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Screening of marine *Actinomycetes* for inhibitory activity against biofilm forming bacteria

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Abstract

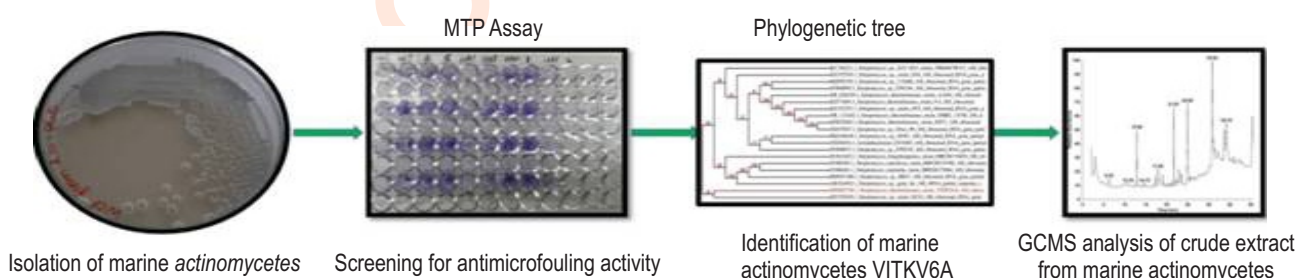
Aim: Marine ecosystem has served as an important source of economically and biotechnologically significant secondary metabolites. In view of this, the present study was carried out to isolate marine *actinomycetes* from rhizosphere soil sample and screen them for anti-microfouling activity.

Methodology: Screening of marine *actinomycetes* was done by agar well diffusion method and microtiter plate assay. The isolates with potent anti-microfouling activity were characterized by biochemical test and scanning electron microscopy. Finally, the isolates were identified by 16S rRNA gene sequencing. The isolate VITKV6A showing maximum activity was subjected to mass cultivation and extraction of bioactive compounds using different solvents. The crude extract, thus, obtained was analyzed by GC-MS.

Results: Forty marine *actinomycetes* were isolated from rhizosphere soil sample of *Rhizophora apiculata*. Three isolates VITKV6A, VITKV7A and VITKV10A showing maximum activity were selected and subjected to morphological, cultural and molecular characterization. These isolates were identified as *Streptomyces thermolineatus*, *Streptomyces variabilis* and *Streptomyces althoticus* by 16S rDNA sequencing. When tested for anti-microfouling activity, the chloroform extract of VITKV6A showed highest activity against all the three biofilm forming bacteria *Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila* used in this study. Gas chromatography-mass spectroscopic analysis of crude secondary metabolite extract from *Streptomyces thermolineatus* sp. (VITKV6A) was carried out and the results of the mass spectrum of compounds showed matches that were phenolic/polar molecules.

Interpretation: The results obtained indicate that bioactive compounds from marine *actinomycetes* would serve as potential anti-microfouling agent for control of microfouling caused in submerged environment.

