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Studies on Isolation, Classification and Phylogenetic Characterization of Novel Antifungal *Streptomyces* sp. VITSTK7 in India

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Abstract: The aim of the study was to screen the antifungal activity of the crude extract prepared from the strain *Streptomyces* spp. VITSTK7 against *Aspergillus* sp. and to characterize the isolate. A total of 8 strains were isolated from the marine sediments collected at the Puducherry coast, India. All the eight strains were primarily screened for antifungal activity against three species of *Aspergillus* namely *A. fumigatus*, *A. niger* and *A. flavus*. Our search resulted in the isolation of a potential strain VITSTK7. The production media was optimized for maximum yield of secondary metabolites. The metabolites were extracted using ethyl acetate, lyophilized and screened for antifungal activity against the three *Aspergillus* species by well diffusion method. A maximum zone of inhibition (21 mm) was observed for *A. fumigatus* in comparison with the standard antifungal antibiotic Nystatin (20 mm). This potential strain was further identified based on Hideo Nonomura classification. A phylogenetic tree was constructed by maximum parsimony method to identify up to the species level. Molecular taxonomy and phylogeny revealed that the strain belonged to the genus *Streptomyces* and was designated as *Streptomyces* spp.VITSTK7. Blast search of the 16s rRNA sequence of the strain with the sequences available in the NCBI data bank showed a maximum similarity of 86% with *Streptomyces longisporoflavus* (DQ 442520) with the bootstrap value of 100. The 16s rRNA sequence of the strain *Streptomyces* spp.VITSTK7 was submitted to the GenBank under the accession number GQ 499369. The secondary structure of 16s rRNA and the restriction sites were also predicted using Genebee and NeBCutter online softwares, respectively.

Key words: *Actinomycetes*, antifungal activity, *Aspergillus fumigatus, Streptomyces* spp. VITSTK7

INTRODUCTION

Among the different types of drug prevailing in the market, antifungal antibiotics are very small but significant group of drugs and have an important role in the control of mycotic diseases. The need for new, safe and more effective antifungal is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immune compromised host (Gupte *et al*., 2002). The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (Bevan *et al*., 1995). This involves the screening of microorganisms and plant extracts, using a variety of models (Shadomy, 1987).

The importance of marine sources for the discovery of novel natural products with a pharmaceutical potential has been proved during the last decade and was highlighted in various excellent review articles (Faulkner, 2000; Haefner, 2003; Blunt *et al*., 2003). Bacteria within the order Actinomycetales (actinomycetes) are common soil inhabitants with an unprecedented ability to produce clinically useful antibiotics (William and Paul, 2006). Most of the microbial antibiotics discovered so far are originated from actinomycete bacteria, only a few of them from soil-derived genera (*Streptomyces* and *Micromonospora*). Actinomycetes produce a wide range of secondary metabolites and more than 70% of the naturally derived antibiotics that are currently in clinical use are derived from marine actinomycetes (Pimentel-Elardo *et al*., 2009). Among the 140 described actinomycete genera, only a few are responsible for the majority of over 20,000 microbial natural products identified so far. In particular, the genus *Streptomyces* accounts for about 80% of the actinomycete natural products reported to date (Bull and Stach, 2007).

In the course of screening of marine actinomycetes for antifungal activity, ethyl acetate extract prepared from the cell free supernatant of the isolate *Streptomyces* spp. VITSTK7 produced antifungal activity against *A. fumigatus* species. In the present study, the authors report the isolation, characterization, and the antifungal activity of the crude extract prepared from the strain *Streptomyces* spp. VITSTK7 against *Aspergillus* species.

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MATERIALS AND METHODS

Isolation of marine actinomycetes: Marine sediment samples were collected at the depth of 600-900 cm from the Bay of Bengal coast of Puducherry, India. The sample was processed further for the isolation and characterization was carried out in Biomolecules research laboratory at VIT University. The samples were dried to minimize the bacterial contaminants. 1 g of soil sample was then serially diluted up to 10^{-6} dilution level. 0.1 mL of the diluted suspension was spread over the surface of starch casein agar medium prepared in 50% sea water to enhance the isolation of actinomycetes (Kuester and Williams, 1964). After 7 days incubation at room temperature white powdery colonies of actinomycetes formed were isolated and sub-cultured on ISP 1 media (Rabah *et al*., 2008).

