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Isolation and extraction of antimicrobial peptides from *Streptomyces minutiscleroticus* and *Streptomyces albogriseolus* from Mangrove soil of Mangalore Coast, Karnataka

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Streptomyces species are the dominant source of microorganisms involved in the production of broad-spectrum antibiotics used by many pharmaceutical and biotechnological sectors. A mangrove ecosystem with a saline and humid atmosphere harbours specific organisms that could produce significant bioactive agents that are applicable for many biomedical applications. The present study aimed to isolate, screen, with characterization and production of antimicrobial peptides from *Streptomyces* spp. from mangrove soil sediments. Mangrove soils were collected from four different sites on the Mangalore coast. The soil was pre-treated for the enrichment of actinomycetes and cultured with the use of five different media which exhibited distinctive morphological, biochemical, and phylogenetic features along with distinctive spore structure and arrangements by SEM image analysis. This led us to the identification of the two *Streptomyces* species, *Streptomyces minutiscleroticus* and *Streptomyces albogriseolus.* The antimicrobial screening was carried out to study their antagonistic property against the test pathogenic bacteria cultures of ATCC. In an attempt to explore peptide molecules with antimicrobial properties from actinomycetes cultures dwelling in the mangrove soil of Mangalore coast, we could enhance the peptide production in ISP2 medium for peptide production by *S. minutiscleroticus* and *S. albogriseolus.* Further, the minimum concentration of 0.01 mg microbial peptide was determined to inhibit the growth of pathogenic bacteria as assayed by broth dilution and plate assays. The LC-MS analysis revealed molecular weights of 25 kDa and 29 kDa antimicrobial peptides from *S. albogriseolus* and 31 kDa antimicrobial peptides were detected from *S. minutiscleroticus.*

Keywords: Antimicrobial peptides, Mangrove soil, Peptides, Phylogenetic analysis, SEM analysis, *Streptomyces albogriseolus*, *Streptomyces minutiscleroticus*.

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Introduction

Streptomyces are Gram-positive filamentous microorganisms widely distributed in a natural ecosystem. The species belonging to this genus are distinguished with features of both bacteria as well as fungi and are grouped under the order Actinomycetales of the domain bacteria. On culture medium, *Streptomyces* exhibit distinct characteristics of aerial and substrate mycelium which differentiates into structures bearing spores. They have a high content of guanine and cytosine in their DNA and are involved in the utilization and recycling of metabolic compounds[1,2]. They are one of the major mediators of the biogeochemical cycling of elements along with the major producers of bioactive molecules such as enzymes and antibiotics[3,4]. The genomic analyses of *Streptomyces* have revealed their potential to produce natural antibiotics in varied environmental conditions[5]. These structural, functional and molecular properties of *Streptomyces* species contribute to their existence in any soil environment after bacteria and fungi with respect to their abundances[6]. To focus further on the occurrence of *Streptomyces*, one of the productive ecosystems selected in the present study is the mangroves of the Mangalore coast.

The mangroves soil sediments of the Mangalore coast have unexplored niches for the identification of *Streptomyces* species that could lead to analyse of certain bioactive compounds of interest. Although Mangrove environment encompasses significant portions of the earth's surface, It can thus be speculated that the organisms growing in the mangrove environment are metabolically diverse from terrestrial organisms and have received considerable attention as a source of natural products[7,8]. The Mangalore coast offers a suitable source for the isolation of the novel strains of *Streptomyces* with

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temperature variation ranging from 27 to 40 °C spanning over the year, along with humid environmental conditions. Among the members of actinomycetes, *Streptomyces* spp. are known to be the dominant genera in mangrove soil and are greatly influenced by variations in temperature, pH, moisture, aeration, and organic content. These factors may contribute to the synthesis of potential primary and secondary metabolites having survival benefits by the microbial community. Therefore, the mangrove ecosystem offers a promising and efficient productive ecosystem in the wetland of tropical latitudes in the coastal area.

