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Screening for Antimicrobial and Antioxidant Property of *Streptomyces* sp VITJS3 Isolated from Bay of Bengal, Puducherry Coast of India

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The main focus of the present investigation was to screen the bioactivity of *Streptomyces* sp VITJS3 isolated from Bay of Bengal, Puducherry coast of India. The ethyl acetate crude extract checked for antibacterial activity showed better zone of inhibition at 20mg/ml concentration against 5 pathogenic bacteria *Staphylococcus aureus* (23mm), *Pseudomonas aeruginosa* (23 mm), *Escherichia coli* (11 mm) and *Salmonella typhi* (22 mm). The antifungal activity of the crude extract showed moderate zone of inhibition at 20mg/ml concentration against *Aspergillus niger* (14mm), *Aspergillus flavus*(11mm), *Aspergillus fumigatus*(15mm) *and Fusarium oxysporum*(12mm). The antioxidant potential of the crude extract evaluvated at 1000, 500, 250, 125, 62.5 and 31.75 µg/ml concentrations exhibited strong reducing power activity with 90% percent inhibition. The GC-MS profile of the JS3 crude extracts revealed the presence of volatile compounds. The morphological, physiological, biochemical properties of the strain VITJS3 was identified and confirmed as *Streptomyces sp.* Thus the study evidence the strain *Streptomyces* sp VITJS3 has various bioactive potential and hence further studies on purification and structure elucidation of the lead molecule will ensure the active compound for therapeutic use.

Key words: Antioxidant activity, Antifungal activity, Antibacterial activity, Marine actinomycetes.

Natural products contribute to the discovery of novel bioactive metabolites[4]. The marine environment is being exploited for its new bioactive compounds with unique structures. Actinomycetes are the filamentous bacteria which has provided many important bioactive compounds of high commercial value and are potent producers of antibiotics. Actinomycetes from marine source have been reported to produce new structurally variant compounds. It has been emphasized that actinomycetes from marine sediments may be valuable source for the isolation of novel strains which could be commercially success. The discovery of novel antimicrobial compounds is currently the thrust area of research where it attempts to overcome the global resistance to pathogenic bacteria[5]. In recent years, numerous pathogenic bacteria and fungi has developed resistance due to extensive use of antimicrobial drugs. On the other hand, free radicals are known to be the major cause of various diseases. Antioxidants are compounds responsible for balancing oxidation processes in the body which delay to inhibit oxidation and reduce the effects of oxidative stress and neutralize free radicals by donating an hydrogen atom from their hydroxyl groups. The deleterious effects in the body are protected by the antioxidants where they quench or scavenge the amount of free radicals generated. Since the free radicals are responsible for causing

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several kinds of diseases like artherosclerosis, antiaging, immunosuppression, inflammation, ischemic heart disease, atherosclerosis, cardiovascular diseases, inflammation, and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Antioxidants may be useful in preventing the deleterious consequences of oxidative stress. Hence there is increasing interest in the protective biochemical functions of natural compounds which are candidates for the prevention of oxidative damage caused by oxygen-free radical species[20]. This study scientifically supports marine environment serves as extreme source for antimicrobial drugs in traditional medicine for the treatment of wide variety of therapeutic diseases.

MATERIALS AND METHODS

Collection of marine soil samples

Marine soil samples were collected from South east coast of India, Puducherry – Thavalakuppam(Lat.11°52"Nand 79" 47'E), at the depth of 50 cm at littoral zone and the samples were stored at 4°C for further use.

Sample collection and isolation

Marine soil samples were air dried for one week prior isolation in order to decrease gram negative bacterial population. 1 g of the soil sample was taken and treated at 60°C for 1 h and mixed with 10 ml of sterile distilled water which was serially diluted and plated. The isolation of actinobacteria was performed on selective media such as actinomycetes isolation agar (AIA), Kuster's agars, Bennett agar, Starch casein agar along with 25% marine water and 25% marine soil extract for effective isolation [2]. Agar plates were supplemented with cyclohexamide and nalidixic acid to inhibit common contaminants and incubated at 27 ° C for 1 - 2 weeks. Further the plates were examined for the presence of actinomycetes. The emerged colonies were sub cultured and stored in refrigerator for further use.

Characterization of marine actinomycetes and taxonomic exploration

The potent actinomycetes were characterized morphologically and biochemically. The morphological cultural characteristics were determined on various ISP medium[19]. Actinomycete strains were examined micromorphologically by slide culture method[21]. Isolates were identified on the basis of their colony morphology, color of sporulating aerial mycelia and the growth of actinomycetes was compared with key guidelines[12].

