Research Article

Antimicrobial Potential of *Streptomyces violaceoruber* VLK-4 Isolated from South Coast of Andhra Pradesh, India

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ABSTRACT

The aim of the present study was to isolate, identify and analyze the phylogenetic relationship of the potent actinomycete strain VLK-4, Isolated from South Coast of Andhra Pradesh, India. The soil samples collected from the South coastal region were pre-treated, diluted and plated on yeast extract, malt extract and dextrose agar medium. The potent strain VLK- 4 exhibiting high antimicrobial activity was identified by employing micro morphological, cultural, physiological and biochemical methods. Phylogenetic analysis of 16s rRNA gene sequence of the strain showed close relationship with *Streptomyces violaceoruber and designated as Streptomyces violaceoruber VLK-4.*

Keywords: Marine actinomycete, *Streptomyces violaceoruber* VLK-4, Antimicrobial activity.

INTRODUCTION

Actinomycetes are prolific producers of thousands of biologically active secondary metabolites. Actinomycetes from terrestrial sources have been studied and screened since the 1950s, yielding many important anti-infective and anti-cancer drugs. Actinomycetes isolated from the marine environment currently receive considerable attention due to the structural diversity and unique biological activities of their secondary metabolites. As the global threat of drug-resistant pathogens continues to rise, new strategies and resources are required to accelerate and advance the drug discovery process. Actinomycetes are Gram positive, free living, saprophytic bacteria widely distributed in different habitats, frequently filamentous and sporulating with DNA rich in G+C (55-75%).

The filamentous actinomycetes providing highly valuable bioactive compounds have been remarkably successful and approximately two thirds of the naturally occurring antibiotics have been purified from them.[1,2] They can produce an array of secondary metabolites known to possess antibacterial, antifungal or antitumor properties. They are considered highly valuable, as they produce various antibiotics and other therapeutically useful compounds with diverse biological activities.[3] They are proven to be rich source of biologically active natural products and a number of actinomycetes, especially those belong to the genus *Streptomyces*, are being extensively used for commercial production of different medically important compounds.[4] Nearly 7,600 bioactive compounds are derived from the genus *Streptomyces.*[5] In the present study we have selected marine habitats from south coast of Andhra Pradesh, India as a source for the isolation of potent actinomycetes with broad spectrum antimicrobial activity.

MATERIALS AND METHODS

Sample collection

Soil samples collected in sterile polythene bags from marine habitats of south coast of Andhra Pradesh, India, were air dried at room temperature (30 ± 2°C) for 2-4 days. The air - dried soil samples were pretreated with calcium carbonate (10:1w/w) and incubated at 30°C for four days.

Isolation

The air-dried samples were subjected to pre-treatments to enrich the actinomycetes population as well as to reduce the unwanted microbial population. Calcium-carbonate pre-treatment was employed.[6] The treated soil samples were suspended in sterile distilled water and 0.1ml of serially diluted sample (10^{-3} dilution) was spread over the surface of yeast extract- malt extract dextrose (YMD) agar medium with pH 7.0[7], containing 3% NaCl supplemented with tetracycline (25 µg/ml) and secnidazole (25 µg/ml). After incubation for a week at 30°C, distinct strains were selected and maintained by sub culturing on yeast extract-malt extract dextrose (YMD) agar slants at 4°C.

The strains were initially screened for antimicrobial activity against test bacteria such as *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* and fungi like *Aspergillus niger, Candida albicans, Fusarium solani* and *F. oxysporum.* Preliminary screening for inhibitory ability of the isolate was tested by Cross streak method. The isolates were inoculated as a single streak in the centre of the Petridish and incubated at 30°C for 3-4 days to permit growth and antibiotic production. Later the test bacteria and fungi were inoculated by streaking perpendicular to the growth of isolate. The plates were incubated for 24-48 hours at 30°C in case of bacteria and 72 hours for fungi. After

incubation, growth inhibition of test bacteria and fungi around the isolate was taken as positive for inhibitory activity.

