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Antimicrobial and larvicidal activity of *Streptomyces* sp.VITPK9 isolated from a brine spring habitat of Manipur, India

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ABSTRACT

The aim of the present study was to assess the antimicrobial and larvicidal activity of Streptomyces sp.VITPK9, isolated from brine spring habitat of Manipur, India. The isolate was identified by molecular taxonomy and phylogeny of the isolate revealed that it belonged to the genus Streptomyces and designated as Streptomyces sp.VITPK9 (JN689333). Blast search of 16S rDNA sequence (1300m bases) of the isolate with sequences available in the GenBank (NCBI) database showed 97% similarity with Streptomyces pseudogriseolus (X80827). The ethyl acetate (EA) extract of Streptomyces sp.VITPK9 showed highest zone of inhibition (24mm) against Candida albicans and (17 mm) against P. aeruginosa. Larvicidal efficacy was studied and found to be high after 24h exposure to EA extract (1000ppm), the mortality rate was observed as A. subpictus (LC_{50}=831.78, r^2=0.930), C. tritaeniorhynchus (LC_{50}=489.21, r^2=0.923), C. gelidus (LC_{50}=151.29, r^2=0.831), R. microplus (LC_{50}=369.21, r^2=0.917) and H. bispinosa (LC_{50}=424.55, r^2=0.943). The purification of the active principles responsible for the biological activity is under process.

Keywords: *Streptomyces* sp.VITPK9, Antimicrobial activity, larvicidal activity.

INTRODUCTION

Natural products have been the successful leads in all aspects including medicines and insecticides. The use of synthetic compounds has many negative effects, including health and ecological imbalance. Rise in resistant pathogens and ticks are alarming issue, which is of major concern in the field of research [1]. Nearly millions of people were affected by vector borne diseases resulting in economic loss of life [2]. There have been several cases of infections cause by resistant bacteria or fungi in many immunocompromised patients. Microbes have been the source of natural products since time immemorial and yielded several novel compounds. Approximately 32,500/- natural products reported are from microbial sources [3]. Half of the compounds in the market are mainly contributed by *Actinomycetales* [4].

Actinomycetes are filamentous Gram positive bacteria with high G+C content. They are ubiquitous in nature. They are known to synthesize a diverse group of promising compounds as secondary metabolites which are currently being used for the treatment of various diseases [5-6]. Nearly 80% of the currently available antibiotics are from *Streptomyces* [7]. Besides antibiotics many novel compounds such as immunosuppressive agents, larvicidal and enzyme inhibitors were also discovered from marine *Streptomyces*. Ivermectin and abamectin produced by *Streptomyces avermectinius* was reported with antihelmintic activity [8]. Recent findings have suggested that *Streptomyces* are potent sources of insecticidal agents. *Streptomyces spinosa* was reported for insecticidal activity [9]. Isolated compounds like (2S,5R,6R)-2-hydroxy-3,5,6-trimethyloctan-4- and 5-(2,4-dimethylbenzyl) pyrrolidin-2-one from *Streptomyces* sp.VITDDK3 [10] and *Streptomyces* sp.VITSVK5 were reported for larvicidal activity [11].

Manipur, being a part of Indo-Burma hotspot biodiversity, not much been exploited for actinomycetes diversity. Reports suggests that the North East region of India including Manipur is still a virgin area and veritable treasure trove for isolation and screening of novel actinomycetes for antibiotics and other novel bioactive compounds [12-13]. *Streptomyces sindenensis* (LS1-128) and *Streptomyces tanashiensis* (A2D) were reported from Loktak Lake (freshwater lake) habitat, Manipur, India [14-15]. Isolation of novel species *Streptomyces manipurensis* has been reported from limestone deposits of Manipur [16]. The present study was undertaken to screen the biological activity namely antimicrobial and larvicidal activity of *Streptomyces* sp.VITPK9 isolated from a brine spring habitat of Manipur, India.