Extraction of secondary metabolite: Kuster's agar was used as a production media for the extraction of crude secondary metabolite. The isolated strain VITSTK7 was inoculated in Kuster's broth and incubated for 7 days. It was centrifuged for 15 min at 10,000 rpm and the supernatant collected was mixed with an equal volume of ethyl acetate and kept for overnight in rotary shaker (100 rpm). The crude extract obtained was dissolved in water and lyophilized (Augustine *et al*., 2005).

Assay for antimicrobial activity: The lyophilized powder was dissolved in DMSO and used for testing anti-*Aspergillus* activity against *Aspergillus fumigatus, Aspergillus flavus and Aspergillus niger* by agar well diffusion method (CLSI M38-A). Standard antibiotic Nystatin (25 µg/disc) was used as a positive control (Kumar and Kannabiran, 2010).

Classification: The potential strain VITSTK7 was subjected to Nonomura classification (Nonomura, 1974) based on aerial mass color, production of melanoid pigment, reverse side pigment, soluble pigment, spore chain morphology, spore surface and carbon utilization test. The color of the mature sporulating aerial mycelium grown on Oatmeal agar medium (ISP3) was recorded as described earlier (Prauser, 1964). Melanoid pigment production was assessed by culturing the strain on ISP 6 and ISP 7 medium (Shirinling and Gottlib, 1966). Production of the reverse side pigments and soluble pigments were assayed on culturing ISP 7 medium. The spore chain morphology and spore surface area were analyzed by direct microscopic examination of the culture area by cover slip culture technique. Utilization of carbon sources arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose were analysed for classification. These carbon sources were separately supplemented (1%) in each ISP 1 medium.

Molecular characterization: The genomic DNA of actinomycetes strain VITSTK7 was isolated using HiPurA bacterial DNA isolation and purification kit (Himedia, India). It was amplified using PCR master mix kit, Medoxmix (Medox, India) by following the procedure given in the user manual (Rainey *et al*., 1996). Universal 16S rRNA primers were used (Forward primer FC 27 and reverse primer RC 1492). The methodology for sequencing was adapted from earlier reports (Mincer *et al*., 2002; Magarvey *et al*., 2004) and the 16S rRNA was sequenced bidirectionally.

Construction of Phylogenetic tree: The 16S rRNA partial gene sequence obtained from the isolate VITSTK7 was compared with other bacterial sequences by using NCBI BLAST search (Altschul *et al*., 1990; Altschul *et al*., 1997) for their pair wise identities. Multiple alignments of this sequence with the sequences available in the data bank were carried out by Clustal W 1.83 version of DDBJ (http://clustalw.ddbj.nig.ac.jp/top-e.html) and the phylogenetic tree were constructed in MEGA 4.0 version (http://www.megasoftware.net) using the neighbor-joining (NJ) method with 100 replicates as bootstrap value and NJ belongs to the distance-matrix method (Joseph *et al*., 2009). The nucleotide sequences were analyzed for estimating the number of nucleotide substitutions between sequences by Tamura-Nei method (Tamura *et al*., 2007). The 16S rDNA sequence was submitted to the GenBank, EMBL (Europe), and the DNA Data Bank (Japan) under the accession number GQ499369.

Secondary structure prediction and Restriction site analysis: The RNA secondary structure of the isolate VITSTK7 was predicted using Genebee online software (http://www.genebee.msu.su/services/rna2_reduced.html) by greedy method and the restriction sites of the DNA of the strain was analyzed by NEB cutter Version 2.0 (http://tools.neb.com/NEBcutter2/).

RESULTS AND DISCUSSION

Assay for antimicrobial activity: Out of eight isolates, VITSTK7 alone showed significant anti-*Aspergillus* activity. The crude compound was extracted using Kuster's agar. Earlier studies antifungal compounds were extracted using Oatmeal Broth (ISP 3) (Joseph *et al*., 2009), YMD (Yeast malt dextrose) broth (Kavitha and Vijayalakshmi 2007) and Starch casein broth (Dhanasekaran *et al*., 2007). But we have optimized the media for antifungal producing compound using the Kuster's Broth. It produced highest zone of inhibition against *A. fumigatus* (21 mm) (Fig. 1) when compared to *A. niger* (12 mm) and *A. flavus* (16 mm). The standard antifungal antibiotic, Nystatin (25 µg/disc) produced an