Peptides with antimicrobial activity are actively derived biological molecules that serve as immune modulators and they facilitate a broad spectrum of antimicrobial activity, besides their antimicrobial function they even act as drug delivery vectors and signalling molecules in many signal transduction pathways for various therapeutic applications. Microbial derived peptides have shown considerable importance as antimicrobial peptides such as bacteriocins from numerous bacteria[9,10]. Antimicrobial bacteriocin peptides are produced by certain Gram-positive and Gram-negative bacteria as one of the survival defence mechanisms in their adaptive environment over other bacteria[11,12]. These antimicrobial peptides are small molecules that are cationic in nature, interact with negatively charged cell membranes on bacteria, and eventually lead to disruption of cellular solutes and cell death[13-16]. Understanding these mechanisms is of importance since there exists a great imbalance between the control of microbial infections and the number of effective molecules to control the resistance variants. Also, according to World Health Organization (WHO), a continuous dosage of antibiotics has led to the acquisition of resistance in pathogens, which has necessitated to identify novel, target-specific molecules to kill drug resistant pathogens[17,18]. Therefore, peptides from reliable sources such as *Streptomyces* from the unique environment as that of mangrove soil may prove useful in identifying antimicrobial peptides. Significant members of the *Streptomyces* genus are characterized for producing antibiotics and other chemotherapeutic agents that have been clinically implicated[19,20]. But limited exploration has been undertaken to discover new molecules such as microbial peptides from the mangrove soil in the Indian coastal areas.

Studies have shown that bioactive peptides from bacteria have different mode of action as antimicrobials and thus promote drug development and discovery[21-23]. In the recent era, there is a need for the potential contribution of biologically active peptides of actinomycetes with multidimensional properties in the clinical world yielding many therapeutic applications[24-26]. Intracellular extraction of *Streptomyces* species is highly beneficial in producing hydrolytic enzymes. Also, studies have revealed that peptides with biological and biocompatibility properties control the cellular process by modulating the pathway specific enzyme synthesis, nucleotide synthesis, protein synthesis and progression to cell death[27,28]. The present investigative study on isolation and identification of *S. minutiscleroticus* and *S. albogriseolus* from mangrove soil of Mangalore coast on evaluating their intracellular peptide extracts showed antimicrobial activity. The strains identified in the study showed distinct morphology as analyzed by slide culturing and SEM analysis. The biochemical characteristics of these isolates showed different enzyme activities. The bioactive peptides with respect to antimicrobial activity were extracted from intracellular homogenate following the standard method for protein extraction. In brief, peptides were separated upon precipitation using ammonium sulfate salt and salt removal by subjecting to dialysis membrane separation. The *streptomyces* peptides were separated into fractions based on a salt gradient using CM-Cellulose chromatography followed by LC-MS spectrometry analysis.

Materials and Methods

Sample collection

The unique habitats of mangroves in the coastal region of Mangalore were identified. The mangroves vegetation was found in places such as the Nethravathi river estuaries, riverbank and estuary at Thannirbhavi around Mangalore coast were selected for collecting the soil samples during January 2019. The soil samples were aseptically collected at the depth of 25 cm and the parameters such as pH and temperature were noted at the time of sample collection.

Pre-treatment of the soil samples

Collected soils were dried at room temperature and sieved to remove any debris. Calcium carbonate (1%) was sprayed over the dried soil and kept for

overnight. The soil was dried further at 60 ºC for two hours and utilized for isolation.

Growth media for isolation of *Streptomyces*

To isolate *Streptomyces* colonies, five different isolation media that were incorporated with appropriate amount of fluconazole (1 mg/mL) and nyastatin (1 mg/mL) were used. The media included in the study were starch casein agar media, starch nitrate agar media, yeast malt extract agar (ISP2), glucose asparagine agar media and Kenknight & Munaier's agar media. The serially diluted soil samples of 0.1 mL were inoculated at the dilution rate of 10^{-3} and 10^{-5} to each of the above media. The plates were inoculated at 30 ºC for 7 days for the appearance of *Streptomyces* colonies. The isolates of *Streptomyces* colonies were standardized to grow on ISP2 agar medium.

