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Identification of antibacterial secondary metabolite from marine *Streptomyces* sp. VITBRK4 and its activity against drug resistant Gram positive bacteria

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Abstract:

Drug resistance by bacterial pathogens becomes a major health problem worldwide. Hence, it is important to search for broad spectrum of antibiotic from natural sources. Marine actinomycetes isolated from marine sediments collected at different sampling sites along the southeast coast of Bay of Bengal, India were investigated for antagonistic activity against selected drug resistant Gram positive bacterial pathogens. All actinomycetes isolates were screened for antibacterial activity against standard drug resistant ATCC strains. The potential isolate which showed higher inhibitory activity against drug resistant pathogens was mass cultured and the ethyl acetate (EA) extract of the cell free culture broth was tested for antibacterial activity. The biochemical, morphological and physiological characterisation of the isolate revealed that it was Gram-positive rod, sporulating and produced grey aerial mycelium. The spore chain morphology, and smooth surface morphology showed that it belongs to the genus *Streptomyces*. Based on Nonomura's key for classification of *Streptomyces* and Bergey's Manual of Determinative Bacteriology, the isolate was identified as *Streptomyces* species and designated as *Streptomyces* sp. VITBRK4. Purification and characterization of EA extract of the isolate by thin layer chromatography (TLC) and HPLC-DAD analysis showed the presence of indolo compound along with few other unidentified metabolites. The result of this study showed that the antibacterial activity of the EA extract against drug resistant strains may be due to indolo compound present in the extract.

Keywords: *Streptomyces* sp. VITBRK4, Methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), Antibacterial activity.

Full Length Original Research Paper

INTRODUCTION:

The prevalence of bacterial antibiotic resistance currently possesses a serious threat to human health worldwide. Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* (VRE) [1] (Menichetti, 2005) and *Mycobacterium tuberculosis* (Loddenkemper et al., 2002)[2] are of much concern. These organisms have developed resistance to several classes of established antibiotics [2] (Menichetti, 2005). The only effective treatment, at present for multiple resistant MRSA infections is vancomycin. Several reports are available about vancomycin resistance by some MRSA isolates[3] (Aeschlimann, and Rybak,1998). *Enterococci,* clinically relevant multiple drug resistant bacteria more often exhibit vancomycin resistance. Linezolid and streptogramin combinations are the new drugs of choice for treating MRSA infections, but linezolid resistance have been reported in VRE and MRSA isolates[4](Tsiodras, et al., 2001). Resistance to these oxazolidinone[5] (Mutnick et al., 2003) streptogramin combinations [6,7](Livermore, 2003; Canu and Leclercq, 2001) and various glycopeptides requires agents with alternative

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targets or modes of action. *S. aureus* is one of the clinically important Gram positive pathogens because of its exceptional virulence, stress tolerance, and capacity to accumulate antimicrobial resistances. MRSA is known as a major nosocomial pathogen which has also developed resistance to many other antibiotics. MRSA and other *S. aureus* strains have been reported to acquire resistance to the last-resort antibiotic, vancomycin[8,9] (Shaw et al., 2005; Zheng et al., 2007). Thus *S. aureus* (MRSA) could fully acquire resistance to vancomycin in the near future.

New drug discovery processes in the past shows that novel skeletons have, in the majority of cases, come from natural sources[10] (Bevan et al., 1995) Actinomycetes are one of the most attractive sources of all types of bioactive metabolites that have important applications in human medicine[11] (Watve et al., 2001). Actinomycetes continue to be useful sources of novel secondary metabolites with a range of biological activities that may ultimately find application as anti-infective, anti-cancer agents or other pharmaceutically useful compounds [12](Bibb, 2005). Hence a study was planned to identify the bioactive compound from the isolate and to study its activity against drug resistant bacterial pathogens.