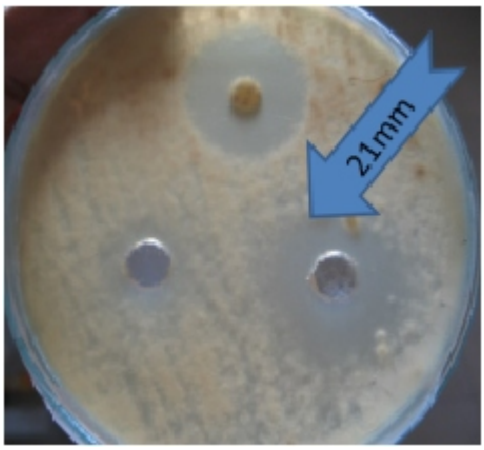

Fig. 1: Antifungal activity of the lyophilized crude (200 µL/well) of *Streptomyces* spp. VITSTK7 against *A. fumigatus.* The zone of inhibition was indicated by arrow and compared with the standard antibiotic Nystatin (25 µg/disc)

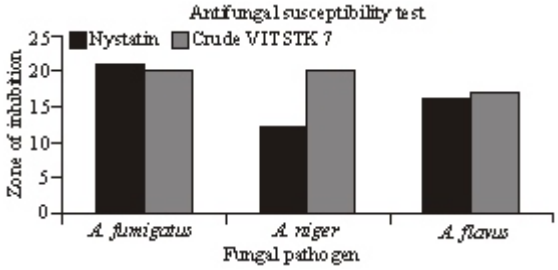

Fig. 2: The zone of inhibition (mm) by the Crude (200 µL/well) of *Streptomyces* spp. VITSTK7 against *A. fumigatus*, *A. niger* and *Aspergillus flavus*. The activity was compared with standard antibiotics Nystatin (25 µg/disc)

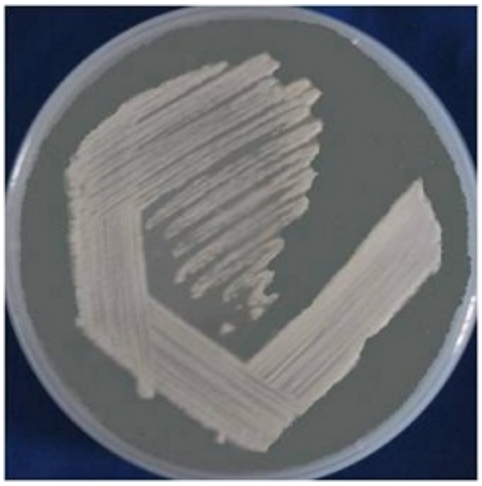

Fig. 3: Aerial mass colour of the Strain *Streptomyces* sp.VITSTK7 was found that white in colour on ISP 3 medium

Fig. 4: Reddish orange reverse side pigment showing on ISP 7 medium

inhibition zone of 20 mm against *A. fumigatus* and *A. niger* and 17 mm against *A. flavus* (Fig. 2). Several marine actinomycetes were reported to possess anti-*Aspergillus* activity, *Streptomyces* sp. PM-32 (Manivasagan *et al.*, 2009) *Streptomyces* sp., *Actinopolyspora* sp. and *Nocardia* sp. (Asha devi *et al*., 2006) and bioactive compounds extracted from actionomycetes also shown to have anti-*Aspergillus* activity, they are 4' phenyl-1-naphthyl-phenl acetamide (Dhanasekaran *et al*., 2007)and Macrolide antibiotic WA 52 (Mohamed *et al*., 2009). Michael *et al.* (1992) and Gomes *et al.* (2000) were isolated chitinolytic actinomycetes from marine origin and found it has antifungal activity.

Classification: The potential isolate VITSTK7 was characterized as per the key for classification and identification of 458 species of *Streptomyces* included in ISP (Nonomura, 1974). The mature sporulating aerial mycelium colour was found to be white (w) in colour (Fig. 3). There was no melanoid production when cultured in ISP 6 and ISP 7 medium. The strain VITSTK7 has the ability to produce a reddish orange color reverse side pigment when cultured in the ISP 7 medium (Fig. 4). The presence of the reverse side pigment is mentioned as "1".

The spore chain morphology was examined under microscopy and it showed Retinaculiaperti (RA) type and due to this it produced open loop and the spore surface was smooth (sm) in nature. It utilized arabinose, xylose, inositol, fructose, sucrose and raffinose as carbon sources. Based on these characteristics the isolate was compared with the other organisms listed in the classification of Nonomura. Most similar organisms *S. pupurogeniscleroticus* (Qian-Cutrone *et al.*, 1999) *S. spiroverticilliatus* (Wenli *et al.*, 2008) and *S. rosciscleroticus* characteristics were compared and tabulated in Table 1.