MATERIALS AND METHODS

2.1 Sampling and isolation

The spring sediments were collected from Ningel, brine spring located in Thoubal District of Manipur, India. It lies between 23° 45' N and 24°45' N latitude and 93°45' E and 94°15' E longitude. Samples (100-150gm) were collected in sterile aluminium sheets (Pochon and Tardiux) and transported immediately to the laboratory where it was dried and used for the isolation of actinomycetes. The isolation process was carried out on Starch Casein Nitrate Agar (SCNA) and Actinomycetes Isolation Agar (AIA) with or without antibiotics with pH 7.2 and the plates was incubated at 27°C for a month [17]. The colonies recognised were stored in ISP4 slants at 4°C and also 20% (v/v) glycerol stock at -80°C.

2.2 Taxonomical characterization of the strain

The morphological studies were carried out according to International Streptomyces Project (ISP) method [18]. Biochemical tests and physiological characterizations including different range of pH, temperature and NaCl concentrations were carried out. 16S rDNA sequencing was carried out as reported earlier [19]. The 16S rDNA nucleotide sequence was also submitted to GenBank, NCBI, USA. The related strains were selected for alignment by software tool CLUSTAL W program available online http://www.genome.jp/tools/clustalw/.

2.3 Fermentation and extraction

The isolate VITPK9 was grown in submerged culture of 250 ml flasks containing 100ml of ISP1. An agar block from fully grown isolate was used to inoculate the flask aseptically. The culture was grown for seven days at 150 rpm and 27°C in rotary shaker [19]. The extracellular metabolite was collected by centrifuging the broth at 4000 rpm for 15 minutes and sterilized by filtration. Then the supernatant was mixed with equal amount of ethyl acetate (1:1 v/v ratio) and incubated for overnight in rotary shaker. The organic crude (EA) extract was extracted using vacuum rotary evaporator and kept for drying at room temperature. A thick brown gummy substance was collected and screened for bioactivity.

2.4 Antimicrobial activity

The EA extract was screened for antimicrobial activity against selected bacterial and fungal pathogens by Kirby-Bauer method [20]. The tested pathogens were *Bacillus subtilis, Pseudomonas aeruginosa, Candida albicans, Candida tropicalis, Aspergillus niger* and *Aspergillus flavus.*

2.5 Larvicidal activity

Different concentrations of the EA extract were tested to study the efficacy against the selected larvae. The tested larvae were *A. subpictus, C. tritaeniorhynchus, C. gelidus, R. microplus and H. bispinosa*. The larvicidal potential was determined by the number of dead larvae after the exposure to different concentration of the extract for 24hrs [11].

2.6 Statistical Analysis

All the experiments were performed in triplicate and represented as mean±standard deviation (S.D). The average larvae mortality were analysed by calculating the LC_{50} value and other statistical values were calculated using the developed software of Reddy [21]. Results were considered significant if $P<0.05$.

RESULTS AND DISCUSSION

3.1 Taxonomical identification:

The growth of the isolate in the culture media and the surface morphology was observed under scanning electron microscope as shown in Figure 1 A and B respectively. The isolate produced a white powdery colony and spore surface was observed as smooth in nature.

