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Antagonistic activity of marine *Streptomyces sp.* VITBRK1 on drug resistant gram positive cocci

Benita Mercy Rajan and Krishnan Kannabiran*

Division of Biomolecules and Genetics, School of Biosciences and Technology, VIT University, Vellore, India

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ABSTRACT

The aim of the present study was to evaluate the antagonistic activity of marine Streptomyces sp. VITBRK1 against Methicillin resistance Staphylococcus aures (MRSA) and vancomycin-resistant Enterococci (VRE). Drug resistance by Bacterial pathogens do exhibit significant tolerance to standard antimicrobials during infections and drug resistance currently a growing problem worldwide. A total of 137 actinomycetes isolated from marine sediments were screened for antibacterial activity against ATCC MRSA and VRE strains. Twenty two isolates exhibited antagonistic activity against standard ATCC strains. The potential isolate which showed activity against drug resistant pathogens was characterized by molecular taxonomic approach and identified as Streptomyces and designated as Streptomyces sp. VITBRK1. The isolate showed significant antibacterial activity against MRSA strains with the zone of inhibition of 24 mm against Staphylococcus aureus (ATCC 29213), 21 mm against Staphylococcus aureus (25923), 21 mm against Staphylococcus aureus (700699) and 13 mm against Staphylococcus aureus (U2A 2150). It also showed antibacterial activity against VRE strains, 25 mm zone of inhibition against Enterococcus faecalis (ATCC 29212), 19 mm against Enterococcus faecium (BM4107), 24 mm against Enterococcus faecium (BM4147) and 23 mm against VRE clinical isolate. HPLC-DAD analysis showed the presence of indolo compounds along with two unknown metabolites. The observed activity of Streptomyces sp. VITBRK1 may be due to the presence of compounds in the isolate. The results of this study suggest that secondary metabolites produced by Streptomyces sp. VITBRK1 could be used against MRSA and VRE strains.

Keywords: antagonistic activity, drug resistance, drug resistant cocci, *Streptomyces* sp. VITBRK1.

INTRODUCTION

Antibiotic resistance caused by methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* [1] and Mycobacterium tuberculosis [2] poses continuous threat to human health world wide. MRSA is a major pathogen associated with both nosocomical and community-acquired hospital infections [3]. Many of these organisms have developed resistance to several classes of established antibiotics [1,2]. In recent years the widespread use antibiotics led to the emergence strains that have systematically acquired multiple resistance genes [4]. *Staphylococcus aureus* strains (59.5 %) causing infections in hospitals were belonged to MRSA worldwide [5]. Enterococci are another group of clinically relevant multiple drug resistant bacteria that has emerged recently, some of which also exhibit vancomycin resistance. The appearance of vancomycin resistant *Enterococci* (VRE) infections has shown great challenge to health care workers. Llinezolid and streptogramin combinations are the new drugs of choice for treating MRSA infections, but linezolid resistance have been reported in VRE and MRSA isolates [6]. Linezolid, daptomycin, and tigecycline are almost universally

active against MRSA AND VRE [7]. But both MRSA and VRE have already demonstrated the capacity to evolve resistance to linezolid and daptomycin [8].

Natural products are novel potential chemical structures possessing antimicrobial activity. At least three of every four current antibacterial agents are related in some way to natural products. The filamentous bacteria of the order *Actinomycetales* (actinomycetes) produced more than 9000 biologically active molecules out of which more than 60 pharmaceutical agents have widely used in the field of medicine [8]. The genus *Streptomyces* was classified under the family *Streptomyceteae*, which includes Gram-positive aerobic members of the order *Actinomycetales* and sub order *Stretomycineae* within the new class *Actinobacteria* and have a DNA G-C content of 69±78 mol%. *Streptomyces* are a prolific source of secondary metabolites yielded many antibiotics; more than 80% antibiotics available in the market are from *Streptomyces*, including, streptomycin, neomycin, tetracycline and chloramphenicol [9].

MATERIALS AND METHODS

Sample collection and isolation of actinomycetes

Marine sediment samples were collected from Chennai, Cuddalore and Marakkanam coasts of the Bay of Bengal at a depth of 400 cm. The sediment samples were dried in laminar air flow for 8-12 h and then kept at 42°C for 10-30 days in a sterile Petri dish and these preheated samples were used for the isolation of actinomycetes. The International Streptomyces Project (ISP) No. I media, Starch casein agar and Bennett's agar with 25% sea water, 25% soil extract 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 7-18 days. All the media were prepared with varying salt concentrations (3, 5, 7, 9, 12, 15, 18 and 21% [w/v]) to isolate the halophilic actinomycetes. The isolates were sub cultured and maintained in slant culture at 4 °C as well as at 20% (v/v) glycerol stock at -80 °C [10].