MATERIALS AND METHODS:

Sample collection and isolation of actinomycetes:

Marine sediment samples were collected at different locations from the southeast coast of Bay of Bengal, India at a depth of 400 cm. The sediment samples were dried and used for the isolation of actinomycetes. Soil samples (1g) were diluted to 9ml with sterile distilled water and 0.1ml of the diluted sample was plated. The following dilutions 10^{-3}, 10^{-4}, 10^{-5} were used for plating. The International Streptomyces Project (ISP) No. I media, with 25% sea water was used for the isolation of actinomycetes and the growth media was supplemented with antibiotics, cycloheximide (25 mg/ml) and nalidixic acid (25 mg/ ml) (Himedia, Mumbai, India). Plates were incubated at 28°C for 7-14 days for the growth of actinomycetes. The isolates were maintained in slant culture at 4°C as well as at 20% (v/v) glycerol stock at -80°C [13](Korn-Wendisch and Kutzner, 1992).

Drug Resistant Bacterial strains:

Gram positive MRSA bacteria *Staphylococcus aureus* (ATCC 29213) and VRE bacterial strain, *Enterococcus faecalis* (ATCC 29212) and were obtained from ATCC culture collection centre.

Primary screening for antibiotic production:

Primary screening was done to detect the production of antibiotics by the following techniques[14] (Suthindhiran and Kannabiran, 2009). Cross streak method/perpendicular plate method was used for the screening of antibiotic production by actinomycetes isolate. Sterile plates of ISP 1 media were prepared and pure culture of the actinomycetes was inoculated as a thick line across the plate at the centre. Inoculated plates were incubated at 28°C for 5-7 days till confluent growth was observed. The test bacterial strains were inoculated perpendicular to the grown actinomycetes colony. These plates were further incubated at 37°C for 24 h and the plates observed for inhibition of growth of test organism. The production of antibiotics by actinomycetes isolates was also measured by well diffusion and disc diffusion methods. Cell free supernatant (30µl) was poured into wells bored

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out of Muller Hinton agar (Himedia, India) plates seeded with the test organism. The plates were incubated at 37°C for 24h and the zone of inhibition was measured. Cell free supernatant (10µl) was applied to sterile disks (6mm in diameter) punched out of Whatman filter paper grade 1[15](Kala and Chandrika, 1993). These disks were applied on Muller Hinton agar (Himedia, India) seeded with a lawn culture of the test organisms following Kirby-Bauer technique. Inhibition zones were expressed as diameters and measured after incubation at 37°C for 24h.

Optimization of media for antibiotic production:

Influence of the various culture media on the antibacterial potential of the isolate was studied by cylinder plug method using ISP 1 supplemented with sea water collected at the sampling site, marine agar, actinomycetes isolation agar, starch casein agar(Himedia, India).

Characterization and identification of the potential isolate:

The classical method described in the identification key by Nonomura[16] (Nonomura, 1974) and Bergey's Manual of Determinative Bacteriology[17](Buchanan and Gibbons, 1974) was used for the identification of the isolate. The morphological, cultural, physiological and biochemical characterization of the isolate was carried out as described in ISP[18] (Prauser, 1964). The morphology of the spore bearing hyphae with the entire spore chain with the substrate and aerial mycelium of the isolate was examined by light microscope (1000x magnification) as well as scanning electron microscope (Hitachi, S-3400N). Culturing media used were those recommended in the International Streptomyces Project (ISP)[19] (Pridham and Gottlieb, 1946). Mycelium was observed after incubation at 28°C for 2 weeks and colours were also determined[20](Gordon et al., 1974). Carbohydrate utilization was determined by growth on carbon utilization medium (ISP 9)[21] (Shirling and Gottlieb, 1966) supplemented with 1% carbon sources at 28°C. Temperature range for growth was determined on inorganic salts starch agar medium (ISP 4) using a temperature gradient incubator. Hydrolysis of starch and milk were evaluated by using the glucose starch agar and skim milk agar respectively. Reduction of nitrate and production of melanin pigment were determined by the method of ISP[22](Williams et al., 1989). All cultural characteristics were recorded after 14 days.