Key words: *Actinomycetes*, Anti-microfouling, Biofilm, 16S rRNA



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Introduction

Biofouling is referred to as the build-up of undesirable living organisms and several organic and inorganic compounds. In most of the cases, biofouling includes microscopic organic impurities or an observable slimy layer comprising bacteria and other microorganisms. This group of bio fouling is called microfouling, or more frequently biofilm, and occurs everywhere in both natural and industrial environments where surfaces are exposed to water (Costerton, 2017). When fully established, biofouling occurring in marine environment also includes macroscopic organisms, like algae and larvae of barnacles and other invertebrates causing problem for submerged structures, such as pipelines, cables, fishing nets, pillars of bridges and oil platforms and other port or hydrotechnical constructions (Raikkin, 2004). Around 40% increase in fuel consumption of ships has been reported due to biofouling (Champ, 2000). In the following, we distinguish between “biofilm”, including only microorganisms, and “biofouling”, referring to fouling with both microscopic and macroscopic organisms. Since biofouling in the marine environment is initiated by a conditioning film consisting of organic compounds followed by initial colonizers such as bacteria and microalgae, curtailing microfouling will be an effective approach to check the subsequent steps leading to biofouling (Abarzua and Jakubowski, 1995). Conventional approaches to marine biofouling control through application of antifouling paints depend on the release of toxins that kill adhering organisms. Hitherto, tributyltin (TBT) and tributyltin oxide are the most active solutions to control biofouling but they are highly toxic (Shan *et al.*, 2011). Antifouling paints encompassing irgarol, chlorothalonil, dichlofluanid and diuron are used against fouling organisms, but these antifoulants have been reported to cause extensive pollution due to their toxic effects on non-target organisms (Chapman *et al.*, 2014; Ji Young and Myoung Sug, 2012). Current efforts have been focussing on substituting environmentally persistent toxins with naturally derived, degradable repulsive, eco-friendly compounds (Yebra *et al.*, 2004). There have been several studies demonstrating the presence of biologically active secondary metabolites in marine organisms such as actinomycetes ((Abid *et al.*, 2016), sponges (Tsukamoto *et al.*, 1997), corals (Clare *et al.*, 1999; Qi *et al.*, 2008) and algae (Dworjanyn *et al.*, 2006). Nevertheless, the supply concern of these antifouling compounds often hinders their commercial application (Dobretsov *et al.*, 2006). Compared to other group of microorganisms marine *actinomycetes* have received little attention in the search for anti-fouling agents. Moreover, the supply of bioactive metabolites and the cultivability of actinomycetes may not be a limiting factor. Therefore, isolation of marine *actinomycetes* capable of producing bioactive compounds with anti-microfouling potential will be a valuable endeavour. The objective of the present study was the isolation, characterization and identification of marine actinomycetes and screening for their anti-fouling potential.

Materials and Methods

Collection of sample: Rhizosphere soil sample of *Rhizophora apiculata* was collected from Pichavaram estuary, Parangipettai, Chidhambaram, Tamil Nadu. Sample was obtained from offshore at a depth of 2 to 3 m. Samples were placed in sterile poly bags, sealed tightly, and transported immediately to the laboratory and kept in deep freezer until further processing.

Isolation of Actinomycetes: Ten gram of soil sample was dispensed in 100 ml of sterile water and kept in shaker for 30 min at room temperature. Suspension dilutions were made upto 10^{-4} dilution. About 100 μ l of suspension was spread on the surface of different agar media and incubated at 28 °C for 7 days. Three different media viz. starch casein agar, yeast malt extract agar, Streptomyces Agar were used for isolation of *actinomycetes* isolates. Selected colonies were purified and maintained on slants of starch casein agar at 4°C for further studies.

Screening of Actinomycetes for anti-microfouling potential: Screening of *Actinomycetes* for anti-microfouling potential was carried out by agar well diffusion assay and microtiter plate assay against three biofilm forming bacteria *Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila* which were isolated earlier (Kavitha and Vimala 2018). Their biofilm forming ability was demonstrated by microtiter plate assay following the procedure of Jordjevic *et al.* (2002).

In agar well diffusion assay, isolates of marine actinomycetes were inoculated in Tryptone Yeast Extract broth and incubated at 28 °C for 7 days. The culture broth was centrifuged at 10,000 rpm for 15 min to obtain the culture supernatant. The test organisms were cultured in Zobell marine broth for 24 hr and a lawn culture was made on Mueller Hinton agar plates. Wells of 6 mm diameter were made on each plate and loaded with the culture supernatant of actinomycetes isolates. The plates were then incubated at 37 °C for 24 hr and the zone of inhibition was measured.

