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Research Article

ISOLATION OF SOIL ACTINOMYCETES INHABITING AMRITHI FOREST FOR THE POTENTIAL SOURCE OF BIOACTIVE COMPOUNDS

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ABSTRACT

An investigation was conducted to evaluate the potential actinomycetes producing bioactive compounds amended in Amirthi forest, Vellore, Tamil Nadu, India. In this present study 5 soil samples were collected from Amirthi forest range for the isolation of actinomycetes. About 31 strains of actinomycetes were isolated and selected based on their morphological features. The selected isolates were allowed to screen for the antagonistic activity by cross streak method. Out of which one showed potency and the crude extract was subjected for antibiogram at the concentration of 20mg/ml against clinical pathogens. The maximum activity was found against *Pseudomonas aeruginosa* (20mm).Simultaneously the crude extract was also evaluated for antioxidant activity. The maximum inhibition was found at 5.0mg/ml concentration with 90.7% . Considering the outcome of the present investigation with both antibacerial and antioxidant activity. Identification of the species was performed following molecular taxonomy by 16S rRNA sequencing and found to be *Streptomyces* sp VITNSJ2.This reports that the unique soil actinomycetes serve as the source to explore bioactive secondary metabolites from forest sources.

Keywords: Soil actinomycetes, antimicrobial activity, antioxidants, secondary metabolites.

INTRODUCTION

Natural products contribute to the discovery of novel bioactive metabolites[1]. Emerging pathogens are highly resistance to drugs and has become more problematic to public health. The discovery of novel antimicrobial compounds is currently the thirst area of research where it attempts to overcome the global resistance to pathogenic bacteria. Actinomycetes are gram-positive, saprophytic bacteria highly distributed in soil[2]. Primary and secondary metabolites produced by these organisms are highly potent, biologically active and remain a powerful source for pharmaceutical discovery [3, 4].Most actinomycetes produce a diverse array of antibiotics including amino glycosides, anthracyclines, peptides, polyenes. Due to pharmacological limitations and prevalence of antibiotic resistant pathogens the search of new antimicrobial drug from actinomycetes are raised. On the other hand, free radicals are known to be the major cause of various diseases. The main characteristic of an antioxidant is its ability to trap free radicals which oxidize nucleic acids, proteins, lipids or DNA [5].The antioxidant has a main property of trapping the free radicals which oxidize DNA; Protein and lipids. All the organisms are protected from the attack of free radical generation which is highly prevented by defense mechanism called antioxidant system. Amirthi forest is situated under the Javvadu/Javadi Hills of Tellai across Amirthi River in Vellore, TN, India. It contains a wide variety of flora and soil is highly rich in organic matter and suitable for the growth of microorganisms [6]. Very few reports are available on their microbial resources which are scarce, imprecise, and not well documented. Due to uniqueness, large geographic variation, different soil types and their contents of this forest, it is quite likely that there is vast distribution of antibiotic producing actinomycetes in this environment.

MATERIALS AND METHODS

Collection of soil sample

Three soil samples were collected at the depth of 5-10 cm in sterile plastic bags from different regions of Amirthi forest, Vellore, Tamil Nadu, India. All samples were transported to VIT University Laboratory, labeled and refrigerated for further investigation.

Isolation and culture condition

1g of the soil samples were weighed and dissolved in 10ml of distilled water and further serially diluted upto10^{-6} dilution,0.1 ml of each dilution were spreaded on starch casein agar uniformly and incubated for 7 days at room temperature [7].The emerging colonies of actinomycetes were subcultured and maintained for future use.

Cross streak method

Secondary screening was done by cross streak method, 31 actinomycetes were allowed to screen for the potential source of active secondary metabolites. In this method a loop full of inoculum was streaked in the middle of the petridish containing modified nutrient agar medium. After inoculation, petriplates were incubated at 28° C for 4 days. 24h old pathogenic bacteria were inoculated perpendicular to the growth line of actinomycetes in the same petriplates at 28°C for 24 h [8]. The inhibitory zones produced by the actinomycetes against the pathogenic bacteria were measured in millimeters (mm).