Optimization of culturing conditions:

To determine the optimal nutritional and cultural conditions and to identify the suitable media for growth, the isolate was inoculated on different culture media (SCA, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7, modified Bennett's agar, sucrose/nitrate agar, and nutrient agar) and the growth was investigated. The effect of cultural conditions like different incubation temperatures (15, 25, 37 and 50 °C), different pH (5.0, 6.0, 7.4 and 9.0) and NaCl concentrations (2, 5, 7, 9 and 12%) on the growth of the isolate was also studied. The carbon and nitrogen sources required were also studied by inoculating the isolates into mineral salt agar with different sugars substituted to starch (D-glucose, sucrose, starch, D-xylose, D-galactose, maltose, L-arabinose, fructose, lactose, and glycerol), organic nitrogen sources like peptone, yeast extract, casein and inorganic sources like ammonium sulphate, ammonium nitrate and urea. The concentrations of carbon sources and carbon utilization tests were done as described earlier [23](Figure et al., 2005). After incubation the dry weight of the mycelium was measured and correlated with the growth of the isolate. Based on the growth of the isolate on

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different media the cultural conditions were optimized.

Fermentation and extraction of secondary metabolites:

Spores at 10^7/ml of the isolate were used to inoculate 1000ml Erlenmeyer flasks containing 200 ml of ISP 1 broth supplemented with 1% (w/v) of glucose and magnesium. After incubation at 30 °C for 24 h in an orbital incubator shaker at 200 rpm, this pre-culture was used to inoculate (5% v/v) a total volume of 15 L culture medium having the same composition as the pre-culture. After six days of incubation the culture broth was filtered to separate mycelium and supernatant. The supernatant was extracted twice with equal volume of ethyl acetate and the combined organic layers were evaporated. The brown gum obtained from the extract was dissolved in 100ml ethyl acetate. The antimicrobial activities were observed only in the ethyl acetate (EA) extract[24](Wu, 1984).

Assay of anti-MRSA activity:

MRSA strains used in this study was sub cultured overnight at 37°C in Mueller Hinton Broth and adjusted to obtain turbidity comparable to 0.5 McFarland standards (1.0 x 108 CFU/ml) before MIC tests. The EA extract was assayed for the anti-MRSA activity using broth micro dilution method in sterile glass test tubes. Prior the MIC test, each EA extract was diluted in sterile ultra pure water. The EA extract was then diluted by doubling dilutions at concentrations ranging from 32-1024 µg/ml. The tested strains were added to sterile Mueller Hinton broth into the test tubes before the EA suspension prepared as described above were added. The bacterial suspension without the EA was used as positive control and extracts in sterile broth were used as negative control. The MIC values were taken as the lowest concentration that showed no turbidity after 24 hours of incubation at 37°C. The turbidity was interpreted as visible growth of the microorganisms. The minimum bactericidal concentration (MBC) was determined by subculture of the tube showing no apparent growth in a sterile Muller Hinton agar plate. The least concentration showing no visible growth on agar subculture was taken as MBC value.

HPLC-DAD screening of ethyl acetate extract for compounds:

A total volume of 15 litters of the culture broth was centrifuged in batches for 15 min at 10000 rpm and the cell free supernatant was extracted with equal volume of ethyl acetate. The solvent fraction was collected and evaporated to dryness in vaccum and re-suspended in 1ml of ethanol. The solvent was allowed to evaporate and the residue was lyophilised. The EA extract was subjected to HPLC-DAD screening (University of Tubingen, Germany). The HPLC-DAD chromatographic system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detector and HP Kayak XM 600 ChemStation (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm. The UV-visible spectrum was measured from 200 to 600 nm. Sample (5 µl) was injected onto an HPLC column (125 X 4.6 mm, guard column 20 · 4.6 mm) filled with Nucleosil-100 C-18 (5 lm). Separation was performed by a linear gradient using 0.1% orthophosphoric acid as solvent A and acetonitrile as solvent B. The gradient was from 0 to 100% solvent B in 15 min at a flow rate of 2 ml/min. Limitations of the method are as follows, polar compounds cannot be separated because of non-retention behaviour on the reversed-phase column. These compounds show front elution. Only compounds having a UV active

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chromophore can be detected. Sugar type compounds (e.g. aminoglycosides) or peptides containing aliphatic amino acids cannot be detected by this method.