To determine anti-biofilm activity of isolates, 40 μ l culture broth of biofilm forming bacterial strains were inoculated into polystyrene 96-well microtiter plates containing nutrient broth (60 μ l) and 100 μ l culture supernatant of marine *actinomycete*. The inoculated microtiter plates were incubated at 37 °C for 3 days. Instead of supernatant, tryptone yeast extract broth was used as untreated control. After incubation, the planktonic cells and supernatant were removed and the adhered cells which formed the biofilm were rinsed and fixed with methanol and air dried. The biofilms were stained with 100 μ l 0.1 % (w/v) crystal violet for 10 min. The stain was discarded and the wells were rinsed with distilled water and solubilized with 99 % ethanol. The optical densities of biofilm were determined using microtiter plate reader (Bio-Rad, USA) at 595 nm which corresponds to the amount of biofilm.

Characterization of Isolates:

Morphological characterization: The morphological characterization of active isolates was carried out by inoculating them in different sterile media viz. starch casein agar (SCA), yeast malt extract agar (YMEA) and *Streptomyces* Agar. Colony morphology was observed with respect to color, aerial and substrate mycelium. Gram staining and scanning electron microscopic analysis of isolates were also carried out.

Biochemical characterization: Gram positive isolates were selected and subjected to biochemical characterization viz. starch hydrolysis, casein hydrolysis, gelatin hydrolysis, urea hydrolysis, citrate utilization test, indole test, Voges-Proskauer test, triple sugar ion test.

Molecular characterization: Molecular identification was carried out by 16S rRNA gene sequencing. Genomic DNA was extracted, and amplified using 8F-(5'AGAGTTTGATCCTGGCT CAG3') with 20 base pairs, and 1541R – (5'AAGGAGGTG ATCCAGCCGCA3') with 20 base pairs. Polymerase chain reaction was performed by adding 5 µl of isolated DNA in 25 µl of PCR reaction solution (1.5 µl of Forward Primer and Reverse Primer, 5 µl of deionized water, and 12 µl of Taq Master Mix). PCR product was sequenced using primers. Sequencing reactions were performed using ABI PRISM BigDye™ Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using 16S rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were suspended in distilled water and subjected to electrophoresis in an ABI 3730 xl sequencer (Applied Bio systems).

Mass culture and extraction of bioactive compounds: Actinomycete isolates showing maximum activity were subjected to solvent extraction by liquid-liquid extraction method. The isolate was cultivated in 500 ml Erlenmeyer flasks containing 200 ml of ISP1 medium. The culture flasks were incubated at 30°C for 7 days on an orbital shaker (150 rpm). The culture broth was then centrifuged for 15 min at 8000 rpm to separate the culture supernatant. The supernatant was then divided into three equal volumes and extracted with equal volume of different organic solvents. Three different organic solvents ranging from polar to non-polar (ethyl acetate, chloroform and pet ether) were screened for effectiveness. The organic phases were evaporated to dryness using a Rota vapor (Laborota 4000).

In order to select the best extraction solvents, based on their anti-microfouling activity, the activity of each crude extract of selected isolates were examined. Briefly, each crude extract was subjected to agar well diffusion assay and microtiter plate assay against biofilm forming bacteria. Solvent showing highest

inhibition diameter and highest anti-biofilm activity was then used for further extraction of active compounds.

Gas chromatography-mass spectrometry (GC-MS) analysis of crude extract: The crude extracts obtained from marine actinomycete isolates were analyzed in GC-MS JEOL (GCMTE II GC-MS, Agilent Technologies 6890N Network GC system for GC) equipped with a HP 5 fused silica column of 50 m × 0.25 mm internal diameter based on the method described by Kushwaha *et al.* (2019). The analysis conditions were 20 min at 100 °C, 3 min at 235 °C for column temperature and 240 °C for injector temperature. Helium was the carrier gas, and split ratio was 5:4. The sample (1 µl) was injected in a split less mode at 300 °C. Run time was 30 min. The compounds were identified by gas chromatography coupled with mass spectroscopy. The molecular weight and structure of the compounds were ascertained by matching with reference compounds available in the National Institute Standard and Technology (NIST).