Production and extraction of antibacterial compounds:

The selected potent antagonistic actinomycete was inoculated in 100 ml starch casein medium and kept at 37°C for 7 days at 150rpm. After incubation the broths were filtered through Whatman No.1 filter paper, bioactive compound was extracted from the solvent phase. The culture filtrate was centrifuged at 5000 rpm for 10 min. Equal volume of ethyl acetate was added at 1:1 ratio [9].The filtrate with bioactive components were concentrated and checked for antimicrobial activity.

Determination of the antibacterial activity

Antibacterial activity of the crude extract was determined by agar well diffusion method. Wells of 5 mm diameter were bored using sterilized gel borer on Mueller Hinton Agar. The 0.1 ml inoculum of test pathogens were spread on the plates and 100 µl of the crude extract 20 mg/ml obtained was tested for their activity against the test pathogens *Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli, Staphylococcus aureus* [10]. Chloramphenicol 25µg/ml was used as positive control and DMSO without extract was used as blank. Plates were incubated at 37°C for 24 h the zone of inhibition was measured.

Free radical scavenging activity

The DPPH radical scavenging capacity of *Streptomyces sp* VITNSJ2 was determined using various concentration (0.1, 0.5, 1.0, 3.0 and 5.0 mg/ml) of crude extract. Ascorbic acid was used as reference compound (0.1, 0.5, 1.0, 3.0 and 5.0 mg/ml). A freshly prepared solution of 0.002% DPPH (1, 1, Diphenyl-2-Picryl hydrazyl) was freshly prepared in methanol. DPPH (2 ml) was added to each tube containing different concentrations of extract (2 ml) and of standard solution (2 ml). The samples were incubated for 20 min at 37 °C in dark place. The absorbance at 515 nm was measured. A blank sample was prepared containing methanol in the DPPH solution[11].All

the test samples were done in replicates and the data were expressed as the percent decrease in the absorbance compared to the control respectively[12].

Molecular characterization of *Streptomyces sp* VITNSJ2

Total genomic DNA was isolated using the phenol Chloroform method **[13].** The 16SrDNA gene was amplified using following primers FC27 (5_-to-3_AGAGTTTGATCCTGGCTCAG) andRC1492 (5_-to-3_ ACGGCTACCTTGTTACGACTT). The reaction mixture was carried out in 25 µl final volume containing 50 ng of DNA, 10 mM dNTPs 2.5 µl of 10× PCR buffer with 1.5 mM $MgCl_2$, 50 pmol of each primer, 200 µg bovine serum albumin (nuclease free) and 0.2 U *Taq* DNA polymerase in an cycler. The thermal cycling as follows: initial denaturation at 80°C for 5 min, followed by 30cycles. denaturation at for 30 sec 94°C, annealing for 30 sec at 55°C, elongation for 60 sec at 72°C, with a final elongation at 72°C for 7 mins [14]. The PCR products were analyzed by agarose gel electrophoresis.

Phylogenetic analysis

The 16S rRNA partial gene sequence data was analysed using BLAST search. The phylogenetic tree was constructed using Gene Bee software .The phylogenic distances was followed by the method [15].

RESULTS AND DISCUSSION

Morphological and cultural characteristics of selected isolate

In the course of screening 31 isolates were selected from each plate based on colony morphology. (Figure 1) and (Table 1) The colonies with powdery appearance had a characteristic feature of concave, convex, flat surface with different colors from grey, white, pink, cream to yellowish. (Figure 2) The cross streak assay was performed, out of which one strain were found to be potent (Figure 3). The morphological, biochemical andtaxonomical characterization represented the features of *Streptomyces* (Table 2, 3).