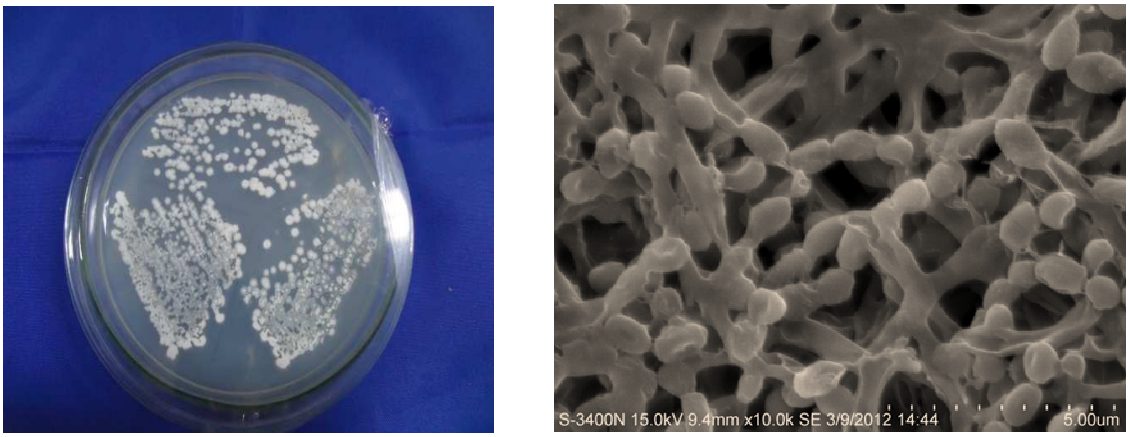

Figure 1. A) Growth of *Streptomyces* sp.VITPK9 in AIA medium B) SEM image of *Streptomyces* sp.VITPK9

The morphological and biochemical results of isolate are given in Table 1. The culturing conditions were optimized with respect to culture media, pH, temperature and NaCl concentration as in Figure 2. Starch or glycerol was found to be the best carbon source and potassium nitrate as the nitrogen-source.

Table 1: Characteristics of *Streptomyces* sp. VITPK9.

Media	VITPK9	Biochemical	VITPK9
AIA	Good growth	Catalase	+
ISP 1	Good growth	Oxidase	+
ISP 2	Good growth	Indole	-
ISP 3	Poor growth	Methyl Red	-
ISP 4	Good growth	Voges proskauer	-
ISP 5	Good growth	Starch hydrolysis	+
ISP 6	No melanin	Gelatin liquefaction	+
ISP 7	Good growth	Citrate utilization	+

ISP = International Streptomyces Project, (+) = Positive, (-) = negative.

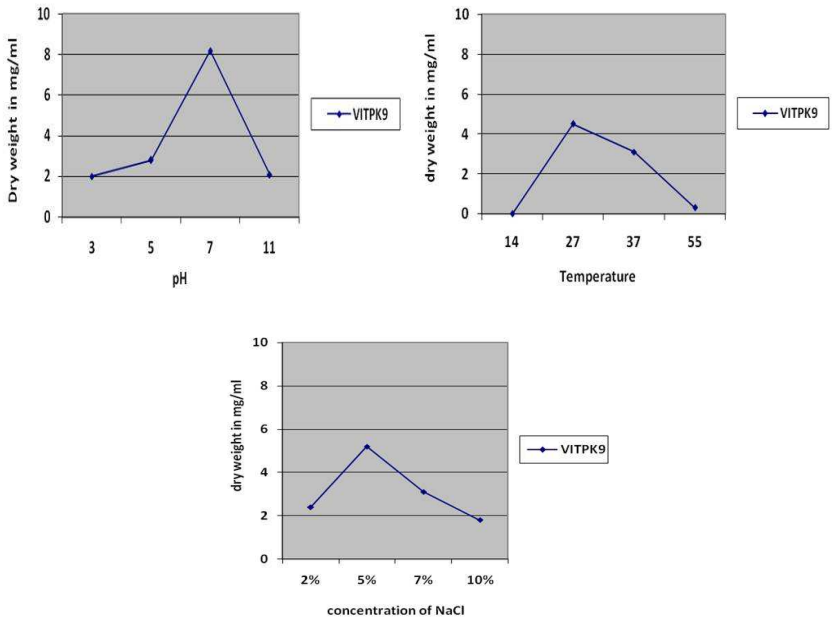

Figure 2: Optimization of culturing conditions of *Streptomyces* sp.VITPK9 with different pH, temperature and NaCl concentration

The 16S rRNA partial gene sequence analysis yielded 16S rDNA nucleotide sequence containing 1300 bases. The blast search of 16S rDNA sequence with sequences available in the GenBank (NCBI) database revealed that the isolate showed 97% similarity with *Streptomyces pseudogriseolus* (X80827). Based on molecular taxonomy and phylogeny the isolate was identified as *Streptomyces* and designated as *Streptomyces* sp.VITPK9. The 16S rDNA nucleotide sequence was submitted to the GenBank under the accession number JN689333 as reported [22]. The phylogenetic tree of the *Streptomyces* sp.VITPK9 is shown in Figure 3.