Results and Discussion

Intense research has been carried out on *actinomyces* isolated from several unexplored extreme environment (Pathom-Aree *et al.*, 2006; Le Roes-Hill *et al.*, 2009; Li *et al.*, 2011). Studies carried out in the past have stated that coastal areas of Tamil Nadu are rich in antifouling compounds producing *actinobacteria*. Manikandan and Vijayakumar (2016) isolated 20 actinomycetes possessing antibacterial activity against biofouling bacteria. Out of these isolates, one potent isolate VS6 was reported to show maximum activity against biofouling bacteria. Harir-Mohammed *et al.* (2017) in his report has demonstrated that *actinomyces* from Sahara soils as potential source of novel antimicrobial compounds.

In the present study, rhizosphere soil sample of *Rhizophora apiculata* was collected and subjected to spread plate technique on different media. In total 40 *actinomyces* isolates were obtained from the rhizosphere soil samples. Starch casein agar yielded good growth and highest number of *actinomycete* isolates compared to other media used in this study. This demonstrates that starch casein agar is the best medium for isolation of marine *actinomyces*. Upon screening these 40 isolates for anti-microfouling activity, 8 isolates demonstrated activity against test organism viz., *Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila*. Among the eight isolates, VITKV6A, VITKV7A and VIT KV10A showed maximum zone of inhibition and higher anti-biofilm activity. The anti-microbial activities of all three isolates were found maximum against *Kocuria rhizophila*. The results of microtitre plate assay demonstrated that the biofilm inhibitory activity of VITKV6A against *Psychrobacter celer*, *Psychrobacter alimentarius* and *Kocuria rhizophila* (OD value of 1.9, 1.2 and 0.9 respectively) was highest compared to VITKV7A and VITKV10A. These three isolates were further subjected to morphological, biochemical and molecular characterization.

Three potent isolates thus screened for anti-microfouling activity were subjected to morphological characterization. Gram staining of isolates showed positive with different morphological structures. They were rod shaped with fungus like branched networks of hyphae. Also, the morphological characterization of isolates on starch casein agar revealed the color of the colony, aerial and substrate mycelium. The aerial mycelium of VITKV6A was whitish grey whereas VITKV7A and VITKV10A showed grey color aerial mycelium. In case of substrate mycelium, it was observed that VITKV6A and VITKV10A showed white mycelium and VITKV7A showed grey mycelium.

The results of biochemical tests were positive for hydrolysis of starch, casein and gelatin, citrate utilization and triple sugar ion test for all the three *actinomycetes* isolates.