Table 1: List of actinomycetes isolated from soil samples of Amrithi forest

Isolates	Aerial Mycelium	Substrate Mycelium	Pigment
VITNSJ1	Blue	Yellow	Yellow
VITNSJ2	Ash	Lite brown	-
VITNSJ3	blue	Ash	-
VITNSJ4	Ash	Yellow	Yellow
VITNSJ5	Ash	White	-
VITNSJ6	Blue	White	-
VITNSJ7	Grey	Brown	-
VITNSJ8	Grey	Grey	-
VITNSJ9	Ash	Lite	Pink
VITNSJ10	Ash	Pink	pink
VITNSJ11	Ash	Grey	-
VITNSJ12	Ash	Grey	-
VITNSJ13	Pink	Pink	-
VITNSJ14	Ash	White	-
VITNSJ15	Grey	White	-
VITNSJ16	White	White	-
VITNSJ17	Ash	Ash	-
VITNSJ18	Ash	Ash	-
VITNSJ19	Ash	Ash	-
VITNSJ20	Ash	Ash	-
VITNSJ21	Blue	White	-
VITNSJ22	White	White	-
VITNSJ23	White	Cream	-
VITNSJ24	Cream	White	-
VITNSJ25	White	White	-
VITNSJ26	Cream	Cream	-
VITNSJ27	White	White	-
VITNSJ28	White	Brown	-
VITNSJ29	Ash	Ash	-
VITNSJ30	white	Ash	-
VITNSJ31	Ash	Ash	-

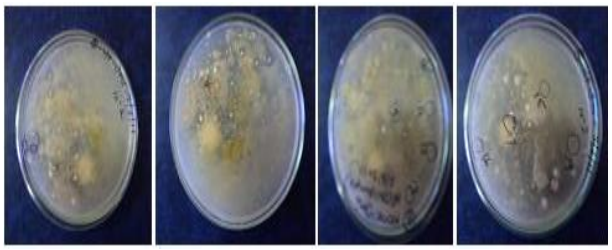

Figure 1: Actinomycetes isolated on Starch casein agar

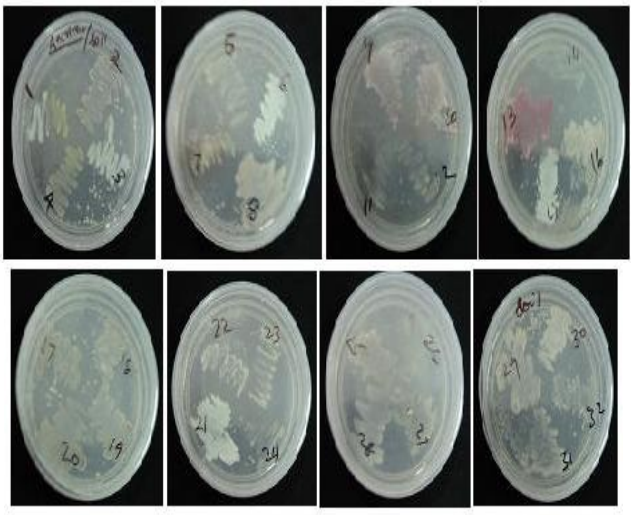

Figure 2 : **Colony morphology of Isolates from Amirithi forest soil samples**

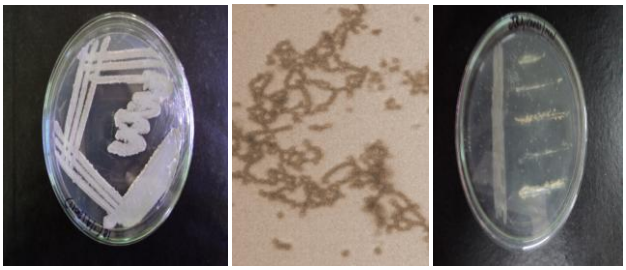

Figure 3: Microscopy, pure culture and cross steak assay of *Streptomyces sp* VITNSJ2 Antibacterial activity

The bioactive potential of the potent strain were extended by testing their suppression potential against gram-positive and gram-negative bacteria. The antagonistic activity of potent isolate was tested by cross streak method and was mass multiplied in liquid medium. Ethyl acetate crude extract were checked for their antibacterial activity by agar well diffusion method namely *Pseudomonas aeruginosa, Salmonella typhi, Escherichia coli, Staphylococcus aureus, Bacillus cereus* where two pathogens were found to be highly susceptible *Pseudomonas aeruginosa* (20mm), *Salmonella typhi* (19mm) and three pathogens were found to be less susceptible viz ., *Escherichia coli,(15) Staphylococcus aureus,(11mm) Bacillus cereus(13mm)* at the concentration of 20 mg/ml (Figure 4) and (Table 4).