Bacterial strains

Gram positive bacterial MRSA strains *Staphylococcus aureus* (ATCC 29213), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 700699) and *Staphylococcus aureus* (U2A 2150) were chosen for this study. VRE bacterial strains, *Enterococcus faecalis* (ATCC 29212) and *Enterococcus faecium* (BM4107) were obtained from ATCC culture collection centre. The drug resistant strains *Enterococcus faecium* (BM4147-*Van A*) were obtained from Institut Pasteur; Paris and the strain VRE is a clinical isolate. The drug resistance profile of the VRE clinical isolate was tested against a spectrum of standard antibiotics and confirmed in accordance with CLSI guidelines (2007).

Screening for antibiotic production

Antibacterial activity of the potential strain was studied by agar plate diffusion assay, 10µl of the cell free supernatant was applied to filter disks (6mm in diameter) [11]. Inhibition zones were expressed as diameters and measured after incubation at 37°C for 24h. All the seven ATCC strains and one drug resistant pathogens were used for the screening of antibacterial activity. 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, Mumbai, India).

Characterization and identification of the potential isolate

The morphological, cultural, physiological and biochemical characterization of the isolate was carried out as described in ISP [12]. The morphology of the spore bearing hyphae with the entire spore chain with the substrate and aerial mycelium of the strain was examined by light microscope (1000x magnification) as well as scanning electron microscope (Hitachi, S-3400N). Media used were those recommended in the International Streptomyces Project (ISP)[13]. Mycelium was observed after incubation at 28 °C for 2 weeks and colours were also determined. Carbohydrate utilization was determined by growth on carbon utilization medium (ISP 9) [14] 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 [15]. All cultural characteristics were recorded after 14 days.

Optimization of nutritional and cultural conditions

In order to optimise the nutritional and cultural conditions and to identify the suitable media for growth, the strain was inoculated in 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 [16,17]. 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 in different media the cultural conditions were optimized.

Fermentation and extraction of secondary metabolites

Spores at 10^7/ml of the strain 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 mycelium was lyophilized, extracted with acetone and concentrated on a rotary evaporator. The supernatant was extracted twice with equal volume of ethyl acetate and the combined organic layers were evaporated to obtain the ethyl acetate extract (EA extract). The brown gum obtained from the extract was dissolved in 100ml methanol/cyclohexane (v/v).

HPLC-DAD analysis of the EA extract

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. A pungent smelling brown gummy substance was obtained. 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 m). 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 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 for antibacterial activity

A total of 137 isolates were isolated from the sediment samples collected at three different sampling sites. Out of 96 isolates from samples collected from Chennai 17 isolates were found to have antibacterial activity, out of 38 isolates from Cuddalore samples 2 were found to be active and all 3 isolates from Marakannam sample was antagonistic to wide range of bacterial pathogens. The organism which produces white powdery and dried colonies suspected to be actinomycetes were sub cultured on ISP-1 agar with sea water. Microscopic identification was carried out to confirm the isolates as actinomycetes before screening for antibacterial activity. All the three screening methods, cross streak, cylinder plate and agar diffusion method employed were found to be effective in detecting antibacterial activity. The potential isolate (VITBRK1) which showed antagonistic activity against methicillin resistant *Staphylococcus aureus* (MRAS) and vancomycin resistant *Enterococci* (VRE) was chosen for further studies.

Antibacterial activity

The culture supernatant (filtrate) of the isolate showed significant antibacterial activity against MRSA strains with the zone of inhibition of 24 mm against *Staphylococcus aureus* (ATCC 29213), 21 mm against *Staphylococcus aureus* (25923), 21 mm against *Staphylococcus aureus* (700699) and 13 mm against *Staphylococcus aureus* (U2A

2150) (Fig. 1 A). It also showed antibacterial activity against VRE strains, 25 mm zone of inhibition against *Enterococcus faecalis* (ATCC 29212), 19 mm against *Enterococcus faecium* (BM4107), 24 mm against *Enterococcus faecium* (BM4147) and 23 mm against VRE clinical isolate (Fig. 1 B).

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.