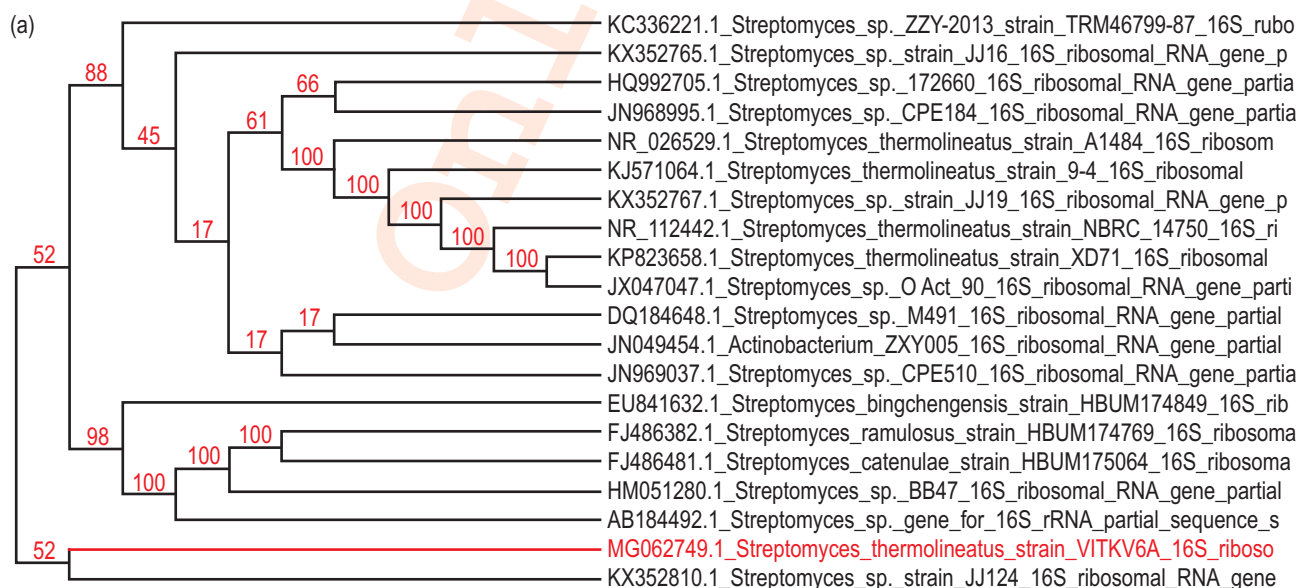
The molecular identification of VITKV6A, VITKV7A and VITKV10A was analyzed by 16S rRNA. Obtained sequences were compared with the GenBank sequence using BLAST and aligned with the sequence retrieved from NCBI GenBank database using the Clustal method. By using neighbor joining tree method phylogenetic tree was constructed (Fig. 1 a, b and c) and the database was deposited in NCBI GenBank with their accession numbers MG062749. 1, MG062750. 1, MG062751. 1, respectively. Depending on the cultural, morphological, physiological, and molecular analysis, the strains were identified as *Streptomyces thermolineatus*, *Streptomyces variabilis*, and *Streptomyces althoticus* respectively.

Several attempts have been made for the isolation of *actinomycetes* from marine environment (Barcina *et al.*, 1987). Recent studies have reported that members from marine environment belonging to several genera viz. *Nocardia*, *Streptomyces* and *Dietzia* (Rainey *et al.*, 1995; Heald *et al.*,

2001), *Streptomyces* (Moran *et al.*, 1995) and *Marinactinospora* (Tian *et al.*, 2009) are potential sources of bioactive compounds.

In a previous study, Xu *et al.* (2009) reported isolation of antifouling compound from *Streptomyces* so UST040711-290. Isolated compound 12-methyltetradecanoid acid which is a branched-chain fatty acid was reported to deter the settlement of larvae, *Hydroides elegans*. A group of five structurally similar compounds were isolated from marine *Streptomyces sp.* and the anti-fouling activity of these compounds were compared with four other structurally related compounds isolated from North Sea *Streptomyces* (Xu *et al.*, 2010). The structure-activity relationships showed that the functional moiety responsible for anti-fouling activity lies in 2-furanone ring. The findings of the study lead to the synthesis of a straight alkyl side-chain compound which was highly effective, non-toxic and capable of inhibiting the settlement of larvae of major fouling organisms. Marine sediments of Red Sea contain novel species of *actinomycetes* whose crude extracts show cytotoxic activity against breast cancer cell lines MDA-MB-231 (Abdelfattah1 *et al.*, 2016). These strains have been identified as members of genera *Streptomyces* and *Nocardioopsis*. An organic extract derived from the marine sponge-associated bacterium *Streptomyces sp.* SBT343 significantly inhibited staphylococcal biofilm formation (Balasubramanian *et al.*, 2017). Marine *actinomycetes* isolated from sea sediments of Andaman and the Gulf of Thailand inhibited biofilm formation by *E. coli* and *S. aureus* by protease activity (Leetanasaksakul and Thamchaipenet, 2018).

Culture filtrates containing secondary metabolites are usually complex mixtures, and the presence of different components may hinder the process of interpretation of data on bioactivity.



