

Production and characterization of antimicrobial compound from marine *Streptomyces* sp. VPTS3-1-a potential soil isolate of Palk Strait, East Coast of India

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Abstract

Sixty-eight morphologically discrete actinobacterial isolates were obtained from marine soil samples of Palk Strait region of Bay of Bengal, Tamil Nadu, and were subjected to the screening of antimicrobial activity against bacterial and fungal pathogens. Of them 37% (25) possessed antimicrobial activity. The potential antimicrobial compound producer was named as *Streptomyces* sp. VPTS3-1 on the basis of phenotypic properties, and the phylogenetic evaluation on the basis of 16S rDNA sequence further categorized the isolate as *Streptomyces afghaniensis* VPTS3-1. The antimicrobial compound was extracted from the *Streptomyces afghaniensis* VPTS3-1 using various solvents, and the antimicrobial efficacies were tested against bacterial and fungal pathogens. Of the solvent extracts evaluated, the ethyl acetate extract exhibited highest antimicrobial activity against all the pathogens tested. On the basis of UV, FT-IR, Mass and ¹H NMR spectral analyses, the compound was identified as highly oxygenated and derivatives of carbohydrates.

Key words: antimicrobial activity, compound purification, marine actinobacteria, screening, spectral analysis.

INTRODUCTION

Actinobacteriae, the slow-growing prokaryotes, are widely distributed in natural ecosystems. They can be found in almost every natural substrate: water, air, soil, foodstuffs, manure and compost (Bizuye *et al.*, 2013). They are Gram positive, saprophytic, free living,

filamentous bacteriae and are vital source for the production of various antibiotics (Al-Dhabi *et al.*, 2018; 2019). They belong to the order Actinomycetales that are found in marine, estuarine environment, terrestrial soil and freshwater (Al-Dhabi *et al.*, 2014). The genus *Streptomyces* produces about 80% of all the known actinobacterial antibiotics (Demain, 2006). In recent years, the rate of discovery of new antibiotics from the genus *Streptomyces* has decreased noticeably [Boubetra *et al.*, 2013]. Thus, many research laboratories around the world are focusing their search for new microbes based antibiotics from non-streptomycete actinobacteria.

Until the 1970s, it was still relatively easy to isolate new compounds from *Streptomyces*, but since 1985 only three new classes of antibiotics have been discovered (Martens and Demain, 2013). One of these compounds is platensimycin, a new class of antibiotic from *Streptomyces platensis* that particularly inhibits biosynthesis of cellular lipid (Wang *et al.*, 2006). Very recently, an antibiotic-producing strain of thermotolerant *Streptomyces* sp. TM32 was isolated from the rhizosphere region of *Curcuma longa* L., a medicinal plant. This is believed to be a new strain of *Streptomyces sioyaensis* that has strong antimicrobial effect against both human and plant pathogens, including *Staphylococcus haemolyticus* MR-CoNS - an antibiotic-resistant strain (Nakaew *et al.*, 2019). It may also reveal as an emerging source for further discovery of valuable and novel bioactive compounds from streptomycetes actinobacteria. In this context, the search for new strains of actinobacteria producing novel antimicrobial compounds is inevitable. Moreover, attempt to explore the actinobacteria from saltpan and mangrove regions of marine environments in India have been limited so far. With this background, the present study deals with isolation of streptomycete strains in the marine soil samples collected from Palk Strait, East Coast of India and characterization of potent strains capable of synthesizing novel antimicrobial compounds and purification and identification of the novel antimicrobial compounds.



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MATERIALS AND METHODS

Study area: Ten soil samples were collected along the region of Palk Strait Coast including Point Calimere seashore, Vedaranyam saltpan, Muthupet mangrove, Thondi saltpan and Tuticorin saltpan, east coast of India.

Isolation of actinobacteria: Each soil sample was serially diluted up to 10^{-6} . About 0.1 mL of the aliquot was spread over the starch-casein agar (SCA) plates and incubated at $28 \pm 2^\circ\text{C}$ for 7–10 days. The colonies of actinobacteria developed over the medium were purified and maintained in SCA medium for further process.

Screening of actinobacteria for the antimicrobial activity: The streak plate method was employed for determining the antimicrobial properties of actinobacterial isolates. Actinobacterial cultures were first cultivated in a straight line on SCA medium for 5–7 days at 30°C . After the incubation period, the test microorganisms (*Salmonella typhi*, *Staphylococcus aureus* and *Cryptococcus neoformans*) were inoculated by perpendicular streaking to the actinobacteria. After incubation at 37°C for bacteria and 27°C for yeasts, the antimicrobial activity was determined by measuring of inhibition zone between test microorganisms and the actinobacterial cultures (Lahoum *et al.*, 2016). The zone of inhibition was measured after 24–48 h. Based on the inhibition zone, the antimicrobial compound producing actinobacterial isolates were selected.