Antioxidant activity

The antioxidant activity of the crude extract was determined according to the DPPH radical scavenging assay. The ethylacetate crude extract with different concentration (0.1, 0.5, 1.0, 3.0 and 5.0 mg/ml) showed activity, however it is comparable to the reference used and remained highly significant. The highest reducing potential was found at the concentration of 5mg/ml with 90.7% inhibition (Figure 5, 6). The percent inhibition was plotted against the concentration compared with the activity of known standard antioxidant.

Table 2: Morphological, cultural and biochemical properties of *Streptomyces sp.* VITNSJ2

Morphological properties							
Sporophore morphology	Spore surface	Colour of aerial mycelium	Colour of substrate mycelium	Spore mass	Gram staining	Acid fast staining	Motility
Spiral	Smooth	Ash	Light brown	Ash	Gram positive	Non-acid fast	-

Cultural Properties							
Starch casein agar	ISP medium 1	ISP medium 2	ISP medium 3	ISP medium 4	ISP medium 5	ISP medium 6	ISP medium 7
Good	Moderate	Moderate	Excellent	Good	Good	Good	Good

Biochemical Properties			
Melanin	Starch	Gelatin	Haemolysis
-	+	-	-

Table 3: Physiological, Antibiotic resistance, carbon and nitrogen utilization tests of *Streptomyces sp.* VITNSJ2

Antibiotic resistance									
Streptomycin	Tetracyclin	Bacitracin	Kanamycin	Ampicillin	Gentamicin	Rifampacin	Vancomycin	Trimethoprim	Fluconazole
S	R	R	S	R	S	R	R	R	R

Effect of Temperature				
15°C	28 °C	37 °C	45 °C	50 °C
+	+	+	+	-

Effect of pH				
5	6	7	8	9
-	-	+	+	-

NACL tolerance(%W/V)				
0.5	1	2	3	4
+	+	-	-	-

Carbon source (1% w/v)							
D-glucose	Sucrose	D-galactose	Mannose	Maltose	Lactose	Mannitol	L-Rhamnose
+	+	+	+	+	+	+	+

Nitrogen Sources(1%w/v)								
Cysteine	Arginine	Therionine	Alanine	Asparticaci	Glycine	Histidine	Lysine	Phenyl alanine
-	+	+	+	+	+	+	+	+

Table 4: Antibacterial profile of ethyl acetate extract of VITNSJ2

	Zone of inhibition (mm) Organisms				
	P. aerugin	***S.typhi***	***E.coli***	***B.cereus***	***S.aureus***
Ethyl acetate crude extract 20mg/ml	**20**	**19**	**15**	**13**	**11**
Chloramphenicol Standard 25µg/ml	**21**	**25**	**28**	**20**	**26**

