growth; ‘-’: No growth

TABLE 4: ANTIBIOTIC SUSCEPTIBILITY/ RESISTANCE OF VLKK-10

Antibiotics (µg/disc)	Susceptibility/Resistance
Gentamicin (10µg)	S
Vancomycin (30 µg)	S
Tetracycline (30 µg)	S
Penicillin (10 µg)	R
Chloramphenicol (50 µg)	S
Clindamycin (25 µg)	R
Imipenem (10 µg)	S
Cefixime (30 µg)	R
Ampicillin (10 µg)	S
Amikacin (10µg)	R

‘S’ – Sensitive; ‘R’ – Resistant

Molecular Characterization of the Potent Strain VLKK-10: Based on morphological, cultural, physiological, biochemical and molecular characteristics, the strain is identified as *Streptomyces* and deposited at NCBI genbank. The partial sequence was aligned and compared with 16S rRNA gene sequence available in the GenBank database by using the multi sequence advanced

BLAST comparison tool available in the website of NCBI. The Gen bank accession number is OR144182. The phylogenetic analysis of 16S rRNA gene sequence was aligned using the CLUSTAL W program from the MEGA 6 Version (18). Phylogenetic tree was constructed using MEGA software Version 6 using maximum parsimony method **Fig. 2**.

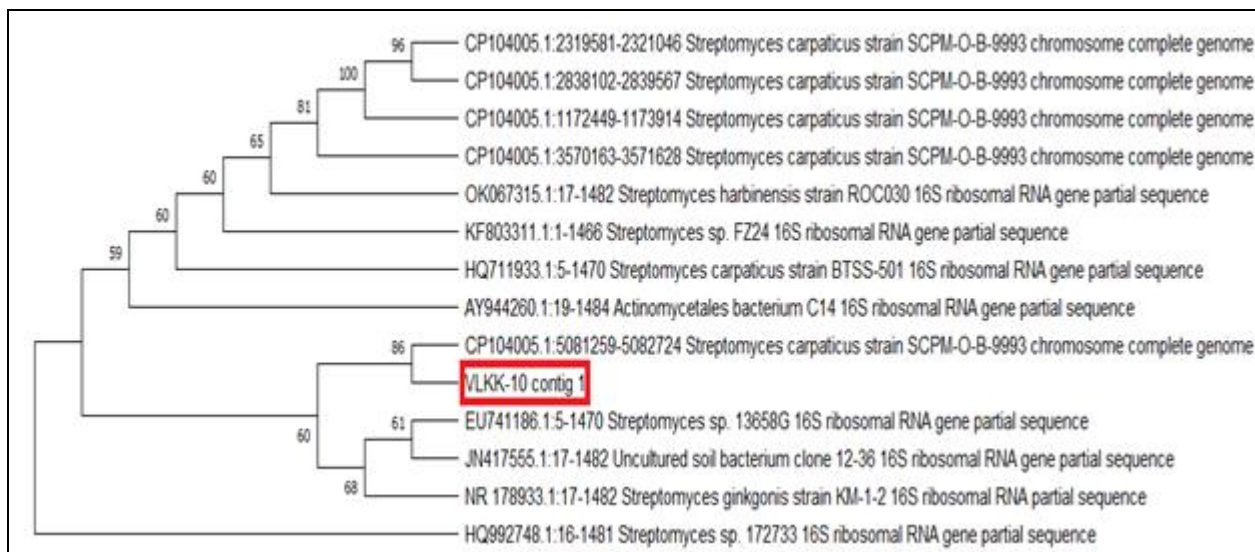


FIG. 2: MAXIMUM PARSIMONY TREE BASED ON PARTIAL 16S rRNA GENE SEQUENCE SHOWING RELATIONSHIP BETWEEN STRAIN VLKK-10 AND RELATED MEMBERS OF THE GENUS *STREPTOMYCES*

Growth Pattern and Antimicrobial Profile of *Streptomyces carpaticus* VLKK-10: The growth curve and antimicrobial profile of *Streptomyces carpaticus* VLKK-10 were studied at regular intervals up to 10 days in batch culture. The stationary phase of strain extended from 168 h to 192 h of incubation **Fig. 3**. The bioactive metabolites obtained from 8-day-old culture showed good antimicrobial activity against *Pseudomonas aeruginosa*, *E. coli*, *Candida*

albicans and *Aspergillus niger* **Table 5**. The secondary metabolites obtained from 5-day-old culture extracts of *S. violaceoruber* VLK-4 (19), *S. albogriseolous* VJMS-7²⁰ and *S. cellulosa* VJDS-7²¹ exhibited broad spectrum antimicrobial activity, while the metabolites extracted from 6-day-old culture extracts of *S. vinaceusdrappus* VJMS-4, *S. rediverticillatus* VJMS-8²² showed high antimicrobial activity.

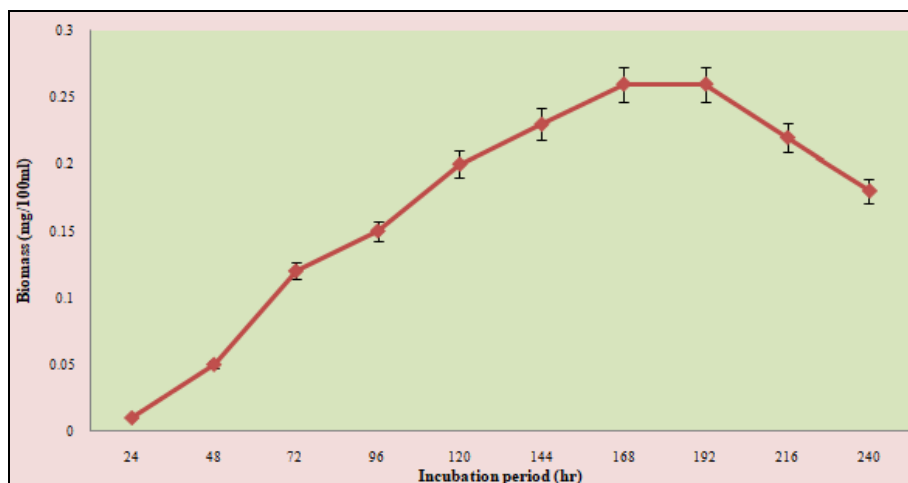


FIG. 3: GROWTH PATTERN OF *STREPTOMYCES CARPATICUS* VLKK-10

TABLE 5: ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *STREPTOMYCES CARPATICUS* VLKK-10

Test organism	Zone of inhibition (mm)
Bacteria	
<i>Staphylococcus aureus</i>	18.11±0.15
<i>Escherichia coli</i>	20.21±0.21
<i>Xanthomonas campestris</i>	14.10±0.11
<i>Pseudomonas aeruginosa</i>	21.09±0.12
<i>Bacillus subtilis</i>	14.10±0.27
<i>Streptococcus mutans</i>	16.28±0.18
Fungi	
<i>Candida albicans</i>	19.10±0.15
<i>Aspergillus niger</i>	16.08±0.26
<i>Penicillium citrinum</i>	09.26±0.13

Statistical Analysis: Readings were taken as the mean ± standard deviation of the three replicates.

CONCLUSION: The present investigation highlights the isolation and antimicrobial potential of *Streptomyces carpaticus* VLKK-10. It is evident from the present study that mangrove habitats of SPSR Nellore district serve as good source for the isolation of potent actinobacteria with broad spectrum antimicrobial activity. Further studies on chemical characterization of bioactive compounds of the strain are in progress.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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