Extraction and purification of antimicrobial compound: Broad spectrum of antimicrobial activity of selected isolates was tested against six different human pathogenic bacteria namely *Salmonella typhi*, *S. typhimurium*, *S. paratyphi B*, *Staphylococcus aureus*, *S. epidermidis*, *Vibrio cholera* and one fungus namely *Cryptococcus neoformans*. Pathogenic bacteria and fungi were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The antimicrobial compound from the selected isolate was separated by using shake flask culture method (Vijayakumar *et al.*, 2012a; Lahoum *et al.*, 2016). The extracted filtrates were tested for their antimicrobial activity by using well-diffusion method (Vijayakumar *et al.*, 2012b). Muller Hinton agar and Sabouraud's dextrose agar medium were used for the determination of antimicrobial activity against test bacteria and yeast, respectively. 25 μL of the extract was added separately into each well and incubated at appropriate temperature. Each test was repeated three times and the antimicrobial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the antimicrobial compound.

The final purification of antimicrobial compound was carried out by the TLC on silica gel 60 (70–325 mesh)

columns. The compound was further eluted with the lower phase of a mixture of chloroform/ methanol/ water (175:100:50), and silica gel column chromatography. 2 g of crude powder was dissolved in 10 mL of ethyl acetate. The solution was passed through a silica gel column in benzene (Dhanasekaran *et al.*, 2008). All the fractions were collected and tested against bacterial and fungal test pathogens.

Characterization of antimicrobial compound

The solubility, melting point, thermo stability and pH stability of the antimicrobial compound was characterized by the standard methods of Harindran *et al.* (1999). The UV spectral measurement of the pure compound was made 200–400 nm by using Shimadzu (UV1601) instrument. The FT-IR spectrum of antimicrobial compound was analyzed by the methods of Fukuda *et al.* (1990). The mass spectrum was recorded using Finnigan MAT 8230 Mass spectrometer under the current (MA) 100 and the temperature at 90°C . ^1H NMR spectra were analysed by Ivanova and Schlegel (1997) method and measured in CDCl_3 on a JEOL GSX-400 NMR spectrophotometer at 400 MHz for ^1H .

Characterization of potential antimicrobial compound producing isolate

Morphological characterization of the strain was observed by the slide culture technique on SCA medium, and the slides were observed under the light microscope and scanning electron microscope. Biochemical and chemotaxonomical characterizations of the isolates were carried out as per the keys prescribed by International *Streptomyces* Project (ISP) and Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Subsequently, 16S rRNA gene sequencing and phylogenetic study was conducted for identification of the isolate *Streptomyces* sp. VPTS3-1 (Thirumurugan and Vijayakumar, 2018).

RESULTS AND DISCUSSION

In the present study, totally 68 strains of actinobacteria were isolated from three different soil types including coastal, mangrove and salt pan environments of five locations namely Point Calimere, Vedaranyam, Muthupet, Thondi and Tuticorin. Number of isolates of actinobacteria was high in mangrove soil (28) followed by, seashore soil (21) and saltpan (19) (Fig. 1). Diversity of actinobacteria isolates was found to increase due to the nutritive status of the respective soil. Nine genera were identified among the 68 isolates of actinobacteria with the genus *Streptomyces* being predominant in all the three environments. Thirty-nine isolates were assigned to the genus *Streptomyces*, 10 to *Actinopolyspora*, 7 to *Saccharopolyspora*, 4 to *Actinomadura*, 3 to *Nocardopsis*, 2 to *Micromonospora*, 1

Table 1. Screening of antimicrobial activity of actinobacterial isolates

S. No.	Actinobacterial isolates	Zone of inhibition (mm)		
		<i>Salmonella typhi</i>	<i>Staphylococcus aureus</i>	<i>Cryptococcus neoformans</i>
Seashore isolates				
1	VPTS1 - 6	8	7	6
2	VPTS1 - 7	7	-	6
3	VPTS2 - 4	7	8	7
4	VPTS2 - 7	6	-	8
5	VPTS3 - 1	16	15	14
6	VPTS3 - 2	10	8	8
7	VPTS3 - 6	4	7	8
Mangrove isolates				
8	VPTM1 - 3	3	12	4
9	VPTM1 - 7	5	10	3
10	VPTM2 - 2	11	-	4
11	VPTM2 - 3	9	-	10
12	VPTM2 - 5	-	9	-
13	VPTM2 - 9	7	-	12
14	VPTM2 - 12	9	8	7
15	VPTM3 - 9	6	10	-
Saltpan isolates				
16	VPTSA1 - 4	12	7	8
17	VPTSA1 - 8	5	11	7
18	VPTSA2 - 2	4	4	9
19	VPTSA2 - 3	8	-	7
20	VPTSA2 - 5	9	-	4
21	VPTSA2 - 7	-	8	-
22	VPTSA2 - 8	10	6	7
23	VPTSA2 - 9	-	10	-
24	VPTSA2 - 10	-	-	6
25	VPTSA2 - 11	-	7	7