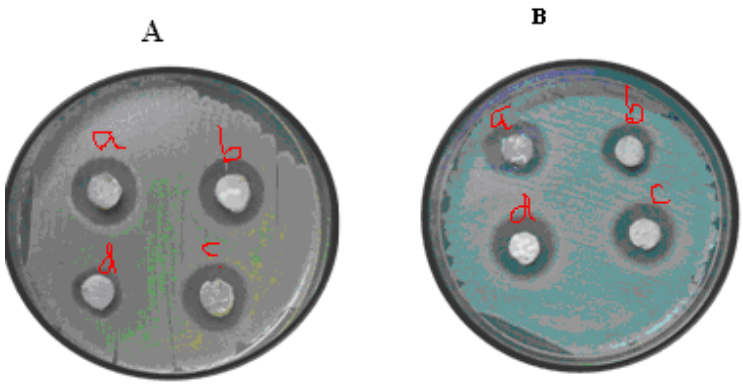

Fig.1. Antibacterial activity of culture supernatant of *Streptomyces* sp. VITBRK1. A) MRSA strains (a-c clockwise & d clinical isolate). (a-d clockwise) B) VRE strains

Reverse side and melanin pigments were absent. Spira-Spirales spore chain morphology was observed under optical microscope at 1000X magnification (Fig. 2. A) And smooth spore surface morphology was observed under scanning electron microscopic (SEM) analysis (Fig. 2).

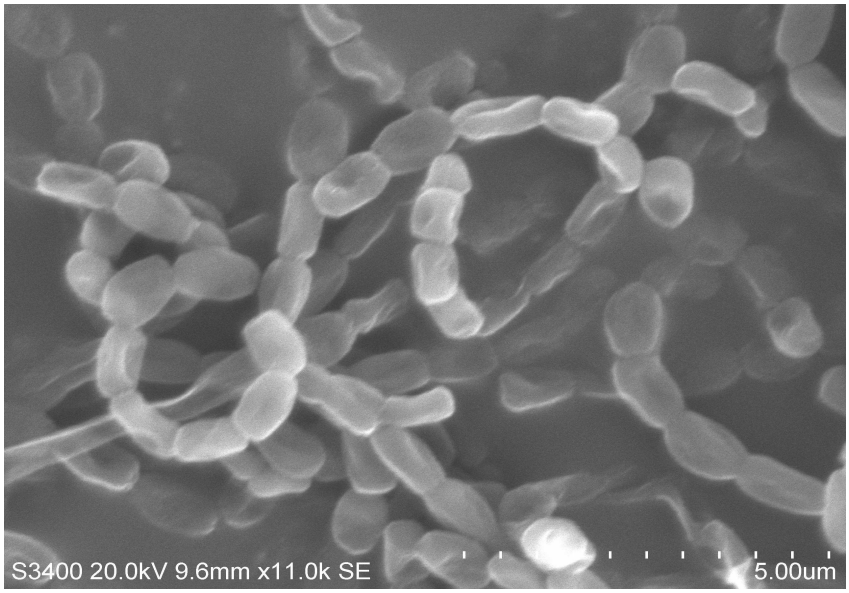

Fig.2. Scanning electron microscopic image of *Streptomyces* sp.VITBRK1. The bar represents 5 µm.

Cultural, physiological and biochemical characterization

The growth of the isolate was maximal in ISP1 medium supplemented with sea water and its growth was equally maximal in actinomycetes isolation agar also. The strain showed maximum growth when cultivated at temperature 28°C; pH 7.4, with sea water 25%.The strain assimilated arabinose, xylose, inositol, mannitol, fructose, sucrose and

raffinose, however the strain did not utilize rhamnose (Table 3). The strain utilized 0.1% of L-asparagine, L-phenylalanine, L-histidine and L-hydroxyprolone as nitrogen source. The strain was halophylic in nature tolerated Na Cl concentrations between 2% to 12%. The strain showed β-haemolysis on blood agar containing 5% sheep blood. 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* and designated as *Streptomyces* sp.VITBRK1.

Identification of compounds by HPLC-DAD analysis

HPLC-DAD analysis of the EA extract of *Streptomyces* sp. VITBRK3 is shown in Fig 3.The peaks of the chromatogram was matched with the reference compound UV-Visible spectrum.