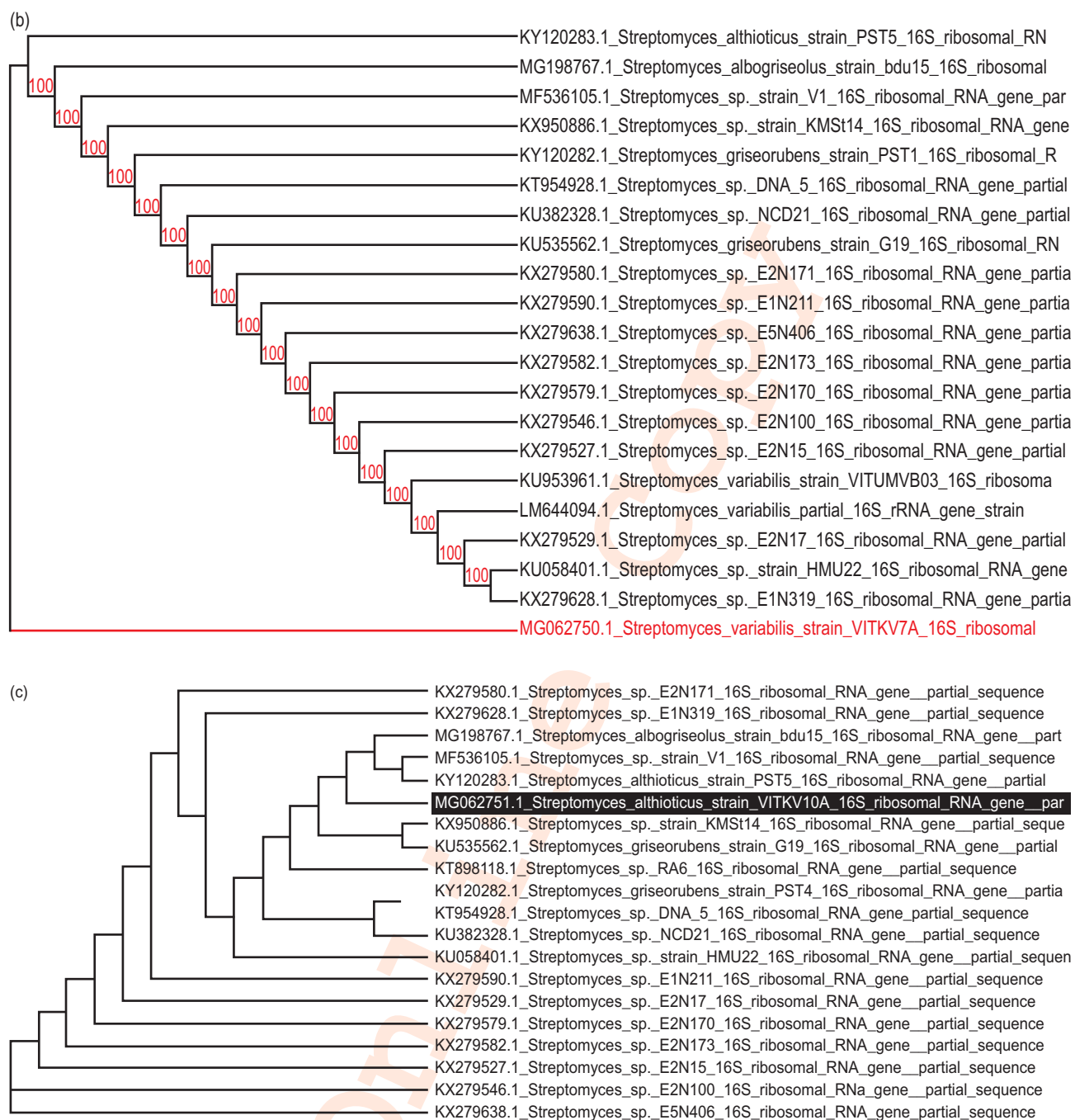


Fig. 1 : Phylogenetic tree of (a) *Streptomyces thermolineatus*; (b) *Streptomyces variabilis* and (c) *Streptomyces althioticus*