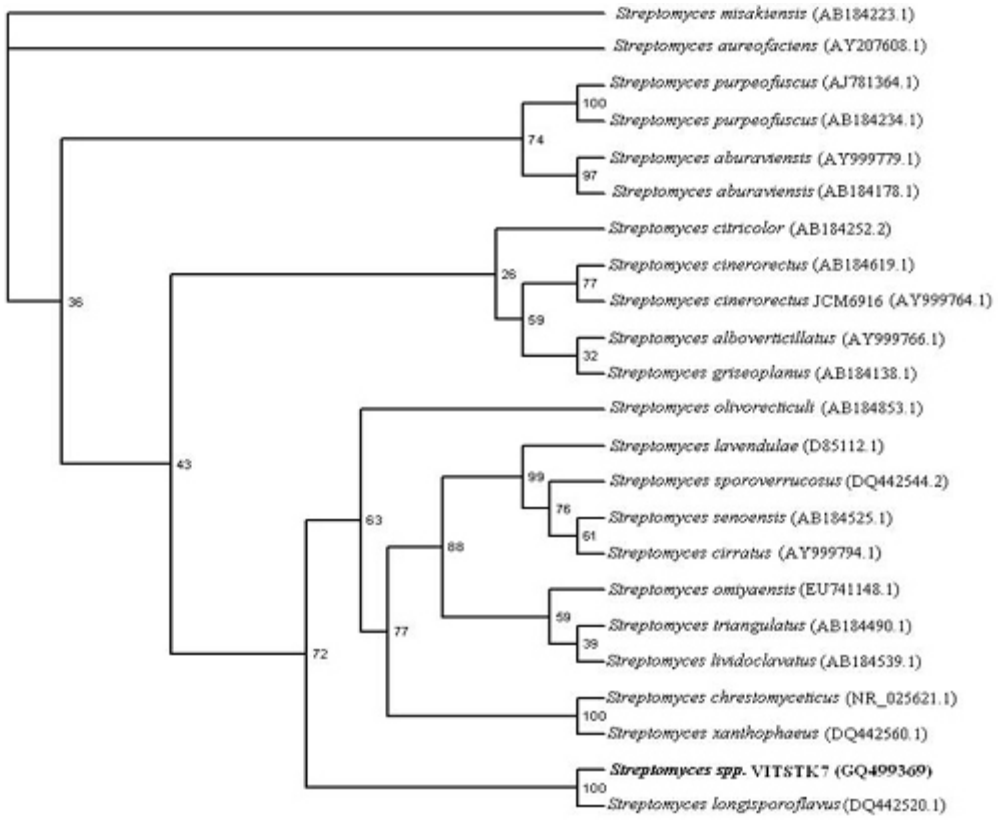

Fig. 5: Phylogenetic tree

Table 1: Nonomura classification of VITSTK7

Characteristics	VITSTK7	*S. purpurogeniscleroticus*	*S. spiroverticillatus*	*S. rosciscleroticus*
Aerial mass colour	W	W	WR	W
Melanoid pigment	0	0	0	0
Reverse side pigment	1	1	1	1
Soluble pigment	0	1	0	0
Spore chain	RA	S	RA	S
Spore surface	sm	sm	sm	Sm
Arabinose	+	+	+	+
Xylose	+	+	+	+
Inositol	+	+	-	±
Mannitol	-	+	-	+
Fructose	+	+	+	+
Rhamnose	-	+	-	+
Sucrose	+	+	±	±
Raffinose	+	+	-	±

By using Nonomura classification, characteristics of the strain VITSTK7 were compared with other sp. of *Streptomyces* which has most similar with that strain. Note: W-white, wr-White and red, 0-absence, 1-presence, RA- Retinaculiaperti, s-Spirales, sm-smooth, +-Utilization of that specific carbon source, --Fails to utilize that specific carbon source and ±-Partial utilization of carbon source.

Phylogenetic tree: The 16 S rDNA sequencing analysis of the isolate yielded 1459 base pairs and NCBI BLAST search analysis showed that the sequence was 86% similar to the sequence of *Streptomyces longisporoflavus* strain NRRL ISP-5165T. The 16S rDNA sequence was submitted to the GenBank, EMBL (Europe), and the DNA Data Bank (Japan) under the accession number GQ499369. A neighbour-joining tree based on 16 S rDNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomyces* family. A phylogenetic tree based on Maximum-parsimony method also showed distinct position (Fig. 5).