Among the 20 isolates tested for biological activity, one isolate designated as VLK-4 was found to be potent as it exhibited high antimicrobial activity. It was identified based on cultural, morphological, physiological and biochemical characters along with molecular approaches. The strain VLK-4 was grown on seven International Streptomyces Project (ISP) media and three non-ISP media to determine morphological, biochemical and physiological and cultural characteristics.[8-11] The morphological characteristics were assessed using scanning electron microscopy (SEM: Model- JOEL-JSM 5600, Japan).

Identification by polyphasic taxonomy

The utilization of carbon and nitrogen sources by the strain was carried out.[12] Biochemical tests including IMVIC, H_2S production[13], nitrate reduction[14], enzymatic activity such as Chitinase[15], L-Asparaginase[16], Cellulase, Lipase, urease, catalase and amylase[17], were also evaluated. The effect of pH (5 - 10), temperature (20 - 50°C) and salinity tolerance of the strain was analysed. In addition, the sensitivity of the strain to different antibiotics was determined by paper disc method.[7]

Molecular identification

The Molecular identification of the potential strains was carried out by amplifying the 16S rRNA gene using the universal primers (actino specific forward primer -5'-GCCTAACACATGCAAGTCGA-3' and actino specific reverse primer-5'-CGTATTACCGCGGCTGCTGG-3')[18]. The amplified product was cloned (HELINI pure fast PCR clean up kit, Helini Biomolecules, India) according to manufacturer's instructions. Sequencing was performed by using automatic DNA sequencer with dye terminator cycle sequencing kit (Applied Biosystems). The 16S rRNA gene sequence (1000bp) was aligned with closely related sequences retrieved from GenBank database of NCBI.

Pair wise sequence alignment

The gene sequence of the strain was aligned using BLAST against the gene library available for potential actinomycete strains in the NCBI and the GenBank. Pair wise evolutionary distances were computed by using MEGA-5.0 software.

Multiple Sequence Alignment

The phylogenetic tree was constructed using the Neighbour-joining method.[19] The closely related homologous strains were identified, retrieved and compared to the sequence of the strain VLK-4 using CLUSTAL W available with the MEGA-5.0 Version.[20]

Gene Bank Acession Number

The sequence of the 16S rRNA gene of the strain has been deposited in National Center for Biotechnology Information (NCBI).

Extraction of crude bioactive metabolites

YMD broth was used as a production medium for the extraction of crude secondary metabolites. The selected potent actinomycetes isolate VLK-4 obtained from the primary screening were inoculated and incubated at 28°C in a rotatory shaker (120 rpm). The crude culture filtrate obtained from a four day old culture was extracted with equal volumes of solvents (viz. ethyl acetate, chloroform, methanol and acetone) independently to extract the antimicrobial compounds. The solvent extracts were evaporated to dryness in a water bath at 40°C and the compound obtained from each solvent was tested for its activity against the test microorganisms by well diffusion method.[21]

Test microorganisms

Test microorganisms employed for the study include Gram positive bacteria - *Staphylococcus aureus* (MTCC 3160), *Streptococcus mutans* (MTCC 497), and *Bacillus subtilis* (ATCC 6633) Gram negative bacteria - *Escherichia coli* (ATCC 35218), *Enterococcus faecalis* (MTCC 439), *Pseudomonas aeruginosa* (ATCC 9027) and Fungi - *Candida albicans* (ATCC 10231), *Aspergillus niger* and *Fusarium oxysporum* (MTCC 3075).

