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Antimicrobial Activity of Marine Actinomycete, *Nocardiopsis* sp. VITSVK 5 (FJ973467)

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Abstract: The aim of the present study was to isolate and to indentify the actinomycetes having antagonistic activity. An actinomycetes strain isolated from marine sediment samples collected at the Puducherry coast of India, showed antibacterial activity against selected microbial pathogens. The nutritional requirements and cultural conditions for maximal growth and yield of secondary metabolites have been optimized under shake-flask conditions. The growth and yield of secondary metabolites was maximal with the use of ISP 1 medium supplemented with sea water, pH 7.4, and incubation temperature of 28° C, salt tolerance of 2% and incubation time of 4-7 days. Based on morphological, biochemical, physiological and phylogenetic characterization, the strain was identified as *Nocardiopsis* sp. VITSVK5 (FJ973467). The petroleum ether extract (1000 µg/ml) obtained from the isolate showed significant antibacterial activity against Gram negative bacteria- *Escherichia coli* (20 mm), *Pseudomonas aeruginosa* (18 mm) and *Klebsiella pneumonia* (15 mm) and Gram positive bacteria- *Enterococcus faecalis* (20 mm), *Bacillus cereus* (13 mm) and *Staphylococcus aureus* (6 mm) when compared with streptomycin (25 µg/disc). The ethyl acetate extract (1000 µg/ml) showed antifungal activity against *Aspergillus fumigatus* (23 mm), *Aspergillus flavus* (15 mm) and *Aspergillus niger* (12 mm) when compared with amphotericin-B (25 µg/disc). The chloroform extract (1000 µg/ml) was very effective against yeasts, *Candida cruzi* (18 mm), *Candida tropicans* (15 mm) and *Candida albicans* (14 mm) when compared to streptomycin (25 µg/disc). In conclusion the isolated strain has broad spectrum of antagonistic activity against Gram positive and Gram negative bacteria and *Aspergillus* sp.

Key words: Actinomycetes, antagonistic activity, antifungal activity, *Aspergillus* sp., *Nocardiopsis* sp. VITSVK5.

INTRODUCTION

Natural products are remains to be the most propitious source of antibiotics (Bull and Stach, 2007). There are approximately 32,500 natural products reported from microbial sources (Antibase data base) including about 1000 derived from marine microbes (Singh and Pelaez, 2008). Several antibiotics were derived from marine actinomycetes (Baltz, 2008) and at present, two-thirds of natural antibiotics are obtained from actinomycetes and it also serves as alternative source of biologically active substances (Behal, 2003). Marine-derived antibiotics are more efficient at fighting microbial infections because the terrestrial bacteria have not developed any resistance against them (Donia and Hamman, 2003). Infectious diseases are leading health problems with high morbidity and mortality in the developing countries (Black *et al.,* 1982). The development of resistance to multiple drugs is a major problem in the treatment of infectious diseases caused by pathogenic microorganisms. This multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistance pathogens. It is indisputable that new drugs, notably antibiotics, are urgently needed to halt and reverse the relentless spread of antibiotic resistant pathogens which cause life threatening infections and risk of undermining the viability of healthcare systems (Tolbot *et al.,* 2006).

Several reports are available on antibacterial and antifungal activity of marine actinomycetes (Suthindhiran and Kannabiran, 2009; Bredholt *et al.,* 2008). Antifungal secondary metabolites have been isolated from akalophylic *Nocardiopsis Dassonvillei* WA52 (Ali *et al.,* 2009), *Nocardia* sp. ALAA 2000 (El-Gendy *et al.,* 2008) and marine *Streptomyces* sp. DPTB16 (Dhanasekaran *et al.,* 2008); and the list of antifungal compounds available was reported by Molinski, 2004; Zang *et al.,* 2005). Of 9 maritime states in Indian peninsula only very few states have been extensively covered for the study of marine actinobacteria for antagonoistic properties against different pathogens (Sivakumar *et al.,* 2007). The Puducherry coast of Bay of Bengal, India was not been studied extensively with respect to antagonoistic properties of actinomycetes. In the course of our screening programme for new antigonistic actinomycetes, the strain, *Nocardiopsis* sp. VITSVK 5 (FJ973467) was isolated from Puducherry coast of Bay of Bengal, India capable of showing antagonoistic acivity against selected microbial pathogens. In the present study, we report the antimicrobial activity of *Nocardiopsis* sp. VITSVK 5 (FJ973467).