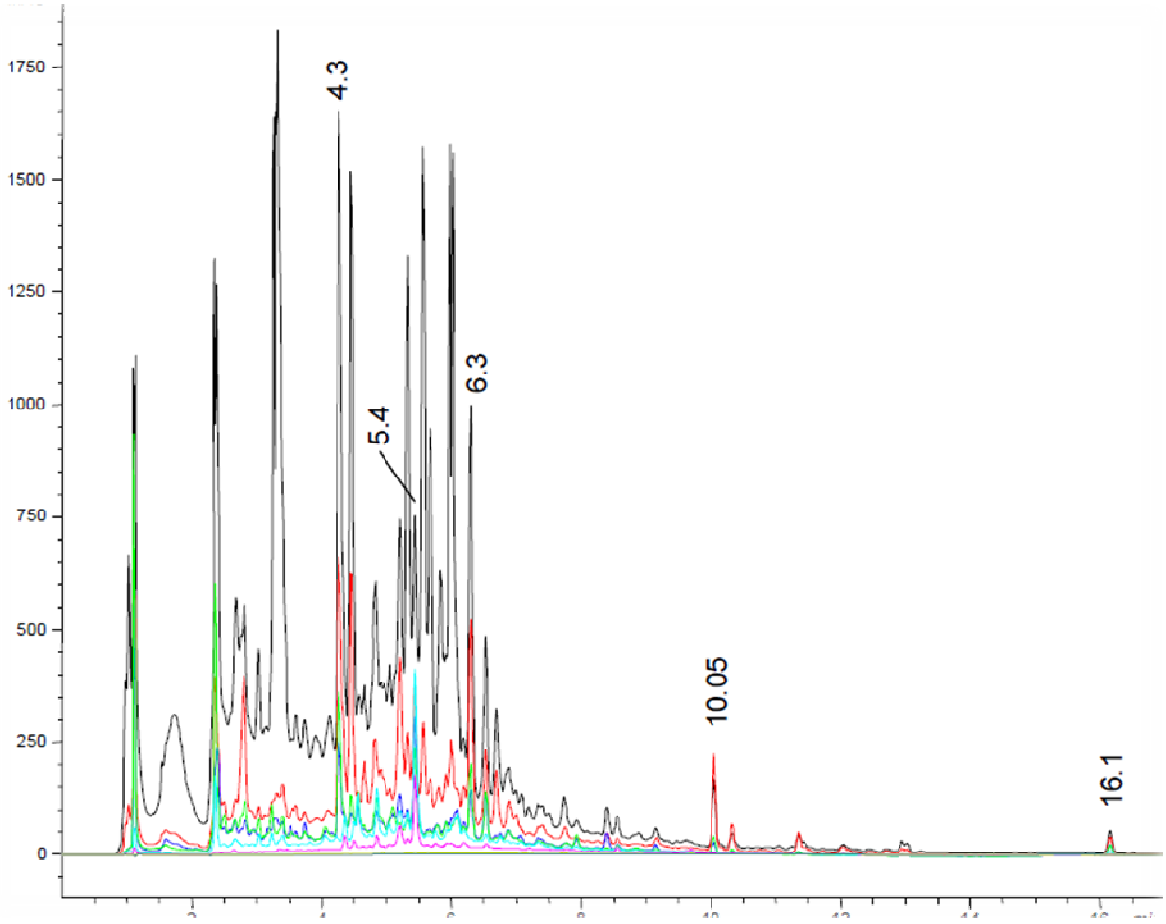

Fig. 3. The HPLC-DAD chromatogram of ethyl acetate extract of *Streptomyces* sp. VITBRK1

The peaks in the chromatogram having the same UV-Visible spectrum and retention time with that of the reference compound was identified and named. In the UV-Visible spectrum three peaks observed were corresponds to the unknown metabolite (Fig. 4 A) (5.4 min), indolo compounds (Fig. 4 B) (10.5, 10.3 and 11.4 min) and the unknown metabolite (Fig. 4 C) (16.1 min).