RESULTS AND DISCUSSION

20 actinomycete strains (VLK-1 to VLK-20) were isolated from the South coast of Andhra Pradesh, India. Among them, one strain found potent was designated as VLK-4. The strain VLK-4 exhibited typical morphological characteristics of the genus *Streptomyces* when grown on the culture media. The cultural characteristics of the strain are represented in table 1. It exhibited good growth on ISP-1, ISP-2, ISP-7, starch casein agar, Czapek-Dox agar and maltose tryptone agar. The growth was moderate on ISP-4 and ISP-5 and nutrient agar media while it was poor on ISP-3 agar. The color of aerial mycelium was white and the substrate mycelium was pale yellow (Figure 1).

Micromorphology of the strain was examined by slide culture method. The culture showed extensively branched aerial mycelium and bear short chain of spores. As its sporophore morphology is of rectus flexibilis type (fig.2), the strain may be placed in the rectus-flexibilis group of *Streptomyces*.[22] Morphological, Physiological and biochemical characteristics of the strain VLK-4 are presented in Table 2.

The physiological tests serves as indispensible tools for classification and identification of actinomycetes.[8] Growth of the strain VLK-4 occurred in the pH range of 5-10 with optimum growth at pH 7. The temperature range for growth was 20-50°C with the optimum growth at 30°C. Tolerance of the strain to NaCl also serves as an important character for species identification. VLK-4 exhibited salt tolerance up to 5% with optimum growth at 3% NaCl, hence the strain could be placed in intermediate salt tolerance group.[23]

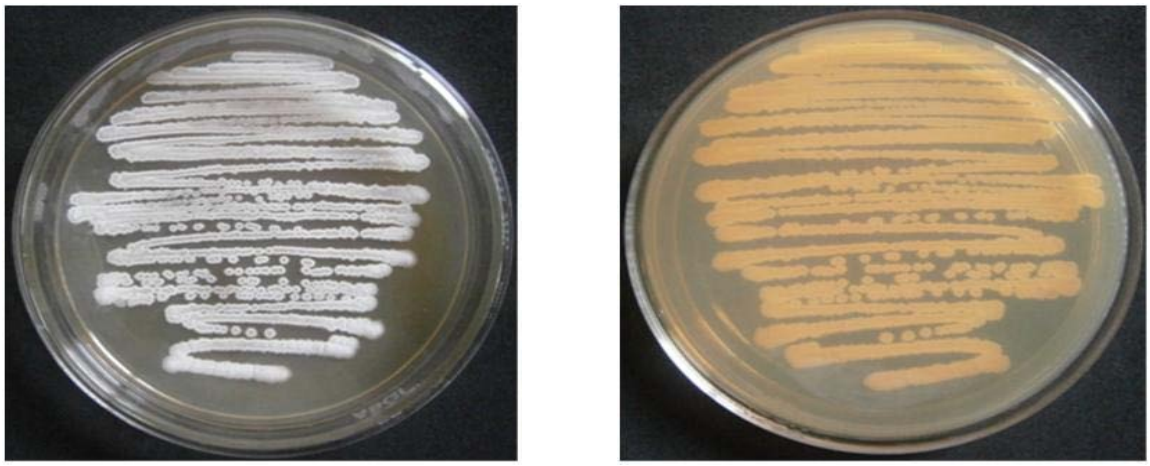

Figure 1: Aerial mycelium and substrate mycelium of the strain VLK-4

Table 1: Cultural characteristics of the strain VLK-4

Medium	Growth	Aerial mycelium	Substrate mycelium	Pigmentation
Tryptone yeast extract agar (ISp-1)	Good	White	Pale yellow	Nil
Yeast extract malt extract dextrose agar (ISp-2)	Good	White	Pale yellow	Nil
Oat-meal agar (ISP-3)	Poor	White	Pale yellow	Nil
Inorganic salts starch agar (ISp-4)	Moderate	White	Pale yellow	Nil
Glycerol asparagine agar (ISP-5)	Moderate	White	Pale yellow	Nil
Tyrosine agar (ISP-7)	Good	White	Pale yellow	Nil
Starch-casein agar	Good	White	Pale yellow	Nil
Czapek-Dox agar	Good	White	Pale yellow	Nil
Maltose tryptone agar	Good	White	Pale yellow	Nil
Nutrient agar	Moderate	White	Pale yellow	Nil