Cross streak method

The actinomycete was cross streaked on modified nutrient glucose agar against wide range of Gram-positive and Gram-negative bacteria. The pathogens were streaked at right angle close enough but not touching the test actinomycete[1,3,17]. The zone of inhibition was measured after two days of incubation.

Fermentation and extraction of antimicrobial compound

The inoculum of the potent strain VITJS3 were prepared on starch casein broth at a seed concentration of 100ml in a 250ml Erlenmeyer flask at an incubation period of 7 days at room temperature and the medium were adjusted to pH 7.2. The physiological parameter of antibiotic production in batch fermentation was standardized for optimum production. The culture filtrate from the fermentation broth was separated and the obtained suspension was centrifuged at 5000 rpm for 10 min. The filtrate was used for solvent extraction process and extracted with 1:1 (v/v) ethyl acetate [18]. The resulting phase suspension was collected and evaporated to dryness under reduced pressure by using a rotary evaporator. The crude extract powder was weighed and tested for antibacterial activity upon clinical pathogens.

Test organisms and inoculum preparation

The pathogens including namely *Staphylococcus aureus* (MTCC No: 7405), *Pseudomonas aeruginosa* (MTCC No: 4676), *Escherichia coli* (MTCC No: 1588), *Salmonella typhi* (MTCC No: 1167) were obtained from Microbial culture collection, IMTECH, Chandigarh – 160036, INDIA. All the pathogenic microbial suspensions were maintained on nutrient broth and allowed to grow up to log phase to a final density of 10^8 CFU/ml at 37°C for 18 h.

Determination of antibacterial activity

The *in vitro* antibacterial activity of the crude extract was determined by agar well plate method. Log phase bacterial cultures were used to determine antibacterial activity of the crude extract. The inoculums were swabbed onto the surface of Mueller-Hinton agar plates. Wells were prepared on agar plates using the help of sterile cork borer

(diameter = 5mm). 100 µl of 20mg/ml concentration of crude extract were introduced into well. Chloramphenicol 25ug/ml was used as positive control. The plates were allowed to diffuse the compounds for 15 minutes and plates were incubated at 37°C for 24h [15]. Extract showing zone of inhibition >15mm were considered to possess antibacterial activity. Each plate was examined and the diameter of the zone was measured and expressed in mm.

Determination of antifungal activity

The antifungal activity was assessed by agar well diffusion assay. The culture medium of sabouraud dextrose broth was inoculated with the fungal strains and was adjusted to 1x 10^8 cFU/ml. 100 µl of the inoculum was placed on the sabouraud dextrose agar and wells were punched and filled with 100 µl *Streptomyces* sp VITJS3 crude extracts and the standard antibiotic miconozole (100 units/disc) was used as positive control. The plates were incubated at 27°C for 72 h. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth.

Free radical Scavenging Activity

The antioxidant activity was determined by DPPH scavenging assay[13].Various concentration (0.1, 0.5, 1.0 & 5.0 mg/ml) of ethyl acetate crude extract were taken in separate tubes. Ascorbic acid was used as reference compound (0.2, 0.4, 0.8.1.0 & 5.0 mg/ml). A freshly prepared solution of 0.002% DPPH (2, 2, Diphenyl-2-Picryl hydrazyl) in methanol was added to each tube containing different concentrations of extracts (2 ml). The samples were incubated in dark place at 37 °C for 20 min and read at 515 nm. The data were expressed as the percent decrease in the absorbance compared to the control. Percentage inhibition of radical scavenging activity was calculated using standard formula

$$\text{Percent DPPH scavenging activity} = \frac{\text{Absorbance (control)- Absorbance (Test)}}{\text{Absorbance (control)}} \times 100$$

Gas Chromatography- Mass Spectrum Analysis

The crude extract of *Streptomyces* sp. VITJS3 was subjected to GC-MS. The analysis was performed using GC SHIMADZU QP2010 system equipped with Elite-1 fused silica capillary column (Diameter: 0.25 mm, Length: 30.0 m, Film thickness: 0.25 µm. Helium gas (99.999%) was used as the carrier gas at a constant column flow rate of 1.51ml/min and an injection volume of 2µl, Ion-source temperature at 200°C and the ionization energy of 70eV with Injector temperature at 200°C. The oven temperature was at 70°C with an increase of 10 min at 300°C. Total GC running time was 35 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 – 1000 m/z. NIST08 and WILEY8 database were used for the identification of the separated peaks and the relative percentage of each component was calculated by comparing its average peak area to the total areas.