RESULTS:

Isolation and screening of actinomycetes:

All actinomycetes isolates were screened for antibacterial activity against drug resistant bacterial pathogens. The isolate which showed antibacterial activity and produces white powdery and dried colonies suspected to be actinomycetes were sub cultured on ISP-1 agar with sea water. Microscopic identification was also carried out to confirm the identity. The screening methods employed showed variable efficacy in detecting antibiotic producers and agar well diffusion method was found to be more effective. The isolate which showed antagonistic activity against MRSA and VRE was chosen for further studies.

Table: 1. Antagonistic activity of *Streptomyces* sp. VITBRK4 on standard drug resistant bacterial strains

Bacterial Pathogens	Zone of inhibition (mm)		
	Culture broth of VITBRK4	EA extract of VITBRK4 (20μg/ml)	Vancomycin (30 μg/disc)
Staphylococcus aureus (ATCC 29213)	21	19	17
Enterococcus faecalis (ATCC 29212)	20	21	18

Bacterial activity:

The culture filtrate supernatant of the isolate showed antibacterial activity against drug resistant MRSA strain *S. aureus* with a zone inhibition of 21mm and 20 mm against resistant *E. faecalis*. The antagonistic activity of the EA extract of the potential isolate is given in Table 1. Ethyl acetate extract produced a zone of inhibition of 19 mm against *S. aureus* and 21mm against *E. faecalis*.

Phenotypic characterization of the isolate:

The isolate was grown on oat meal agar medium (ISP 3), Yeast extract malt extract agar (ISP 2), and Inorganic salt starch agar (ISP 4). It was observed that the mature sporulating aerial mycelium was greyish white in color. Reverse side and melanin pigments were absent. Spore chain and spore surface morphology was observed under scanning electron microscopy (SEM) (Figure 1).

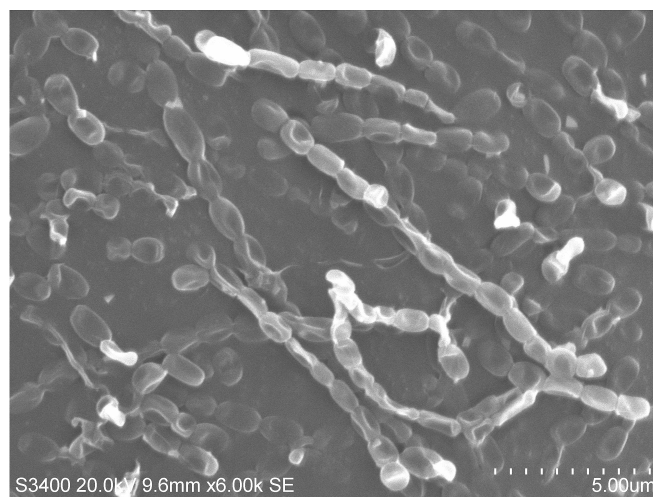

Figure 1: Smooth spore surface morphology of *Streptomyces* sp.VITBRK4 observed under Scanning electron microscope. The bar represents 5 μm.

Cultural, physiological and biochemical characterization:

The growth of the isolate was maximal in ISP1 medium supplemented with sea water and its growth was also equally maximal in actinomycetes isolation agar. The isolate showed maximum growth when cultivated at temperature 28°C; pH 7.4, with sea water 25%.The isolate assimilated arabinose, xylose, inositol, mannitol, fructose, sucrose and raffinose, however it did not utilize rhamnose. The isolate utilized 0.1% of L-asparagine, L-phenylalanine, L-histidine and L-

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hydroxyprolone as nitrogen source. The isolate was found to be halophylic in nature tolerated NaCl concentrations between 2% to 12%. Based on the results of physiological, biochemical and cultural characterization as well as matching the keys given for classification of 458 species of actinomycetes included in International Streptomycetes Project the isolate was identified as *Streptomyces* species and designated as *Streptomyces* sp. VITBRK4.