Slide culture method

Arrangement of sporulation and chain structures were examined by the slide culture method. To the sterile cover-slip at an angle of 40º in the ISP2 agar medium, an 24 h old culture of *Streptomyces* isolates was inoculated at the insertion place of the cover-slip over the glass slide and incubated at 30 ºC for seven days. Then cover-slip along with the growth of *Streptomyces* was carefully removed and transferred to the upward of the other glass slide flooded with lactophenol blue stain, examined under the light microscope at 100 X magnification power and observed spore surface morphology.

Scanning electron microscopy

Selected isolates of *Streptomyces* were grown in ISP2 agar medium and cells were fixed in fixative glutaraldehyde for 2-4 hours at room temperature, repeatedly washed using phosphate saline buffer and dehydrated the cells using acetone, cells were then pre-stained with gold particles and transferred carefully to a stub, observed the complete structure under a scanning electron microscope at 10000X magnification power.

DNA isolation and 16s rRNA gene sequencing of the *Streptomyces* isolates

DNA was extracted according to the standard protocol of CTAB (Cetyltrimethylammonium bromide buffer, 100 mM Tris-HCl, 20 mM EDTA pH. 8.0 1 M NaCl). DNA was confirmed in 1.0% agarose gel electrophoresis. 16s rRNA gene was amplified using forward primer GAAGCGC TCACGGCCTA and reverse primer CGGAGTG TCCATGTTCAGGGAACG. The amplicons were sequenced in *Applied Biosystem sequence scanner software*. The nucleotide sequence was aligned in CLUSTALW, their length obtained was 853bp and the phylogenetic tree was constructed by the neighbour-joining method. The maximum similarity of MY1 and MY16 *Streptomyces* isolates were confirmed using NCBI BLAST tool (https://blast.ncbi.nim.nih.gov/blast.cgi) and the taxonomic percentage level of the isolates was analysed.

Preliminary determination of antibacterial activity by cross streak method

The *Streptomyces* isolates were checked for antibacterial activity by the cross streak method. The isolates were inoculated as a single streak at the centre of the NA agar media. The inoculated plates were incubated at 35 °C for 5-7 days and observed for growth. The test organisms were perpendicularly inoculated to the growth of isolates and incubated for 24 h. The occurrence of growth or no growth surrounding the mangrove *Streptomyces* was compared with the standard antibiotic streptomycin sulphate (0.025 mg).

Extraction of peptides from mangrove *Streptomyces* isolates

The S*treptomyces* isolates were allowed to grow on ISP2 media with maximum growth achieved upon seven days of incubation at 30 °C and rotation at 200 rpm. The cultured broth centrifuged at 10000 rpm and biomasses were harvested. Phosphate buffer (0.1M, pH 7.2) was used to homogenise the biomass to extract the intracellular peptides.

Salting in of intracellular homogenate for peptide precipitation

The intracellular homogenate was precipitated using ammonium sulphate salt until 60% saturation by adding 15.6 g of salt to120 mL crude extract and kept overnight at 4 ºC for complete peptide precipitation. The precipitated peptides were separated after centrifugation at 10000 rpm for 15 min at 4ºC and the pellets were collected using 25 mM phosphate buffer pH 7.2. To remove any salt debris in the pelleted peptide precipitates dialysis was performed using membrane against phosphate buffer pH 7.2 for 48 h. The Lowry's reagent was used to check the content of peptides in the dialyzed extract and peptide quantification was carried out to confirm the presence of peptides.