Solvent extraction offers the ease of liquid handling, the potential for high throughput operation and the potential for adapting to continuous operation (Gailliot, 1998). Selection of the most appropriate solvent is an important step in extraction process. Commonly used water immiscible solvents include alcohols (n-butanol, isobutanol), ketones, acetates (butyl, ethyl, isopropyl), hydrocarbons (toluene, hexanes) and methylene chloride. Common water-miscible solvents are the alcohols

particularly methanol. Both water-miscible and immiscible solvents are used for extracting compounds from the biomass. In this study, culture supernatant of VITKV6A was found to exhibit maximum activity was subjected to solvent extraction using three different solvents (ethyl acetate, chloroform and pet ether). The crude extracts thus obtained were subjected to anti-microfouling activities and the results showed that chloroform extract exhibited maximum zone of inhibition (Fig. 2) and anti-biofilm activity

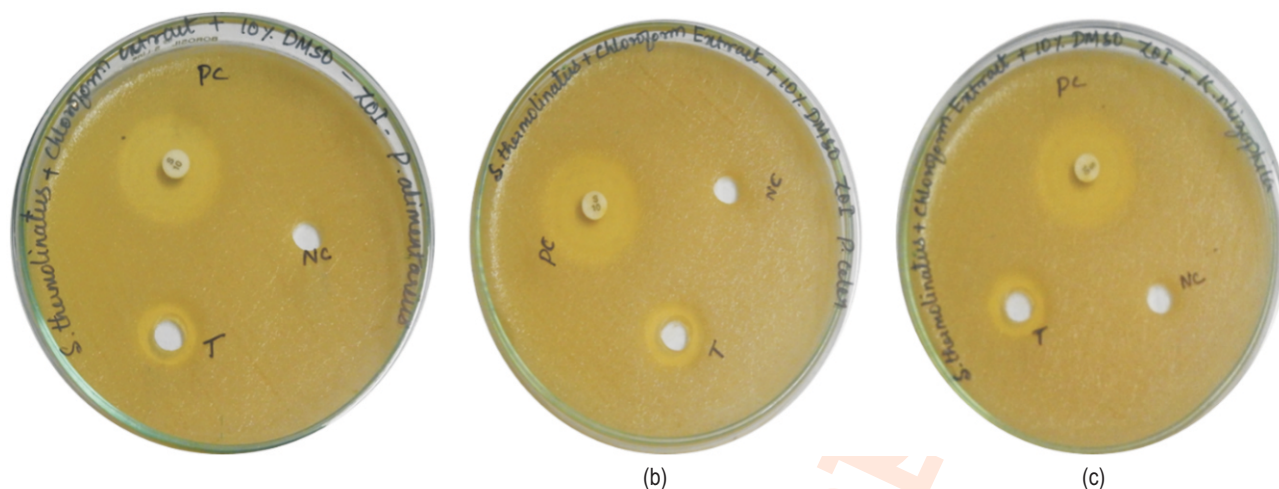


Fig. 2 : Antibacterial activity of the crude extract (Chloroform) of VITKV6A against biofilm forming bacteria by agar well diffusion method