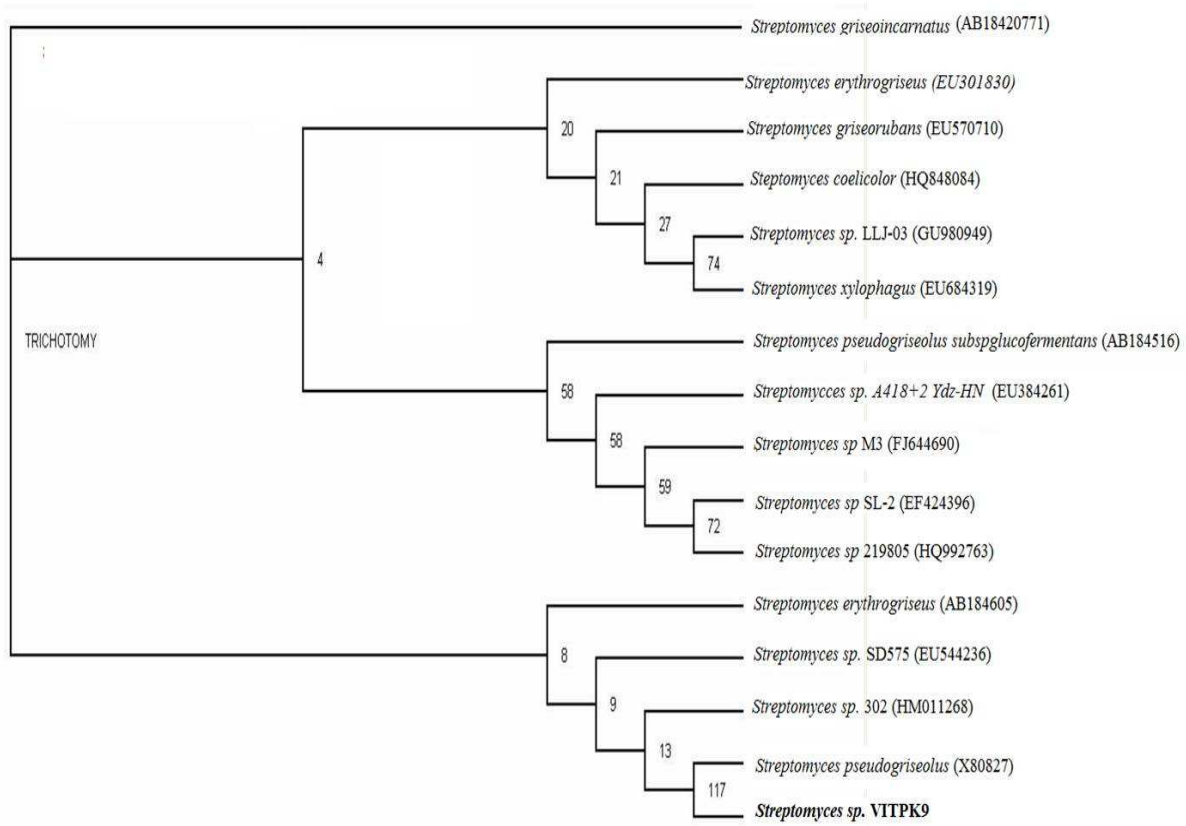

Figure 3: Phylogenetic analysis of *Streptomyces* sp.VITPK9 showed using neighbour-joining tree method using tree view software version. Bootstrap values are represented at the nodes of the tree

.3.2 Antimicrobial screening

The EA extract collected from submerged fermentation were screened for antimicrobial activity. The EA extract was suspended in DMSO at a concentration of 2mg/ml. The standard antibiotics used were chloramphenicol (1mg/ml) against bacterial pathogens and amphotericin B (1mg/ml) against fungal pathogens. The suspended EA extract was loaded according to Kirby-Bauer method. The EA extract showed higher antimicrobial activity against fungal pathogens *C. albicans* (24mm) and (17 mm) against *P. aeruginosa*. The antimicrobial activity of EA extract is given in Table 2.