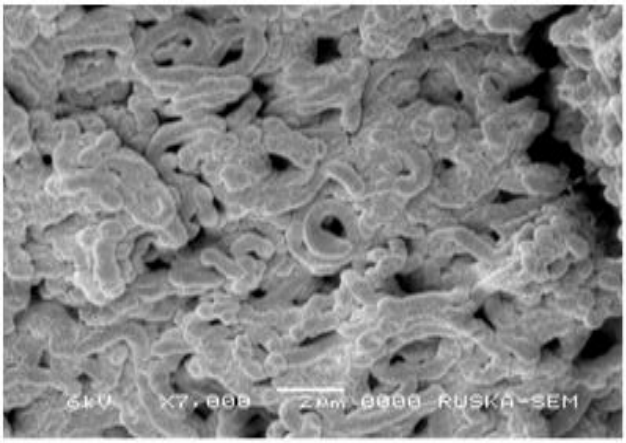

Figure 2: Scanning Electron Microscopic photograph of Streptomyces *violaceoruber* VLK-4 (X 7,000)

The strain VLK-4 had the ability to hydrolyze starch, casein and exhibited positive response to citrate utilization and catalase, urease, cellulase, lipase, L-asparaginase production but negative for indole, methyl red, vogues-proskauer, hydrogen sulphide production and nitrate reduction. It could also produce enzymes like catalase, urease, cellulase, lipase and L- asparaginase. The utilization of starch and casein revealed that the isolate could produce extracellular enzymes such as amylase and casienase respectively. Positive reaction for catalase revealed that the isolate can survive the stress condition generated by reactive oxygen species.

The consumption of different carbon sources by the strain indicated its wide pattern of carbon utilization (Table 3). The strain VLK-4 efficiently utilized the carbon sources such as Dextrose, maltose, fructose, starch, galactose, lactose, sucrose and arabinose but could not utilize xylose and mannitol. Carbohydrate utilization plays a prominent role in the taxonomic characterization of actinomycete strains.[22] The actinomycetes strains tested for resistance to different antibiotics could be useful as a taxonomic aid.[10] VLK-4 was susceptible to ampicillin, gentamicin, kanamycin and clindamycin but resistant to streptomycin and nalidixic acid (Table 4).

Gene sequence of 16S rRNA of VLK -4 was blasted against nucleotide database of the NCBI. It was found that the strain showed a close relation with *Streptomyces violaceoruber* (fig.3). The 16S rRNA sequence was deposited in the Gen Bank database of NCBI under the accession number KF908011. Basing on the morphological, physiological, biochemical characteristics and molecular characterization by 16s rRNA sequencing, the strain VLK-4 has been identified as *Streptomyces violaceoruber* VLK-4.

Table 2: Morphological, Physiological and Biochemical characteristics of strain VLK-4

Character	Response
Morphological characters	
Mycelial form	Branched
Sporophore morphology	Rectiflexible
Color of aerial mycelium	white
Color of substrate mycelium	Pale yellow
Physiological characters	
Gram reaction	+
Acid-fast reaction	-
Production of melanin pigment	-
Range of temperature for growth	20-50°C
Optimum temperature for growth	30 °C
Range of pH for growth	5-10
Optimum pH for growth	7
NaCl tolerance	5%
Biochemical characters	
Catalase production	+
Arginine hydrolase	-
Hydrogen sulfide production	-
Nitrate reduction	-
Starch hydrolysis	+
Methyl red test	-
Voges-Proskauer test	-
Indole production	-
Citrate utilization	+
Casein hydrolysis	+
Enzymatic activity	
Amylase	+
Cellulase	+
Caseinase	+
Chitinase	+
L-Asparaginase	+
Lipase	+
Urease	+

The antimicrobial efficiency of the isolate *Streptomyces violaceoruber* VLK-4 was evaluated by using four different solvents such as ethyl acetate, chloroform, methanol and acetone. Among the solvents used ethyl acetate extract exhibited highest antimicrobial activity whereas the other solvent extracts showed moderate to minimum activity against all the microbes tested. The ethyl acetate extract of the isolate VLK-4 showed maximum activity against *Streptococcus mutans* and *Staphylococcus aureus* followed by *Bacillus subtilis, Escherichia coli, Candida albicans* and *Pseudomonas aeruginosa* (Table 5).