Assay Anti- MRSA activity:

Determination of minimum inhibitory concentration (MIC) by broth dilution method was performed by using the two fold concentration increments of the extract incorporated into the broth. This is a modified method of the CLSI M7-A4 protocol. The complete inhibition of visible growth was taken as the MIC for the MRSA strain. The least concentration 1024 µg/ml showing no visible growth on agar subculture was taken as MBC value.

Purification of the secondary metabolite:

After seven days of incubation 1.5 L of culture broth yielded 1mg of the brown coloured crude extract. The total volume of crude collected from 15 litters of crude was stored in air tight container at 4°C till further use. Multiple wave length monitoring using HPLC-DAD was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm (Fig. 2) The UV-visible spectrum was measured from 200 to 600 nm. The screening revealed the presence of indolo compounds with the retention time of 6.7 min (Figure 3).

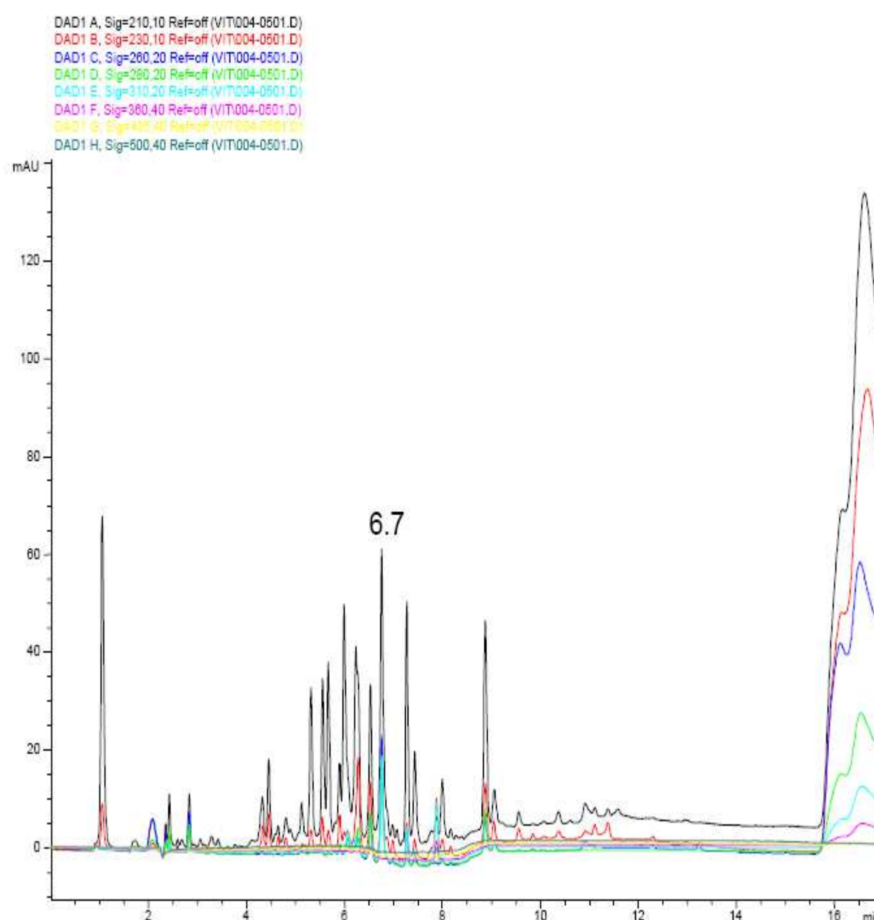

Figure 2: The HPLC-DAD chromatogram of ethyl acetate extract of *Streptomyces* sp. VITBRK4.