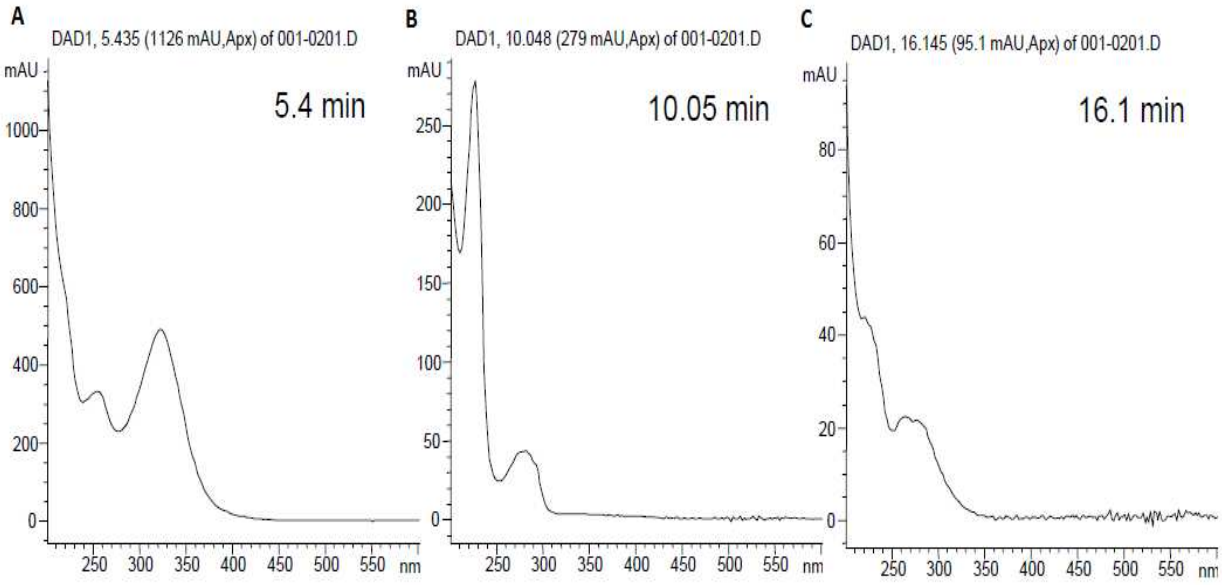

Fig. 4. The HPLC-DAD chromatogram of ethyl acetate extract of *Streptomyces* sp. VITBRK1 A) Peak at 5.4 min represents unknown metabolite B) Peak at10.05 min corresponds to indolo compounds and C) Peak at 16.1 min represents unknown metabolite

DISCUSSION

The potential isolate *Streptomyces* sp.VITBRK1 having antagonistic activity against drug resistant MRSA and VRE strains was isolated from marine sediment samples collected at the Marakkanam coast of Bay of Bengal, India. The culture supernatant of the isolate showed significant antibacterial activity against drug resistant standard MRSA and VRE strains. Fractionation of EA extract by HPLC DAD resulted in identification of indolo compounds along with two unknown metabolites. Indolo compounds have been shown to have significant antibacterial activity against drug resistant Gram positive and Gram negativebacterial pathogens.

One new 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 *S.aureus* [18]. Indole-3-carboxylic acid extracted from actinomycete *Acrocarpospora* sp. strain, FIRDI 001 exhibited significant antibacterial activity against a group of Gram-positive: *Staphylococcus aureus* subsp. *aureus* (BCRC 10451), and *Bacillus subtilis* subsp. *subtilis* (BCRC-10255), Gram-negative: *Pseudomonas aeruginosa* (BCRC-11633), *Klebsiellapneumoniae* subsp. *pneumoniae* (BCRC-16082) and *Escherichia coli* (BCRC-11634) [19]. Chlorinated bisindole pyrroles extracted from a novel marine actinomycete, NPS12745 has been shown to possess broad-spectrum activity against both Gram-positive and Gram-negative organisms [20]. A new indole alkaloid, streptomycindole have been extracted from *Streptomyces* sp. DA22, associated with South China Sea sponge *Craniella australiensis* [21].

Actinomycetes strain designated as BT-408 producing polyketide antibiotic SBR-22 having antibacterial activity (20mm) against methicillin resistant *Staphylococcus aureus* [22]. It was reported that an active compound, laidlomycin obtained from *Streptomyces* sp. CS684 showing antibacterial activity against methicilin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococci* (VRE) strain [23]. Neocitreamicins I and II, novel antibiotics with activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci have been reported from *Nocardia* [24]. Ethyl acetate extract obtained from the isolate exhibited significant inhibition against standard bacterial pathogens, as well as multidrug resistant clinical isolate. In a recent report a compound, 2-(2',4'-Dibromophenoxy)-4,6-dibromophenol isolated from the marine sponge *Dysidea granulosa* (Bergquist) collected at the coast of Lakshadweep islands, Indian Ocean, exhibited potent and broad spectrum *in-vitro* antibacterial activity, especially against methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), vancomycin resistant *Enterococci* (VRE), vancomycin sensitive *Enterococci* (VSE) and *Bacillus* spp.[25].

CONCLUSION

The results of this study showed that *Streptomyces* sp. VITBRK1 is potential actinomycetes isolate capable of producing indolo antibacterial compounds capable of acting against drug resistant MRSA and VRE strains.

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