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MATERIALS AND METHODS

This study was carried out during December 2008 to June 2009 in the Biomecules research laboratory, School Biosciences and Technology, VIT University, Vellore, India.

Sample collection and isolation of actinomycetes: Marine sediment samples were obtained from different locations at the Pudduchery coast of India. From each location, 15 g of sample was collected at 50 to 100 cm depth from the surface. These samples were placed in small pre-labeled plastic bags and tightly sealed. It was pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37 ˚C for 4 days and subjected to serial dilution (up to 10^{-6} dilution) by adding 1 g of soil sample in 10 mL of distilled water. About 1.0ml of of diluted sample was plated on actinomycete isolation agar by pour plate technique and incubated at 28 ˚C for 7-10 days. After incubation the powdery colonies were subcultured on ISP 1 medium mixed with sea water/starch caesine agar supplemented with antibiotics, cycloheximide (25 µg/ml) and nalidixic acid (25 µg/ml) (Himedia, Mumbai, India).

Phenotypic characterization:

Aerial mass colour and reverse side pigments: The mature sporulating aerial mycelium colour was recorded in Oat meal agar (ISP 3), Yeast extract malt extract agar (ISP 2), Inorganic salt starch agar (ISP 4), Glycerol asparagine agar base (ISP 5), Tyrosine agar base (ISP 7), Starch casein agar and Czapek dox agar (Das *et al.,* 2008). The reverse side pigments of the colony, namely distinctive (+) and not distinctive (-) was tested using Peptone yeast extract iron agar (ISP 6) (Das *et al.,* 2008). Production of melanoid pigments was tested on ISP 1 and ISP 7 medium.

Spore chain and surface morphology: The spore bearing hyphae and spore chain was determined by direct examination of culture under microscope 1000x magnification by cover slip method using a well grown sporulated culture plate. The spore surface morphology of the mycelium was observed in 14 days old culture under scanning electron microscope (Das *et al.,* 2008).

Physiological and biochemical characterization: The ability of the isolate to utilize various carbon and nitrogen sources were studied by the method recommended in International Streptomyces project. Carbon sources like glucose, mannitol, fructose, xylose, sucrose, raffinose, inositol, arabinose and rhamnose were tested on Carbon utilization agar (ISP 9) supplemented with 1% carbon sources (Nonomura, 1974). The ability of the isolate to utilize various nitrogen sources like leucine, histidine, tryptophan, serine, glutamic acid, lysine, arginine, methionine and tyrosine for growth were also tested.

Sodium chloride tolerance and cultural conditions: Different concentrations of sodium chloride (0, 2, 5, 7 9 and 12%) was added to the starch casein medium and plated. The plates were incubated at 28 ˚C for 7 -14 days and salt tolerance was tested. The growth of the isolate on ISP 4 media incubated at different temperatures (4, 15, 25, 28, 37, 42 and 50˚C) and at different pH (5, 6, 7, 8, and 9) was tested to determine the optimal temperature and pH.

Molecular taxonomy, sequencing and phylogenetic analysis: The DNA was isolated by HiPurA bacterial DNA isolation and purification kit (Himedia, India) and amplified by PCR using a master mix kit, Medoxmix (Medox, India) as per user manual. The primers and the PCR conditions were adapted from Rainey *et al.* (1996). The primers and the methodology for the sequencing were adapted from Mincer *et al.* (2002); Magarvey *et al.* (2004). The sequencing was carried out in both the sense and antisense directions. The similarity and homology of the16S rRNA partial gene sequence was analyzed with the similar existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using BLAST search. The DNA sequences were aligned and phylogenetic tree was constructed by neighbor joining method using ClustalW software (Saitou and Nei, 1987). A bootstrap analysis of 1000 replicates was carried out. The secondary structure and the restriction sites in the 16s rDNA sequence of the isolate were predicted using the bioinformatics tools Genebee and NEBCutter (version 2.0) and bioinformatics tool available online www.genebee.msu.su/services/rna2_reduced.html.