- = No antimicrobial activity

to *Actinomyces*, 1 to *Actinoplanes* and 1 to *Microbispora*. Similar type of diversity report of the actinobacteria has already been reported by many workers from most parts of the world. The predominance of streptomycetes was reported earlier in Palk Strait region of Bay of Bengal (Vijayakumar *et al.*, 2007), Mount Everest region soil (Gurung *et al.*, 2009); South China Sea gorgonian corals (Zhang *et al.*, 2013) arid and desert habitats of Iran (Mohammadipanah and Wink, 2016) and mangrove sediment in South Sumatra, Indonesia (Rozirwa *et al.*, 2020).

In the present study, among the 68 isolates of actinobacteria only 25 (37%) isolates were found to possess antimicrobial potentiality. The percentage of

occurrence of antagonistic actinobacteria also varied *i.e.*, saltpan soil (53.63%), followed by seashore soil (33.33%) and mangrove soil (28.57%) (Table 1; Fig. 1). Among the antimicrobial compound producers, the isolate VPTS3-1 exhibited more antimicrobial activity against all of the pathogens tested. This isolate had higher activity against test bacteria than the fungal pathogens. In a similar study, twenty-five strains of *Streptomyces* isolated from around hundred soil samples were screened for the antimicrobial activities against Gram's positive and Gram's negative bacteria and some fungi by Khaliq *et al.* (2013). Among them, only 4 isolates exhibited wide spectrum antimicrobial activity. Of which, three isolates had good antibacterial

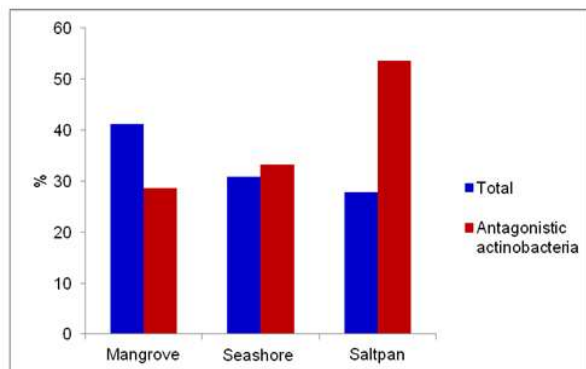


Fig. 1. Distribution of total and antagonistic actinobacteria

activities against Gram positive and Gram negative bacteria including drug resistant *S. typhi* and *E. coli*. Isolate SK-5 was found to be very active against some indigenous *Trichoderma* and *Aspergillus* species (Khaliq *et al.*, 2013).

The antimicrobial activity of the different solvent extracts of strain VPTS3-1 against 7 pathogens (six bacteria, one fungus) was noteworthy. The compound of VPTS3-1 in ethyl acetate solvent showed more antimicrobial efficacy than other solvents tested. The antimicrobial efficacy of the isolate VPTS3-1 compound was maximum against *V. cholerae* (24 mm) followed by *S. aureus* (22 mm), *Cryptococcus neoformans* (20

Table 2. Antimicrobial efficacy of crude extract of actinobacteria isolate VPTS3-1

Name of the pathogens	Zone of inhibition (mm)				
	Alcohol	Chloroform	Distilled water	Ethyl acetate	Methanol
<i>Salmonella typhi</i>	8	6	6	18	11
<i>S. typhimureium</i>	9	7	6	18	9
<i>S. paratyphi</i> B	10	6	9	14	13
<i>Staphylococcus aureus</i>	15	7	10	22	16
<i>S. epidermidis</i>	7	8	9	14	10
<i>Vibrio cholerae</i>	15	14	9	24	15
<i>Cryptococcus neoformans</i>	3	4	7	20	8

Table 3. Characterization of antimicrobial compound derived from the isolate VPTS3-1

S. No.	Name of the test	Result
1	TLC Rf value (cm)	0.38
2	Colour	Light brownish
3	Nature	Viscous
4	Solubility	Alcohol, chloroform, distilled water, ethyl acetate & methanol
5	Extractability	
	Alcohol	Light yellow
	Chloroform	Colour less
	Distilled water	Light yellow
	Ethyl acetate	Dark brown
	Methanol	Brownish yellow
5	Petroleum ether	Colour less
6	Melting point (°C)	180
7	pH stability	07-Aug
8	Temperature stability (°C)	20-40
9	UV-spectrum	200 - 205
	(200 - 400 nm)	
10	IR spectrum (cm ⁻¹)	3400.0, 2911.0, 1655.5, 1111.1
11	Mass (m/z) ion peak	326
12	¹ H NMR (1-10 ppm)	1 - 4