SDS- Polyacrylamide gel electrophoresis

Non-denaturing SDS PAGE was carried out in a 12.5% polyacrylamide gel. The electrophoretic run was performed at 4 °C. Peptides were stained using 0.1% coomassie brilliant blue R-250 (w/v) in a mixture of 10% acetic acid and 25% methanol for 30 minutes. Once the staining is complete, the gels were destained using 10% acetic acid solution and the molecular weights of the peptides were compared.

CM-cellulose Ion exchange chromatography

Further peptide purification was carried out by ion-exchange chromatography on Carboxy methyl (CM)-cellulose purchased from *Sigma chemicals*, Bangalore. The dialyzed peptides sample was loaded onto a CM-cellulose column (1X15 cm in length, 3.14 mm in diameter) resin that was pre-activated using 0.5N NaOH in orbital shaker for 30 min followed by washing and activation using 0.5N HCl. The treatment was repeated until the pH of the resin reached neutral and pre-equilibrated with 0.1 M sodium phosphate buffer pH 7.2. The bound peptides were eluted with stepwise NaCl salt concentration gradient (0.1, 0.5, 1.0, 1.5 and 2.0 M) in 0.1 M sodium phosphate buffer pH 7.2 at a flow rate of 1.0 mL/min. The eluted fractions were recorded at 280 nm and the highest absorbance fractions were pooled to obtain different peak fractions.

Antibacterial activity of the CM-cellulose peak fractions

Well diffusion method was performed to check the antibacterial activity of the CM-cellulose pooled fractions. The test bacteria were inoculated on NA media followed by well loading with 1 mg/mL of pooled fractions. The antibacterial activity was examined after incubating at 37 °C for 24 h and examined for the zone of inhibition around the wells.

LC-MS/MS analysis

Peptide peak elutions obtained from CM-Cellulose chromatography that showed antibacterial activity were examined on an ultra-performance liquid chromatography system (Acquity UPLC from Waters Corporation, USA) coupled to a mass spectrometer with ESI-QT of-MS capabilities (Milford, MA, from Waters Corporation, USA). The analysis was processed by ethylene bridged hybrid (C18, 4.6 mm x 50 mm 2.6 µm) column using 0.1% formic acid in water as an aqueous phase, 0.1% formic acid as mobile phases and acetonitrile as an organic modifier. A focused gradient was delivered at a flow rate of 0.4 mL/min. 5 µL of the sample was injected and column oven temperature was kept at an optimal level throughout the chromatographic run (22 °C). Finally, peptide mass range and area of the desired peptide were analysed using waters corporation's mass lynx software (V4.1, Milford, MA, USA).

Results

Growth comparison of *Streptomyces* strains on different media

Actinomycetes isolates MYI and MY16 grown on *Streptomyces* specific media showed varied mycelium growth with both aerial and substrate mycelium. The colouration of colony growth, production of pigments, and hyphal textures were examined as shown in Table 1.

Culture morphology of *Streptomyces* strains

The molecular identification of *S. minutiscleroticus* with GenBank accession number MW479445 showed grey aerial mycelium and yellow to brown substrate mycelial colouration with powdery texture when grown on different media. *S. albogriseolus* GenBank accession number MW479446 showed yellow to brown substrate mycelial colouration with

Table 1 — Morphology of *Streptomyces* grown on different media

Isolates	Media	Mycelial colour aerial substrate		Shape	Elevation	Texture	Spore arrangement
MY1	ISP2	grey	brown	circular	flat	powdery	spira; long closed spiral filaments
	GAA	grey	brown	circular	raised	powdery	
	SNA	grey	brown	circular	flat	powdery	
	SCA	grey	yellowish grey	circular	flat	leathery	
	KMA	grey		circular	flat	powdery	
MY16	ISP2	grey	brown	irregular	raised	powdery	rectus; straight and branched flexible filaments
	GAA	white	white	circular	flat	powdery	
	SNA	grey	grey	circular	raised	velvetty	
	SCA	grey	grey	circular	flat	slimy	
	KMA	grey	brown	circular	flat	powdery	

Note: ISP2; yeast malt extract agar, GAA; glucose asparagine agar media, SNA; starch nitrate agar, SCA; starch casien agar, KMA; kenknight munnaier agar

the production of soluble violet colored pigments Fig. 1a-b.