Fermentation and preparation of crude extract: The selected antagonistic isolates was inoculated into ISP 1 broth, and incubated at 28ºC in a shaker (200-250 rpm) for seven days. After incubation the broths were centrifuged at 6000 rpm for 15 min and the cell free supernatant was filtered through Whatman No.1 filter paper. The filtrate was transferred aseptically into a conical flask and stored at 4ºC for further assay. To the culture filtrate, equal volume of solvents, ethyl acetate, chloroform and petroleum ether were added and centrifuged at 5000 rpm for 10 min. The crude extract was then concentrated in rotary vacuum and lyophilized using a freeze drier (Thermo, USA) for 5 hours at 5 ºC. The crude extract obtained from different solvents was tested for antimicrobial activity against selected pathogens.

Microbial pathogens: Bacterial pathogens, *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 10273), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (MTCC 430) and *Staphylococcus aureus* (ATCC 25923) were used. Fungal pathogens, *Aspergillus fumigatus* (ATCC No 46645), *Aspergillus flavus* and *Aspergillus niger* (ATCC No. 16404) were used. Yeasts, *Candida cruzi, Candida tropicans* and *Candida albicans* (ATCC 10231) were used.

Assay of antimicrobial activity: The antibacterial activity of secondary metabolites (25 µg/ml) extracted with different solvents was tested by agar diffusion assay.

The plates were incubated at 37ºC for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite when compared to controls. Antifungal activity of the crude extract was determined by using the standard method CLSI M38-A (formerly NCCLS). The fungal cultures were maintained in 0.2% dextrose medium and the optical density of 0.10 at 530 nm was adjusted using spectrophotometer. Each fungal inoculums were applied on plate and evenly spread on Sabouraud's Dextrose agar (HiMedia, India) using a sterile swab. Agar diffusion assay was followed to evaluate the antimicrobial activity along with amphotericin-B. The Petri plates were incubated at 30ºC for 2 days. At the end of the 48 h, inhibition zones formed in the medium were measured in millimeters (mm). All experiments were done in three replicates.

RESULTS

The sampling site Puducherry is located on the southeast coast of India [Latitude (N) 12º20` and Longitude (E) 79º95`]. A total of 25 strains were isolated from the marine sediment samples and designated as VITSVK1-VITSVK25 based on their colony morphology observed on the master plate. The isolate were small to medium sized, grayish white to pure white in colour, round, powdery, with regular margin and pigmented in golden yellow colour. Among the isolates one isolate (VITSVK5) which showed significant antimicrobial activity against selected bacterial and fungal pathogens were selected and characterized by polyphasic taxonomy.

The cultural and morphological characteristics of the isolate in different media are given in Table 1. The actinomycete isolate showed excellent growth and abundant aerial mycelium formation on Yeast extract agar (ISP1) supplemented with sea water and Starch casein agar. Good growth was seen in Actinomycete isolation agar, Tyrosin agar and Czapez's agar whereas only moderate growth was seen on yeast extract agar (ISP 2), Oat meal agar (ISP 3), Inorganic salt agar (ISP 4) and Glycerol aspergine agar (ISP 5). Yeast extract agar (ISP1) supplemented with sea water was used as the optimal media for the culture of the isolate.

The isolated strain is a Gram positive, non-motile actinomycete. The aerial mycelium is unbranched, white in color with sparse substrate mycelium with brownish orange reverse pigment and the aerial and substrate mycelium are medium dependent. In the optimized Yeast extract agar (ISP1) medium supplemented with sea water the isolate produces white spore mass. The spores are smooth, appeared as long chain and oblong in shape (Fig.1). The scanning electron microscopic study of the morphology of the spore-bearing aerial hyphae appears zigzag in nature (Fig. 2).