RESULTS

In the present study an attempt was made to isolate antagonistic actinomycetes species from marine sediment samples of Southern coastal area, Puducherry, India. This was collected at the depth of 50-500 cm. The bioactive potential of the strain *Streptomyces* sp VITJS3 was studied on the basis of its morphological and biochemical properties.

Table 1. Morphological properties of *Streptomyces sp*. VITJS3

Morphological properties						
Sporophore morphology	Spore surface	Colour of aerial mycelium	Colour of substrate mycelium	Gram staining	Acid fast staining	Motility
Spiral	Smooth	Ash	Pink	+	Non-acid fast	-

Table 2. Biochemical Properties of *Streptomyces sp*. VITJS3

Biochemical Properties							
Melanin	Starch	Gelatin	Haemolysis	Tween	Casein	Esculin	Gelatin
-	+	-	-	+	+	+	-

The morphological characterization of the strain was done based on the, color of aerial and substrate mycelia, shape, ornamentation of spore surface.(Table 1).The taxonomic studies of the isolate VITJS3 revealed that it belongs to *Streptomyces* sp. The pure culture and microscopy of *Streptomyces* sp. VITJS3 were examined and presented (Fig.1, 2). The biochemical and cultural properties the of strain VITJS3 was characterized (Table 2, 3).The effect of pH, temperature, NaCl,

Table 3. Cultural properties of *Streptomyces sp*. VITJS3

Medium	Growth
ISP medium 1	Good
ISP medium 2	Very good
ISP medium 3	Good
ISP medium 4	Good
ISP medium 5	Good
ISP medium 6	Good
ISP medium 7	Good
ISP medium 8	Good
ISP medium 9	Good
Bennett agar	Good
Nutrient agar	Moderate
Czapex Dox agar	Moderate
Kenknight's agar	Moderate
Actinomycete isolation agar	Very Good
Starch casein agar	Very Good

Table 4. Effect of temperature on *Streptomyces sp*. VITJS3

Effect of Temperature				
15°C	27 °C	37 °C	45 °C	50 °C
-	+	+	-	-

Table 5. Effect of pH on strain *Streptomyces sp*. VITJS3

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

Table 6. Effect of NACL tolerance on *Streptomyces sp*. VITJS3

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

Table 7. Effect of carbon source on *Streptomyces sp*. VITJS3

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

Table 8. Effect of nitrogen source on *Streptomyces sp*. VITJS3

Nitrogen Sources(1%w/v)								
Cysteine	Arginine	Threionine	Alanine	Aspartic acid	Glycine	Histidine	Lysine	Phenyl alanine
-	-	-	-	+	-	+	-	-

Table 8. Antibacterial activity of *Streptomyces sp*. VITJS3

Organisms	Reference drug(Reference drug 25µg/ml)	Crude extract of *Streptomyces* VITJS3 20mg/ml
P. aeruginosa	25	23
S. typhi	25	22
E. coli	20	11
S. aureus	21	23

Table 9. Antifungal activity of *Streptomyces sp*. VITJS3

	Fusarium oxysporum	*Aspergillus flavus*	*A. niger*	*A. fumigatus*
Concentration (mg/ml)	20mg/ml			
Crude Extract VITJS3	12	11	14	15
Miconozole(100units/disc)	22	35	32	40

carbon and nitrogen sources were optimized (Table 4, 5, 6, 7, 8) The preliminary screening of the strain was confirmed by cross streak assay (Fig. 3) The antibiotic was extracted from the fermentation broth using ethylacetate solvent and which was subsequently evaporated to obtain the crude extract. Further the antibacterial activity of ethyl acetate crude extracts at the concentration of 20mg/mL well was assayed by agar well diffusion method against pathogens. *Staphylococcus aureus* (23mm), *Pseudomonas aeruginosa* (23 mm), *Escherichia coli* (11 mm) and *Salmonella typhi* (22 mm) showed better zone of inhibition. (Table 9) (Fig 4). Ethylacteate crude extract showed