Table 2: Antimicrobial activity of VITPK9 against selected fungal and bacterial

Pathogens	*Streptomyces* sp.VITPK9 Zone of Inhibition (mm)	Standard antibiotics Zone of Inhibition (mm)
Fungal pathogens		
C. albicans	24 ± 1.0	18
C. tropicalis	18.57±0.67	13
A. niger	12.97±0.06	12
A. flavus	12.37± 0.07	13
Bacterial pathogens		
P. aeruginosa	17±0.17	18
B. subtilis	10.83±0.21	22

Values are mean ±S.D (n=3).

3.3 Larvicidal activity

Different concentration of the EA extract was taken ranging from 62.5-1000 ppm. The larvicidal activity was evaluated by counting the mortality of the larvae. The activity was found to be concentration dependent. Higher the concentrations of the EA extract, higher the rate of mortality of larvae. The EA extract showed a maximum mortality against *Culex gelidus* with the LC_{50} value of 151.29 ppm among the selected larvae in the Table 3.

Natural drugs have been the propitious sources of leads to meet the urgent requirement of new drugs, especially antibiotics and other biopharmaceutical compounds [23]. As stated earlier in 1999, the chemical novelty associated with the natural products is higher than that in synthetic chemistry [24]. The decline in the discovery of new drugs in pharmaceutical companies and rise of new pathogens or insects are threat to humans [25]. The development of new analytical and preparative techniques helps to study the diverse classes of compounds produced by microorganisms.

Various screening approaches and compound profiling techniques are being developed to improve and ease the discovery of novel compounds [26]. Rise of resistant pathogens and insects are the growing menace to the world that dictates the intensive need for the discovery of novel leads.

Table 3: Larvicidal activity of ethyl acetate extract of *Streptomyces* sp.VITPK9 against larvae

Species	Concentrations (ppm)	% mortality[a] (ppm) ± SD	LC_{50} (LCL–UCL) (ppm)	Slope	r^2
A. subpictus	1000	54			
	500	39			
	250	19	831.78 (580.7-1189.7)	10	0.930
	125	10			
	62.5	2			
C. tritaeniorhynchus	1000	68			
	500	50			
	250	34	489.21(384.4-622.5)	19	0.923
	125	19			
	62.5	9			
C. gelidus	1000	100			
	500	87			
	250	66	151.29 (124.1-184.3)	42	0.831
	125	42			
	62.5	27			
R. microplus	1000	76			
	500	59			
	250	39	369.21(303.5-449.0)	21	0.917
	125	21			
	62.5	14			
H. bispinosa	1000	75			
	500	51			
	250	37	424.55 (346.5-520.3)	22	0.943
	125	22			
	62.5	10			

There are many historical examples in which natural products serves as the best medicine. It was Selman Waksman (1941), who discovered the unrivalled capacity of actinomycetes to produce exploitable natural products [27]. Among the actinomycetes, *Streptomyces* contributes the largest class to produce high number of antibiotics known till date. Starting from actinomycin, streptomycin, rapamycin, frigocyclinone are produced by *Streptomyces* sp. [28]. Besides antibiotics, many other novel compounds which are of biopharmaceutical interest have also been identified. For instance, *Streptomyces spinosa* was reported for insecticidal activity [9]. Recent reports of *Streptomyces* sp. VITSTK7 proofs that the ethyl acetate extract could be promising source for using as an eco-friendly approach to control ticks and mosquitoes [29].

In our present study, *Streptomyces* sp.VITPK9 was isolated from unexplored habitat of Manipur and its biological activity was investigated. The isolate produced white aerial and substrate mycelium on Starch Casein Nitrate Agar and Kuster's Agar characteristic of *Streptomyces* species. It also grows well on AIA, ISP 4, ISP 5, ISP 6, ISP 7 ISP 9 and Kuster's agar media. The isolate fail to produce any diffusible pigments. It utilises all sources of carbon like D-glucose, D-fructose, D-sucrose, mannitol, starch and glycerol. The isolate have the ability to degrade starch, casein and urea. To our knowledge, it is the first report that *Streptomyces* species isolated from brine spring of Manipur produced both antimicrobials and larvicidal activity.

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