The physiological and biochemical characteristics of the isolate are given in Table 2.The physiological properties showed that no melanoid pigments were produced on of the media used, and the isolate used the following as carbon sources, glucose, mannitol, fructose,

Table 1: Cultural characteristics of *Nocardiopsis* sp. VITSVK5 (FJ973467) on different culture media

Media	Growth	Aerial mass colour	Pigmentation
Yeast extract malt extract agar (ISP 2)	Moderate	White	-
Oat meal agar (ISP 3)	Moderate	White	-
Inorganic salt agar (ISP 4)	Moderate	Gray	-
Glycerol-Aspergin agar (ISP 5)	Moderate	Gray	-
Tyrosin agar (ISP 7)	Good	White	Brownish orange
Starch casein agar	Abundant	White	Light yellow
Czapez's agar	Good	White	Golden yellow
Yeast extract agar (ISP 1) + sea water	Abundant	White	Brownish orange
Knight's agar	Good	White	Light yellow
Actinomycetes isolation agar	Good	White	Golden yellow (dark)

Table 2: Caharacteristics of *Nocardiopsis* sp. VITSVK5 (FJ973467)

Tests	
Grams stain	+
Aeril mycelium	White
Motility	Non motile
Colony color	White
Spores	-
Soluble pigment	-
Melanin	-
Starch hydrolysis	+
Carbon source (1% w/v)*	
D-glucose	+
Sucrose	+
D-galactose	+
Mannose	+
Maltose	+
Starch	+
L-Rhamnose	+
Mannitol	+
Inositol	+
Nitrogen source (1% w/v)*	
Glutamic acid	++
Leucine	++
Methionine	++
Histidine	++
Serine	+
Tryptophan	+
Effect of Temperature*	
15°C	-
28 °C	++
37 °C	+
45 °C	-
Effect of pH*	
5	-
6	+
7	++
8	++
9	-
Effect of NaCL concentration (w/v)*	
2%	++
5%	+
7%	+
9%	-
12%	-

*Growth of the strain was measured as dry weight of the mycelium.

xylose, sucrose, raffinose, inositol, arabinose and rhamnose for good growth. It exhibited good growth on various nitrogen sources like leucine, histidine, tryptophan, serine, glutamic acid, lysine, arginine, methionine and tyrosine. It was found to be negative for indole, VP, citrate, nitrate reduction and catalase. The isolate was found to be positive for urease test showing their ability to hydrolyse urea and positive for MR test. The isolate showed excellent growth and abundant aerial mycelium formation at pH 7-8, temperature 28˚C and less growth and aerial mycelium formation at lower and higher pH values, temperatures used on the Yeast extract agar (ISP1) supplemented with sea water. The isolate showed excellent growth and abundant aerial mycelium formation at 2% NaCl concentration and the growth was decreased at higher concentrations of Na Cl. The blast search of the 16S rDNA sequence (1447 base pairs) of the isolate showed maximum (98%) similarity with *Nocardiopsis* sp. E-143 (FJ 764792) and phylogenetic tree was constructed with bootstrap values (Fig. 3). Due to non availability of physiological, biochemical and cultural characterists of the closest phylogenetic neighbours, we are unable to compare the characteristic features. Based on the molecular taxonomy and phylogeny the strain was identified as *Nocardiopsis* sp. and designated as VITSVK5. The RNA secondary structure (Fig 4) of 16s rRNA gene of *Nocardiopsis* sp.VITSVK5 showed the free energy of the predicted structure is-360.6 kkal/mol. The nucleotide sequence of 16s rDNA of 16 S rRNA gene partial sequence was deposited in the GenBank under the accession number (FJ 973467).