Fig. 1. Pure culture plate of *Streptomyces* sp VITJS3 on starch casein agar

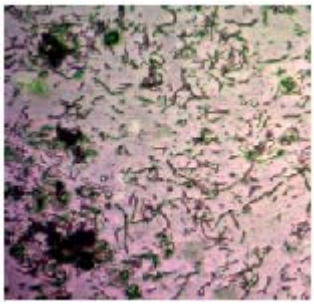


Fig. 2. Light microscopy of *Streptomyces* sp VITJS3

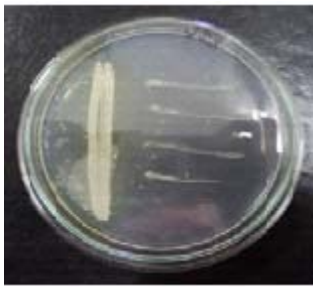
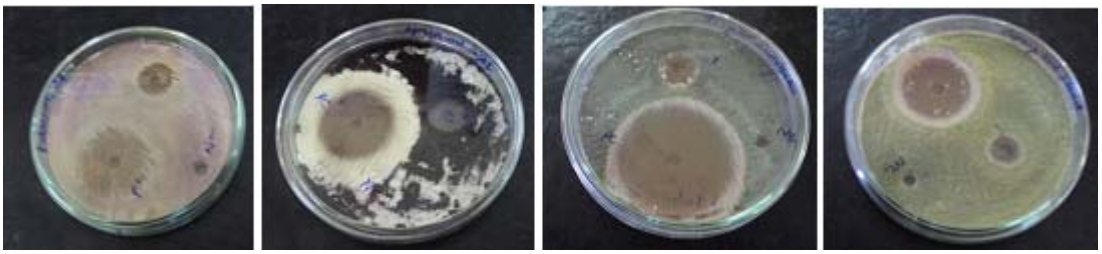


Fig. 3. Cross streak assay of *Streptomyces* sp VITJS3 on modified nutrient agar

(1)*Fusarium oxysporum* (2)*Aspergillus niger* (3)*Aspergillus flavus* (4)*Aspergillus fumigatus*

Fig. 4. Antibacterial activity of *Streptomyces* sp VITJS3 crude extract



(1)*Fusarium oxysporum* (2)*Aspergillus niger* (3)*Aspergillus flavus* (4)*Aspergillus fumigatus*

Fig. 5. Antifungal activity of *Streptomyces* sp VITJS3 crude extract

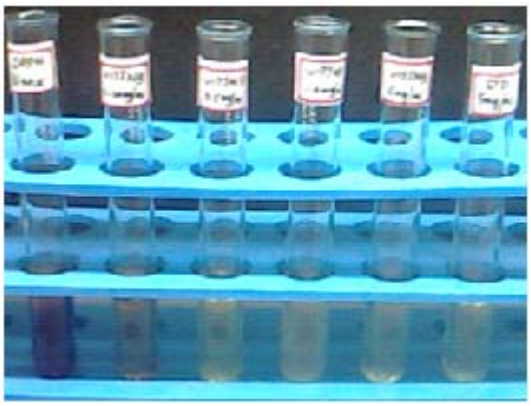


Fig. 6. Antioxidant activity of *Streptomyces* sp VITJS3 crude extract

moderate antifungal activity. Among the fungi tested *Aspergillus niger* (14mm) *showed* better zone of inhibition. The three fungal strains were found to show moderate activity against *Aspergillus flavus* (11mm), *Aspergillus fumigatus* (15mm), *Fusarium oxysporum* (12mm). (Table 10) (Fig 5). The antibacterial and antifungal spectrum of the crude extract from the isolate JS3 was compared with standard antibiotics. The ethyl acetate crude extract was found to exhibit strong reducing power property. The antioxidant activity of the VITJS3 ethyl acetate crude extract showed highest activity however its activity is comparable and remained highly significant with the reference used. (Table 11). (Fig 6,7) Identification of the volatile