Table 3: Utilization of the carbon sources by the strain VLK-4

Carbon Source	Response
Dextrose	+++
Maltose	++
Xylose	-
Sucrose	+
Lactose	+
Arabinose	+
Fructose	++
Galactose	++
Starch	++
Mannitol	-

(+++) - Efficient, (++) - Good, (+) - Moderate, (-) - Poor

Table 4: Antibiotic susceptibility/resistance of VLK-4

Antibiotics	µg/disc	Susceptibility/Resistance
Nalidixic acid	30	R
Streptomycin	10	R
Clindamycin	10	S
Gentamicin	10	S
Ampicillin	10	S
Kanamycin	30	S

Table 5: Antimicrobial Activity of *Streptomyces violaceoruber* VLK-4 by using different solvents

Test microorganisms	Zone of Inhibition (mm)			
	A	B	C	D
Staphylococcus aureus MTCC 3160	16	22	17	16
Streptococcus mutans MTCC 497	14	25	15	15
Bacillus subtilis ATCC 6633	17	20	16	19
Escherichia coli ATCC 35218	18	20	18	17
Pseudomonas aeruginosa ATCC 9027	10	19	16	10
Candida albicans ATCC 10231	12	20	16	10

A-Chloroform extract; B-Ethyl acetate extract; C-Methanol extract; D-Acetone extract

CONCLUSION

Based on the screening results, it is evident that marine habitats of South coast of Andhra Pradesh, India serve as a good source for the isolation of potent actinomycetes with broad spectrum antimicrobial activity. Further studies regarding the extraction and purification of bio active compounds produced by *Streptomyces violaceoruber* VLK-4 are in progress.

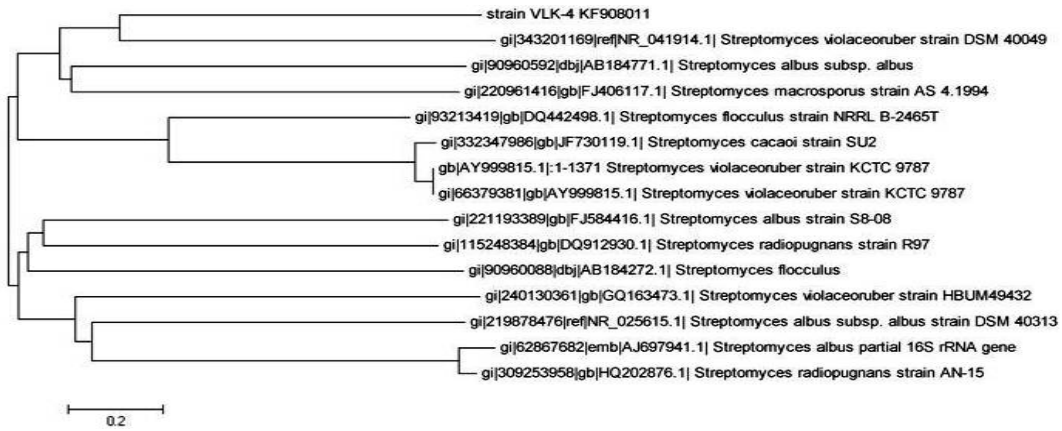

Figure 3: Neighbour-joining tree based on 16S rRNA gene sequences showing relationships between the strain VLK-4 and related members of the genus *Streptomyces* 16S rRNA gene clade.

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