The isolate exhibited a marked antagonistic activity against all the bacterial and fungal pathogens (Table 3). The petroleum ether extract (1000 µg/ml) obtained from the isolate showed significant antimicrobial activity against selected Gram negative bacterial pathogens, *E. coli* (20mm), *P. aeruginosa* (18mm), *K. pneumonia* (15mm); and Gram positive bacteria *E. faecalis* (20mm) and *B. cereus* (13mm) when compared with the standard,

Table 3: Antibacterial activity of the crude extract (cell free supernatant) of the isolate, *Nocardiopsis* sp. VITSVK5 (FJ973467)

Microbial pathogens	Zone of inhibition (mm)	
	VITSVK 5 (1000 µg/ml)	Antibiotics (25µg/disc)
Bacterial pathogens		Streptomycin
Gram negative bacteria		
Pseudomonas aeruginosa	18	14
Klebsiella pneumonia	15	18
Escherichia coli	20	14
Gram positive bacteria		
Enterococcus faecalis	20	21
Bacillus cereus	13	6
Staphylococcus aureus	6	21
Fungal pathogens		
Candida albicans	14	23
Candida cruzi	18	32
Candida tropicans	15	18
		Amphotericin-B
Aspergillus niger	12	9
Aspergillus flavus	15	-
Aspergillus fumigatus	23	12

Values are average of three experiments

Fig. 1: Phase-contrast micrograph of *Nocardiopsis* sp. VITSVK5 (FJ973467) showing young hyphae and terminal single spore on the substrate mycelium. The diameter of substrate mycelium is 0.4 to 0.7 µm. Bar 1 µm.

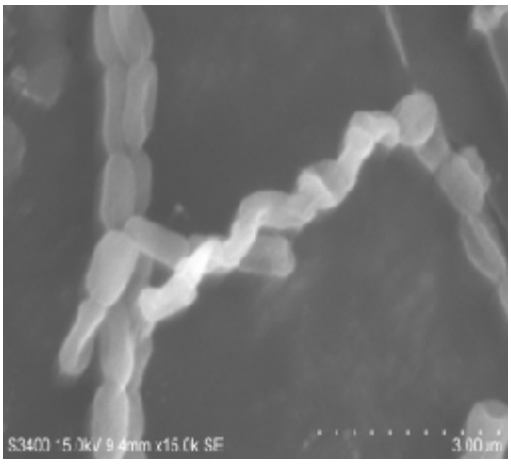

Fig. 2: Scanning electron micrograph of matured single spores of *Nocardiopsis* sp. VITSVK5 (FJ973467) 8 days of incubation in ISP 9 medium. The size of the spore is 0.6–0.9 µm. Bar 1 µm.

streptomycin (25 µg/disc). The antibacterial activity exhibited by the crude extract was equivalent to that of the activity of streptomycin. The ethyl acetate extract (1000 µg/ml) showed high antifungal activity against *A. fumigatus* (23mm), *A. flavus* (15mm) and *A. niger* (12 mm) when compared with the standard, amphotericin-B (25 µg/disc). The antifungal activity of the crude extract was higher than the activity of amphotericin-B. The chloroform extract (1000 µg/ml) was very effective against yeasts, *C. cruzi* (18 mm), *C. tropicans* (15 mm) and *C. albicans* (14 mm) when compared to streptomycin (25µg/disc). The anticandidal activity was less than the activity of streptomycin. The most susceptible Gram negative bacterial species is *E. coli* and Gram positive bacteria species is *E. faecalis*. *A. fumigatus* is more susceptible fungal pathogen when compared to other fungal pathogens studied. The antagonistic activity of the crude secondary metabolite extracted from the isolate exhibited comparable activity with that of stantard antibiotics.