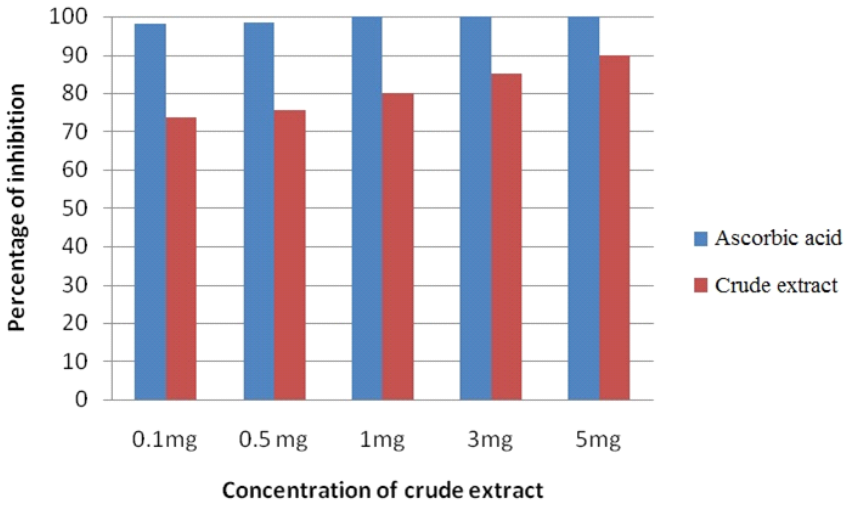


Fig. 7. Comparable antioxidant activity of *Streptomyces* sp VITJS3 crude extract with standard ascorbic acid

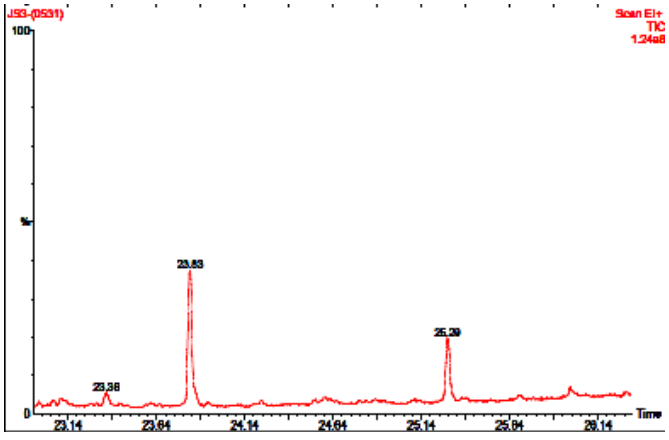


Fig. 8. GC-MS chromatogram of *Streptomyces* sp VITJS3 crude extract

components was carried out using GC-MS analysis. The spectral data were compared with NIST08s, WILEY8 and FAME library. The spectrum of the unknown component was compared with known components. The crude extract of *Streptomyces* sp. VITJS3 exhibited 17 compounds with 2 major constitutes namely Hexacosano, Acetate (MW-424),,Chloroacetic acid,Tetradecyl Ester(MW-290). (Fig 8).Hence the purification and structure elucidation might explore the utilization of the lead molecule for its biotechnological potential and the study reveals that the actinomycetes from marine sources have beneficial effects by virtue of their bioactivities.

DISCUSSION

In recent years, numerous pathogens have developed resistance due to the indiscriminate use of commercial antimicrobial drugs. It has already been envisaged that marine environment are extreme source to provide exciting new bioactive compounds. The phenotypic description of the strain strongly suggests that it belongs to genera *Streptomyces* which was isolated from puducherry coastal area. The strain *Streptomyces* VITJS3 showed potential antioxidant and antimicrobial activity. The results of the present study support previous observation that marine sediments are the sources of metabolically active *Streptomyces*[11], which indicate well defined adaptation of marine actinomycetes requires seawater for growth[8]. Similarly the studies on marine *Streptomyces sannanensis* exhibited good antimicrobial activity against clinical pathogens[16]. Certain *Streptomyces* strains showed strong antibacterial activity against gram positive and gram negative bacteria. The better antifungal activity was observed against *Aspergillus niger* [6, 14]. *Streptomyces* sp. PM-32, showed good activity against both the Gram-positive and Gram-negative bacteria (*S. aureus* and *E. coli*) and weak antifungal activity (against *A. niger* and *C. albicana*)[9]. The broad-spectrum of antibacterial activity from the strain *Streptomyces strain* SLO-105 evidenced against Gram-positive bacteria (*Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Micrococcus luteus*) and antifungal activity against *Aspergillus niger* [10,7]. In the present observation strain *Streptomyces VITJS3* exhibited promising bioactive potential. Hence the findings provides a new insight towards the development of good candidates for bioremediation, pharmaceutical, and bioactive natural products

CONCLUSION

Marine actinomycetes are the major sources to produce biologically active compounds and it is necessary for exploring actinomycetes strains for new potentially compounds. In the field of drug discovery many researchers have focused on the antimicrobial and antioxidant potential compounds during the screening process of new microbial metabolites. The compounds with antioxidant potential play main role in cell damage during inflammatory process and prevent damage by scavenging free radicals and have great relevance in the therapeutics for infectious diseases and prevention. Therefore these pathological and clinical backgrounds have prompted to investigate novel and potent bioactive compounds from actinomycetes which are ultimately used for therapeutic purpose.

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