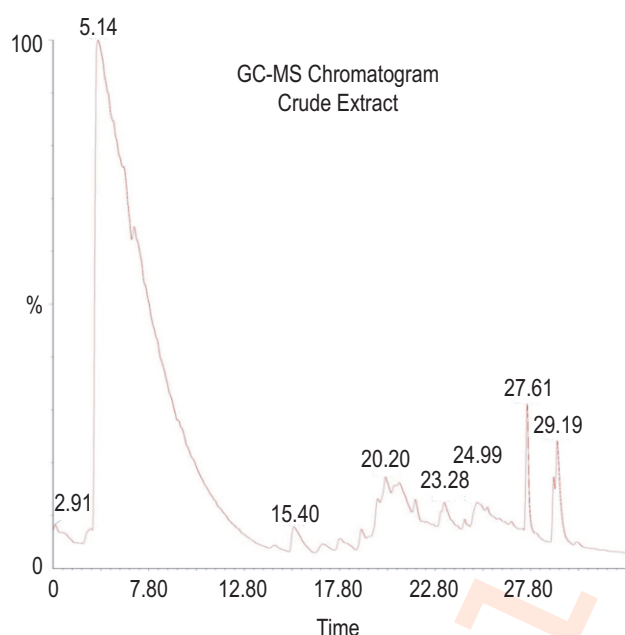


Fig. 3 : Chromatogram from Gas Chromatography - Mass Spectrometry analysis of the crude extract

against all the three biofilm forming bacteria. The zone of inhibition measures 14 mm, 15 mm and 16 mm for VITKV6A against *P. celer*, *P. alimenterius* *K. rhizophila* respectively. The results of antibiofilm activity were found in accordance with the zone of inhibition.

GC-MS analysis of crude secondary metabolite extract from *Streptomyces thermolinitus* (VITKV6A) showed multiple (>10 peaks) compounds throughout the chromatogram. The chromatogram of the GC-MS analysis is shown in Fig. 3. Among the observed peaks, five peaks at 20.20 min, 23.28 min, 24.99 min, 27.61 min and 29.19 min RT (Retention Time) were significantly major with regard to their intensity. The area

percentage compositions of these five major peaks in the studied crude extract was 5.085, 2.258, 4.085 and 3.815, respectively. NIST library search of the mass spectrum of these 5 compounds showed matches that were phenolic/polar molecules. NIST library match did not show any non-polar/hydrocarbon derivatives, suggesting that the studied crude extract mainly consists of polar molecules, corresponding to their initial extraction using chloroform solvent. The molecular weight and mass spectrum fragmentation pattern of these 5 compounds were completely different, with molecular weight ranging between 441.28 g mol⁻¹ to 87.95 g mol⁻¹. The GC-MS analysis along with NIST library match analysis provided an understanding of the molecular weight and nature of secondary metabolites in the crude extract, which can support further purification and identification of the active moiety. Research carried out by (Rangel-Sanchez *et al.*, 2014) reported that the phenolic compound isolated from the Avocado roots possessed marked antifungal activity against a wide range of fungi. Studies carried out in the past have reported that phenolic compounds possess antioxidant activity take part in ROS scavenging. Phenol, 2, 4-bis (1, 1-dimethylethyl)-has been identified in *Streptomyces cavouresis* KUV39 isolated from vermicompost samples collected in India (Narendhran *et al.*, 2014). The study also demonstrated that this compound could be probably responsible for the antioxidant and cytotoxic properties of *Streptomyces cavouresis* KUV39. Further, phenol, 2, 4-bis (1, 1-dimethylethyl) - has also been isolated from *Streptomyces* sp. MUM256 and identified to exhibit potential antioxidant activity (Tan *et al.*, 2015). A novel *Streptomyces antioxidantans* reported by Ser *et al.* (2016) was found to produce phenol, 2, 4-bis (1, 1-dimethylethyl) which was detected in the extract.

Further studies on the optimization, isolation and identification of individual compound in order to have an insight into its interaction with the diverse marine ecosystem are in progress. This would also help in assessing the commercial applicability of anti-fouling compound.

This study investigated the potential of marine actinomycetes in producing anti-micro fouling compounds and endorsed that rhizosphere soils of plants in mangrove forests can be considered as a distinctive natural source of anti-micro fouling agents. Thus marine actinomycetes could be a rich source of environmentally benign anti-micro fouling compounds.

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