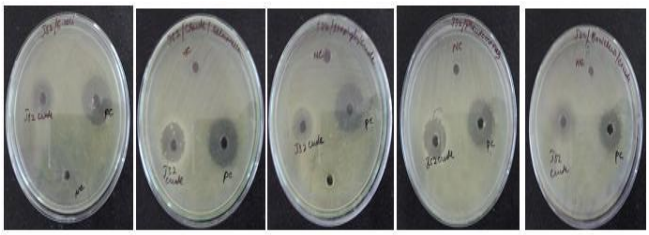

Figure 4: Antibiogram of crude extract VITNSJ2

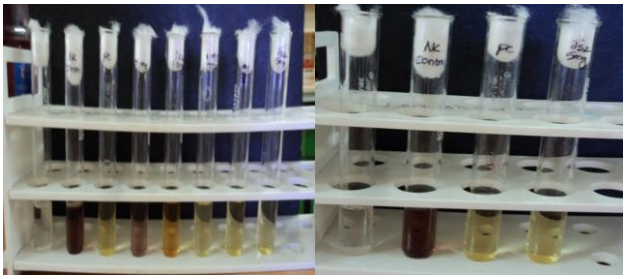

Figure 5: DPPH scavenging activity of *Streptomyces sp* VITNSJ2 crude extract

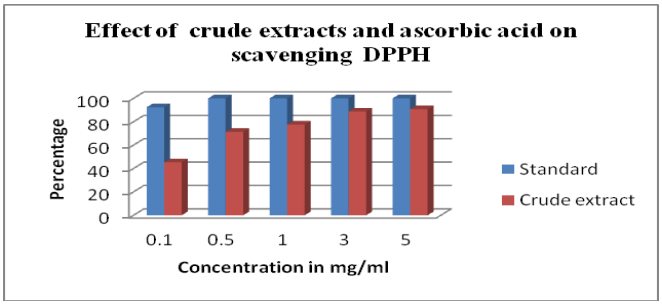

Figure 6: Antioxidant profile of ethyl acetate extract VITNSJ2Molecular and Phylogenetic relatedness

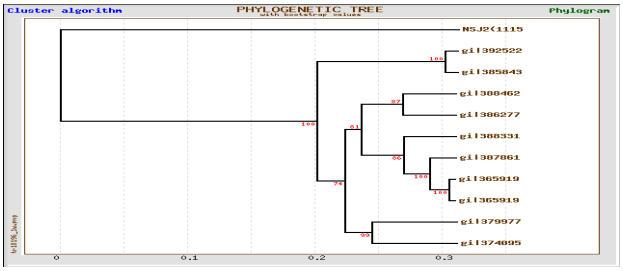

Figure 8: Phylogenetic trees based on 16S rDNA gene sequences of *Streptomyces sp* VITNSJ2

PCR amplification of the genomic DNA with actinomycete specific forward and reverse primers resulted in 1115 bp amplicon. The 16S rRNA sequence thus obtained was subjected to BLAST search using in the NCBI data base. The BLAST search result of the partial 16S rRNA gene sequences of *Streptomyces sp* VITNSJ2 showed 98% similarity to the isolate *Streptomyces rochei* strain D164. The sequences was submitted to Genbank with accession number: JX156416 and the phylogenetic tree constructed based on Gene Bee software (Figure 7, 8).

In order to discover novel compounds. The search should be more and efficient for promising candidates. For this purpose forest region soils were chosen for the study. The actinomycetes exist in various habitats in nature. Actinomycetes dwelling the terrestrial environment are more diverse and unique with the ability to produce unique chemical entities. The terrestrial ones from the soil have been extensively used for the production of secondary metabolites useful to human. This interest is undoubtedly linked to reports that soil actinomycetes are proving to be a productive resource for the discovery of new medicines. Actinomycetes in the forest environment have much role in decomposition as well nutrient regeneration, assessment of microbial diversity in theAmrithi forests are not attempted so far. But very few reports has described the antimicrobial activity .This is the foremost study attempted to showcase the knowledge on reporting with both antibacterial and antioxidant producer from *streptomyces sp* VITNSJ2 in Amrithi forest. The commercially used microbes of pharmaceutical and biotechnology importance are still prevailing by the conservation of the forest region. Considering the outcome of the present investigation, it was concluded that Amrithi forest is a rich source for deriving economically important actinomycetes. The antibiogram results indicated that the forest soils are source for hyperactive actinomycetes antagonistic against the pathogenic bacteria. Thus there is definite scope for bioprospecting of antagonistic actinomycetes from forest soil ecosystem, once appropriate further studies are undertaken.

CONCLUSION

Actinomycetes dwelling in the terrestrial environment are more diverse and unique with the ability to produce different chemical entities. The actinomycetes observed in the current study may be part of the entire diversity dwelling in the forest environment. Our searches for antagonistic actinomycetes have showed the identification of *Streptomyces sp* VITNSJ2 with antibacterial activity and antioxidant activity. The identified potent strain is the prolific producers of bioactive compounds. Further more intensive studies are needed to respect structural characterization to utilize potential source for the drug discovery.

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