Slide culture technique and scanning electron microscopic analysis

The slide culture technique for spore chain arrangement pattern of *S. minutiscleroticus* GenBank accession number MW479445 showed rectus; straight and open-branched flexible filaments. The slide culture technique for spore chain arrangement pattern of *S. albogriseolus* GenBank accession number MW479446 showed spiral, long closed circular filaments as shown in Fig. 2a-b. The *S. minutiscleroticus* GenBank accession number MW479445 was analyzed by gold stained fixation and observed under a scanning electron microscope with characteristics pattern of spore arrangements. As shown in Fig. 3a, The SEM image revealed that the internal structure of the sporangium shows clear compact coiled or parallel oriented spores which are bisporous arranged as tubular or cylindrical shaped with long chains of biverticillate circular open spiral filaments arranged in bunches of 20 µm in size. *S. albogriseolus* GenBank accession number MW479446 in Fig. 3b showed polysporous forms of long flexous spore chains with hooks and streched irregular spirals. The matured spores exhibited biverticillate spore arrangements of 20 µm in size.

DNA extraction and 16s rRNA gene analysis

The 16s rRNA gene sequence of MY1 and MY16 isolates according to GenBank database and BLAST tool analysis with multiple sequencing alignment was assesed by constructing phylogenetic tree to display the generic level of the isolates by the neighbour-joining method. The Taxonomic position of MY1 and MY16 isolates are closely related to members of *Streptomyces* genus. The MY1 isolate showed 92% identity for *S. minutiscleroticus,* GenBank accession

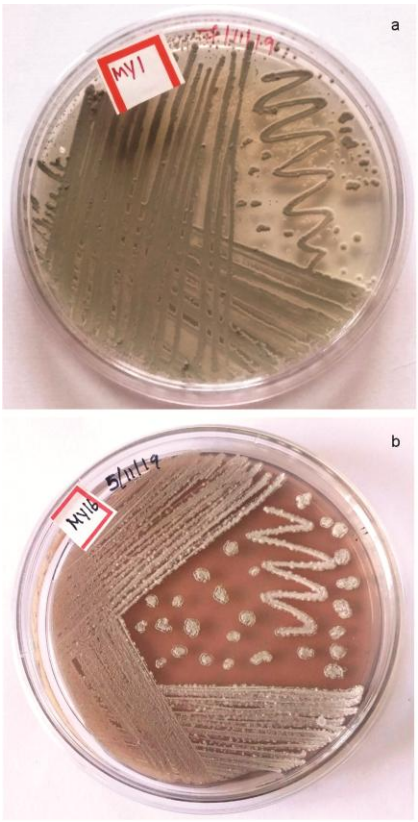

Fig. 1 — Pure cultures of a) *Streptomyces minutiscleroticus* (MY1) and, b) *Streptomyces albogriseolus* (MY16), grown on starch casein agar media.