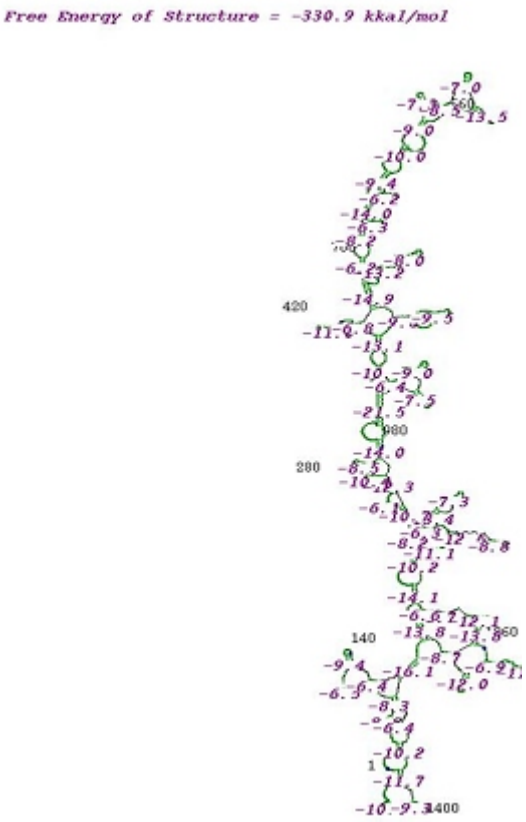

Fig. 6: Secondary structure prediction of 16s rRNA of the strain *Streptomyces* sp. VITSTK7 was done using Genebee online software

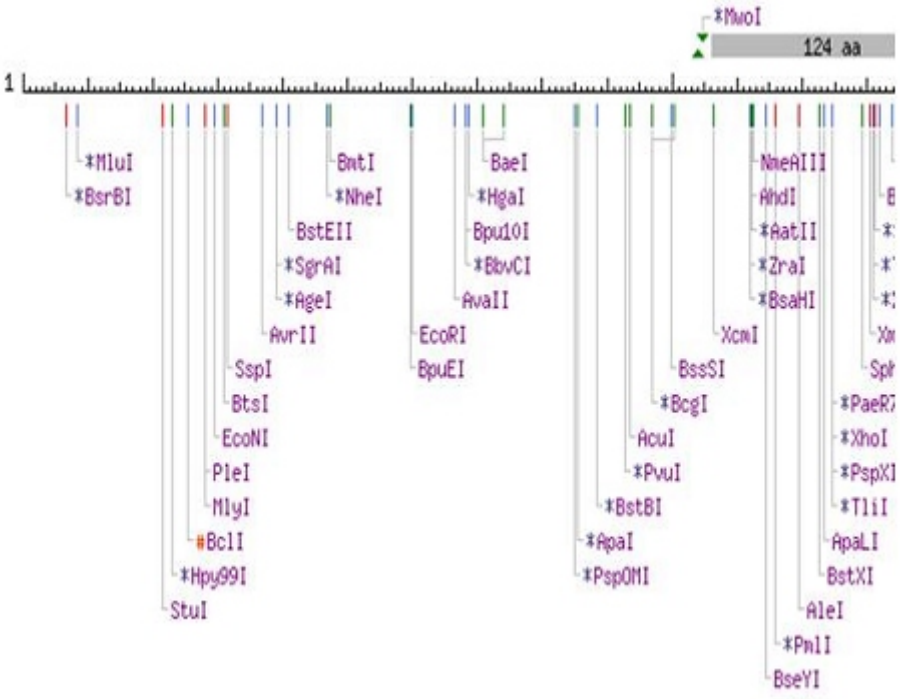

Fig. 7: Restriction sites of the Strain *Streptomyces* sp. VITSTK7 were predicted using NEB cutter

Secondary structure prediction and Restriction site analysis: The RNA secondary structure was predicted for 16s r RNA of *Streptomyces* sp. VITSTK7 (Fig. 6). It showed that the free energy of structure is -330.9 kkal/mol, threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4 and conservativity is 0.8.

The prediction of restriction sites of the strain VITSTK7 showed the restriction sites for various enzymes such as BsrB I, Age I, EcoR I and BssSI etc (Fig. 7). It shows GC and AT content of 56 and 44%, respectively

CONCLUSION

From this study we have concluded that Antifungal substances were produced by marine actinomycetes isolated from Puducherry coast of India and designated as *Streptomyces* sp. VITSTK7. In phylogenetic relation it shows 86% similarity with *Streptomyces longisporoflavus* with Bootstrap value of 100. This strain has produced more inhibition zone on *Aspergillus fumigatus* when compared with that of standard antibiotic Nystatin. So this could be a drug of choice for against fungi.

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