Table 4. Characteristics of selected antagonistic isolate VPTS3-1

S. No.	Name of the test	Result
Morphological characteristics		
1	i) Sporophore morphology	Spiral
	ii) Spore surface	Smooth
2	Colour of aerial mycelium	Ash
3	Colour of substrate mycelium	Light black
4	Spore mass	Ash
Biochemical characteristics		
5	H ₂ S production	-
6	Nitrate	+
7	Urease	+
8	Catalase	+
9	Oxidase	-
10	β-Lactamase	-
11	Melanin	-
12	Starch	+
13	Gelatin	+
14	DNA	+
15	RNA	+
16	Lipid	-
17	Casein	+
18	Haemolysis	-
19	Triple sugar iron	+
Chemotaxonomical characteristics		
20	Di-amino pimelic acid	+
21	Cell wall sugars	-
Molecular characteristics		
22	Length of the 16S rDNA	691 bp
23	Sequence primer - Forward primer	3' TGCCAGCAGCCGCGTAATA 5' -
24	Reverse primer	5' CCGCCTACGACGTCITTA 3' -
25	Accession number	DQ845201
26	Similarity to	<i>Streptomyces afghaniensis</i> AJ399483
27	Percentage of similarity	98.50%

mm), *Salmonella typhi* (18 mm), *S. typhimureium* (18 mm), *S. paratyphi* B (14 mm) and *S. epidermidis* (14 mm). The other solvent extracts had a moderate to minimum antimicrobial activity against the pathogens tested (Table 2). There are many reports on the successful extraction of antimicrobial compounds from actinobacteria using ethyl acetate as an organic solvent and the compounds to exhibit higher inhibitory effect against most of the pathogens tested (Thirumurugan and Vijayakumar, 2015; Cholarajan and Vijayakumar, 2016; Rabia Boukhalfa *et al.*, 2017; Thirumurugan *et al.*, 2018). In contrary to this, Saravana Kumar *et al.*

(2014) reported that the methanol extract of agriculture field actinobacteria showed good activity against tested bacterial and fungal pathogens.

As reported in our earlier report, single separated band was observed in TLC. The R_f value of the isolate VPTS3-1 compound was 0.38 cm. On the basis of UV, FT-IR, mass and ¹H NMR spectral studies and other physico-chemical properties, the antimicrobial compound was identified as highly oxygenated and derivatives of carbohydrates (Table 3). Similar compound has already been reported by Vijayakumar (2006) and Vijayakumar *et al.* (2012a). Likewise, the

antimicrobial compounds, namely, N-isopentyltridecanamide was reported from *Streptomyces labedae* ECR77 (Thirumurugan and Vijayakumar (2015), (3R)-1,3-butanediol from *Streptomyces* sp. MSL(Bindhu Madasu *et al.*,2017) and methyl-4,8-dimethylundecanate from *Streptomyces albogriseolus* (Thirumurugan *et al.*,2018).

Morphological characteristics of the isolate VPTS3-1 *viz.*, ash colored aerial spore mass and blackish reverse side colour were recorded. Development of spiral spore chains on the aerial and substrate mycelia and smooth spore surface of the strain undoubtedly placed it under the genus *Streptomyces*. Biochemical characteristics of the isolate are given in table 4 also supported the generic level identity of the isolate. Presence of LL-diaminopimelic acid (DAP) and the absence of characteristic sugars in their cell convincingly categorized the cell wall of this strain belonged to the cell wall type-I of streptomycetes. The generic level identity of the isolate was confirmed with Bergey's Manual of Determinative Bacteriology. The 16S rDNA sequence of the *Streptomyces* sp. VPTS3-1 was processed and deposited (accession number: DQ845201) in the biological databases namely NCBI/EMBL/DDBJ. The phylogenetic investigation of the sequence revealed that the 691 bp sequence of the isolate was closely related (98.5%) to the existing species of *Streptomyces afghaniensis* AJ399483 (Table 4). Therefore, on the basis of 16S rDNA sequence and phylogenetic relatedness, the potential antimicrobial compound producer was named as *S. afghaniensis* (VPTS3-1). The characterization, identification and optimization of antimicrobial compound production by the *S. afghaniensis* (VPTS3-1) was already reported in our earlier study (Vijayakumar *et al.*, 2012a). The studies with characterization and identification of actinobacteria using ISP protocol and 16S rDNA sequencing were reported by many workers (Saha *et al.*, 2013; Dharumadurai *et al.*, 2014; Sarika *et al.*, 2021). The results of the present study revealed that the marine actinobacteria isolated from Palk Strait region of East Coast of India could be a potential source of novel antimicrobials.

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