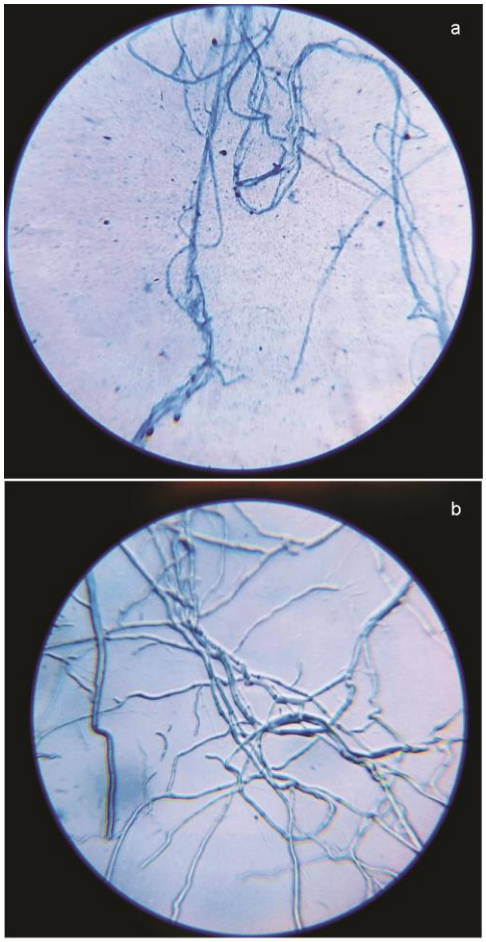

Fig. 2 — Slide culture technique images, a) *Streptomyces minutiscleroticus* MY 1 and, b) *Streptomyces albogriseolus* MY16.

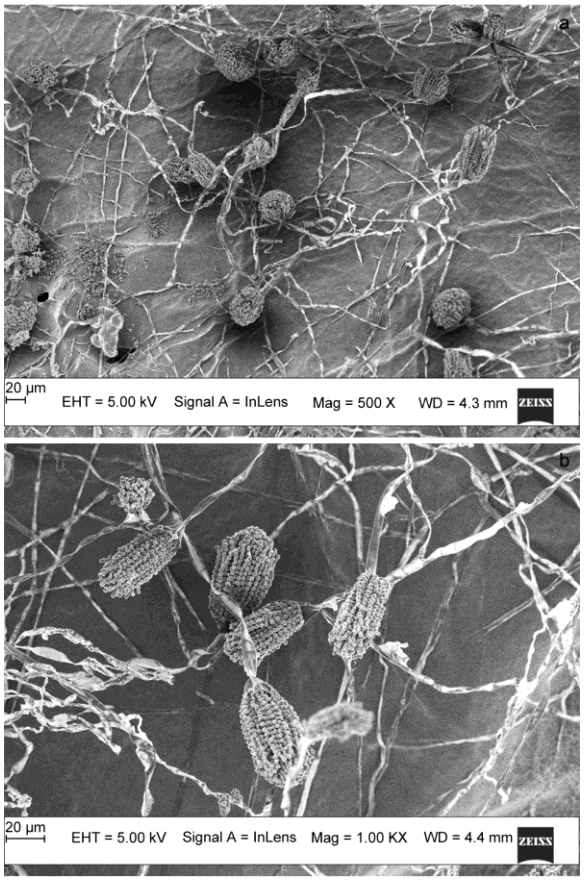

Fig. 3 — Scanning electron microscopic images, a) *Streptomyces minutiscleroticus* MY 1 and, b) *Streptomyces albogriseolus* MY16.

number MW479445 and MY16 isolate resembles 100% relatedness to *S. albogriseolus* and GenBank accession number MW479446 has been documented in database for the mangrove *Streptomyces* isolates depicted in Fig. 4 and 5.

Biochemical characteristics of *Streptomyces* strains

Both the isolates showed positive for hydrolysis of starch, gelatin and casein implicating their involvement in the production of amylase and protease enzymes since these isolates were selected from mangroves region where there is a maximum turnover of organic compounds by producing required enzymes. *S. minutiscleroticus* and *S. albogriseolus* showed positive enzyme production for proteases, ureases and cellulases, positive reaction for indole production, nitrate reduction, MR-VP positive and hydrogen sulphide producton. In contrast, *S. minutiscleroticus* showed negative results for catalase activity and for citrate utilization whereas *S. albogriseolus* showed positive results for the citrate utilization test and no catalase activity.