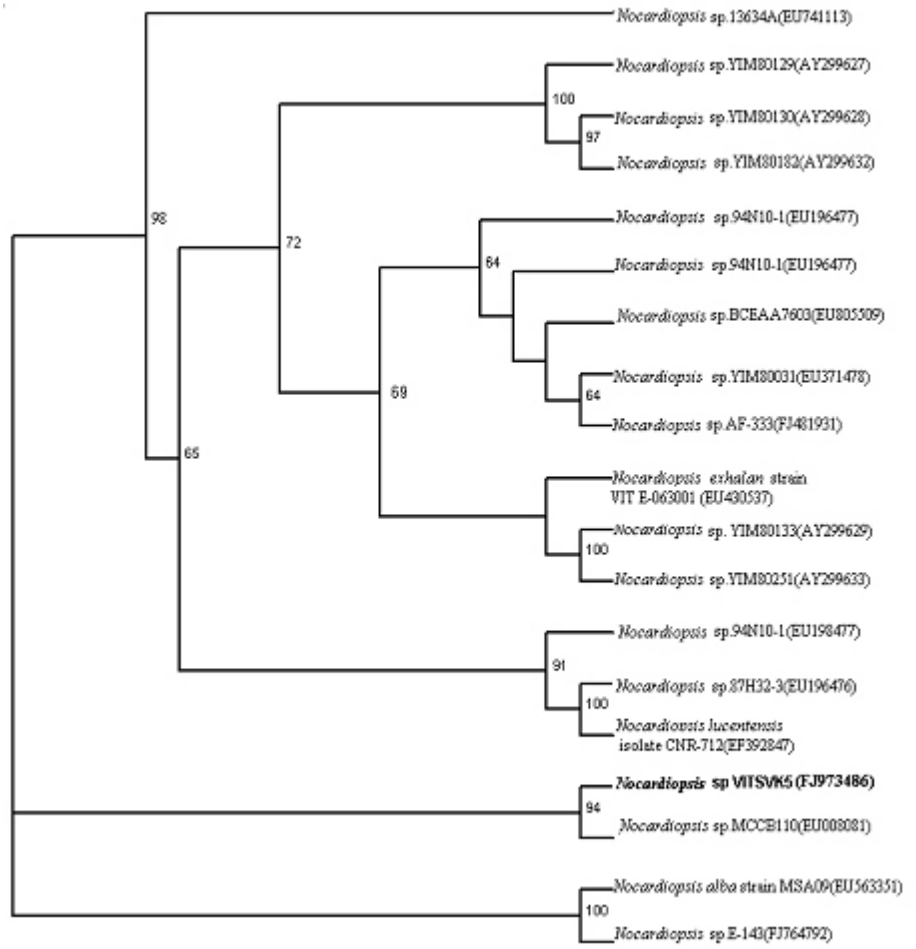

Fig. 3. Relationships between *Nocardiopsis* sp. VITSVK5 (FJ973467) and members of the genus *Micromonospora* on rooted neighbour-joining tree based on 16S rDNA sequences. The numbers at the nodes indicate the levels of bootstrap support based on the analyses of 1000 resampled data sets; only values over 50% are given. The scale bar indicates 0.01 substitutions per nucleotide position.

DISCUSSION

Screening of marine actinomycetes for antagonistic activity resulted in isolation and identification of the potential strain, *Nocardiopsis* sp. VITSVK5 (FJ973467). In the course of our screening for pharmacologically active agents from marine actinomycetes, we found that petroleum ether and ethyl acetate culture broth of *Nocardiopsis* sp. VITSVK5 (FJ973467) was found to be active against selected microbial pathogens. Marine bacteria are emerging as an exciting species for the discovery of new classes of therapeutics and it could provide the drugs needed to sustain us for the next 100 years in our battle against drug resistant infectious diseases (Williams, 2009). Marine organisms have produced enormous antibiotics of diverse chemical structures (Molinski, 2004). Actinomycetes account for >45% of all bioactive metabolites discovered in nature (Berdy, 2005).

The genus *Nocardiopsis* was described by Meyer (1976) and Nocardia belongs to the family Thermonosporaceae is a non-streptomycete group of actinomycete. This new genus was characterized according to its mode of sporulation, molecular genetic studies, numerical taxonomic and chemotaxonomic analysis (Grund and Kroppenstedt, 1990). *Nocardiopsis* genus is an aerobic actinomycete that includes several species (Rainey *et al.,* 1996). According to the key of McCarthy (1989) *Nocardiopsis* is easily differentiated from other stains, *N. africana, N. coeruleofusca, N. longispora, N. mutabilis, N.flava, N. dassonvilli* and *N. prasina.* Several antibiotics have already been isolated from *Nocardiopsis* species.A new pyranonaphthoquinone antibiotic, griseusin D was isolated from the cultural fluid of the alkaphilic *Nocardiopsis* sp. which exhibited weak antifungal activity against *Alternaria alternate* (Li *et al.,* 2007). From Indian marine sediment samples few potential bioactive *Nocardiapsis* have been reported. A protease-producing, crude oil degrading marine *Nocardiopsis* sp.NCIM 5124 have been reported (Dixit and Pant, 2000). A biosurfactant producing marine actinobacteria, *Nocardiopsis alba* MSA 10 have been