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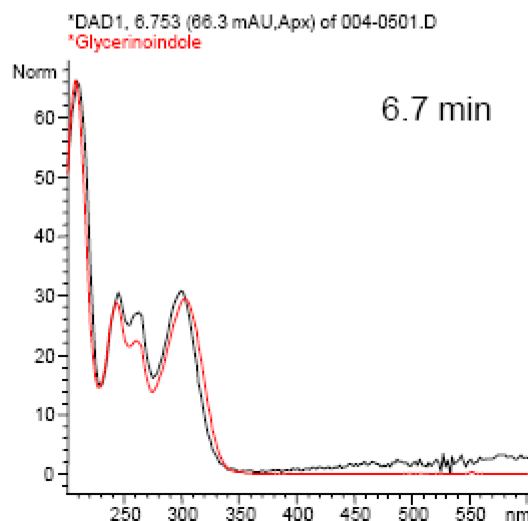

Figure 3: HPLC-DAD chromatogram of ethyl acetate extract of *Streptomyces* sp. VITBRK4. Peak at 6.7 min corresponds to glycerinoindole.

Discussion

Streptomyces sp. VITBRK4 isolate having antagonistic activity against drug resistant MRSA and VRE was isolated from marine sediment samples collected at the southeast coast of Bay of Bengal, India. The culture filtrate and EA extract obtained from the isolate exhibited significant activity against standard bacterial pathogens. The EA extract of *Streptomyces* sp. VITBRK4 not only inhibits MRSA but also VRE, forming 19mm and 21mm inhibition zones respectively. Few reports are available about the strains isolated from marine sediments which exhibited antibacterial activity against drug resistant bacterial strains. Actinomycetes strain designated as BT-408 producing polyketide antibiotic SBR-22 having antibacterial activity (20mm) against MRSA have been reported[25](Sujatha et al., 2005). Indole compounds extracted from microorganisms have been shown to possess antibacterial activity against drug resistant pathogens. The purified indole antibiotic from *Xenorhabdus nematophilus* was reported to be effective against both Gram-positive and Gram-negative bacteria at low to moderate concentrations causing a severe inhibition of RNA synthesis, accompanied by a less severe effect on protein synthesis[26] (Sundar and Chang, 1993). 2-(1H-indole-3yl) acetic acid isolated from *Streptomyces* sp. (No.195-02) from south China Sea was reported to be active against bacterial pathogens[27](Li et al., 2008). Indole-3-carboxylic acid extracted from actinomycete *Acrocarpospora* sp. strain, FIRDI 001 exhibited significant antibacterial activity against a group of Gram-positive: *S. aureus* subsp. *aureus* (BCRC 10451), and *Bacillus subtilis* subsp. *subtilis* (BCRC-10255), Gram-negative: *Pseudomonas aeruginosa* (BCRC-11633), *Klebsiella pneumoniae* subsp. *pneumoniae* (BCRC-16082) and *Escherichia coli* (BCRC-11634)[28] (Cheng et al., 2009). Chlorinated bisindole pyrroles extracted from a novel marine

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actinomycete, NPS12745 has been shown to possess broad-spectrum activity against both Gram-positive and Gram-negative organisms[29] (McArthur et al., 2008). Iindole alkaloid, streptomycindole extracted from *Streptomyces* sp. DA22 has been shown to possess broad-spectrum activity against both Gram-positive and Gram-negative organisms[30] (Huang et al., 2012). An alkaloid, 3-((6-methylpyrazin-2-yl) methyl)-1H-indole obtained from the deep-sea actinomycete *Serinicoccus profundi* sp. nov. has been shown to possess antibactarial activity against *S. aureus* [31](Yang et al., 2013).

CONCLUSION:

The results of this study suggest that the isolate *Streptomyces* sp. VITBRK4 is a potential isolate capable of producing indolo compound as a lead antibacterial compound and could be used against drug resistant bacterial pathogens.

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