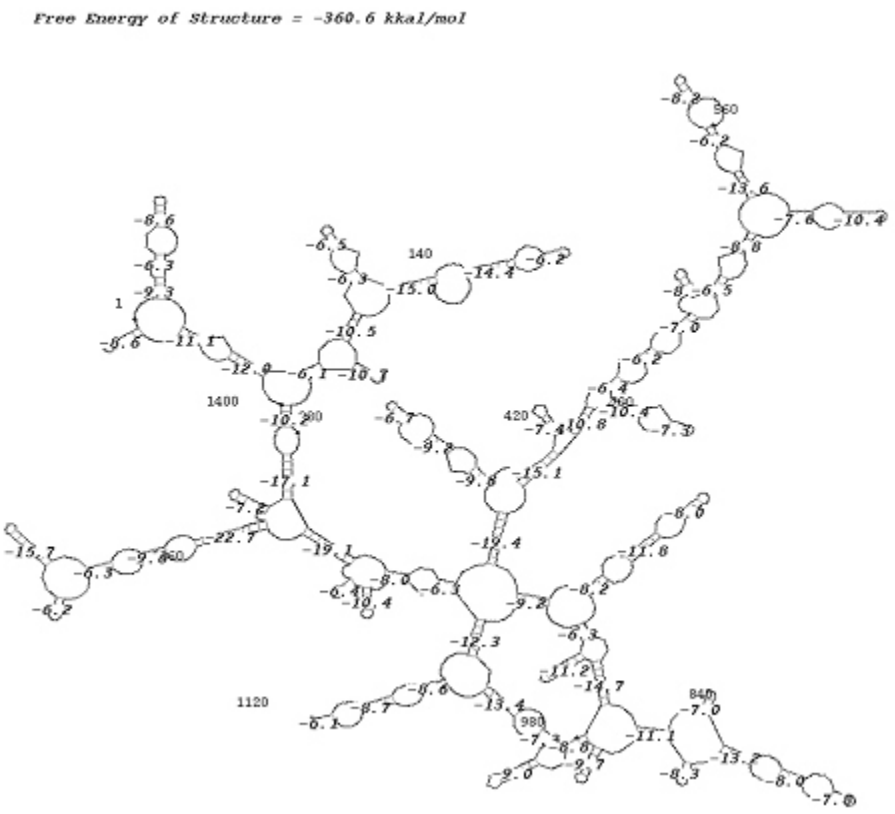

Fig. 4. Secondary structure prediction of 16s rRNA isolated from *Nocardiopsis* sp. VITSVK5 (FJ973467)

reported (Gandhimathi *et al*., 2009). The sponge-associated actinomycetes, *Nocardiopsis dassonvillei* MAD08 having 100% activity against multidrug resistant pathogens have been reported (Selvin *et al.,* 2009). They have isolated 11 antimicrobial compounds in *N. dassonvillei* MAD08 and also isolated an anticandidal protein of molecular weight 87.12 kDa and it has been reported that this is the first strain that produces both organic solvent and water soluble antimicrobial compounds. Isolation of *Nocardiopsis* sp have been reported in shore marine environment and mangrove ecosystem at 8 different locations of Kerala, West Coast of India (Remya and Vijayakumar, 2008). *Nocardipsis* sp. could be used as potential probiotic bacteria for shrimp aquaculture (Ninawe and Selvin, 2009).

The wide-spectrum of microbiosis exhibited by cell free extracts obtained with petroleum ether and ethyl acetate showed that *Nocardiopsis* sp. VITSVK5 (FJ973467) is antagonistic actinomycete and it supports the evidence that marine sediment sample is an important source of biologically active compounds. The compound(s) present within this extract showed activity against *E.coli*, *E. faecalis*, *C. cruzi* and *A. fumigatus* strains among others. The antagonistic secondary metabolite produced by this actinomycete needs to be studied further to identify its chemical nature and characterization of its biological activity.

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