Antibacterial activity of *Streptomyces* strains by Cross streak method

The *S. minutiscleroticus* and *S. albogriseolus* showed antibacterial activity against *Bacillus cereus* ATCC 10876, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumonia* ATCC 9621, *Salmonella typhimurium* ATCC 23564, and *Escherichia coli* ATCC 8739. As shown in Fig. 6, both the

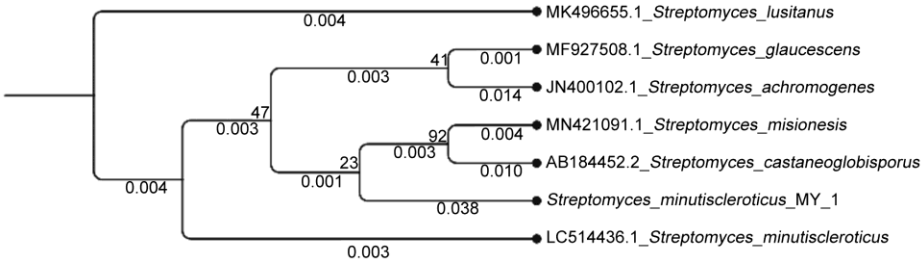

Fig. 4 — Dendogram indicating phylogenetic relationship of the genus *Streptomyces*. MY1 isolate shows 92% relatedness to *Streptomyces minutiscleroticus* constructed by neighbour joining method.

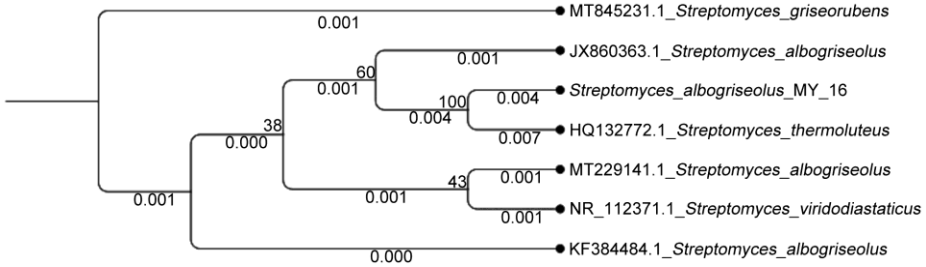

Fig. 5 — Dendogram indicating phylogenetic relationship of the genus *Streptomyces*. MY16 isolate shows 100% relatedness to *Streptomyces albogriseolus* constructed by neighbour joining method.

Streptomyces organisms inhibited the growth of the selected test organisms.

Peptide extraction to determine antibacterial activity

Streptomyces isolates were cultured in production media ISP2 for bioactivity determination. After a maximum growth on the 14th day of the fermentation the produced biomass was separated. The biomass was separated from the media broth and homogenized for intracellular homogenate extraction using 0.1 M phosphate buffer. Intracellular homogenate of the isolates was subjected to salting-in using ammonium sulphate up to 60% saturation. This step resulted in the precipitation of protein components present in the homogenate. These precipitated fractions were salted out by using a dialysis membrane against phosphate buffer and the fractions were resolubilized in a buffer as dialysate extract. An aliquot of the dialysate extract was estimated for the presence of protein by Lowry's method of estimation followed by SDS- PAGE separation. By performing SDS-PAGE technique, the peptides were separated on a 12.5% resolving gels that were visualized as distinct bands after Coomassie Brilliant blue staining procedure. Molecular weights of the peptide bands were determined when compared with the standard molecular weight marker as shown in Fig. 7a.

CM-Cellulose chromatography and LC-MS/MS analysis

Dialysate extracts obtained from the *Streptomyces* isolates were eluted using CM-Cellulose chromatography by the stepwise salt gradient using NaCl (0.1 to 2.0M) and eluted fractions were analyzed at 280 nm using Thermo scientific multiscan sky spectrophotometer. This showed prominent peaks and peak fractions were pooled into five fractions. The Pk4 and Pk5 samples from *S. albogriseolus* eluted using 2.0 M NaCl showed a significant antibacterial activity when tested against the test organisms by the well diffusion method Fig. 8. Also,

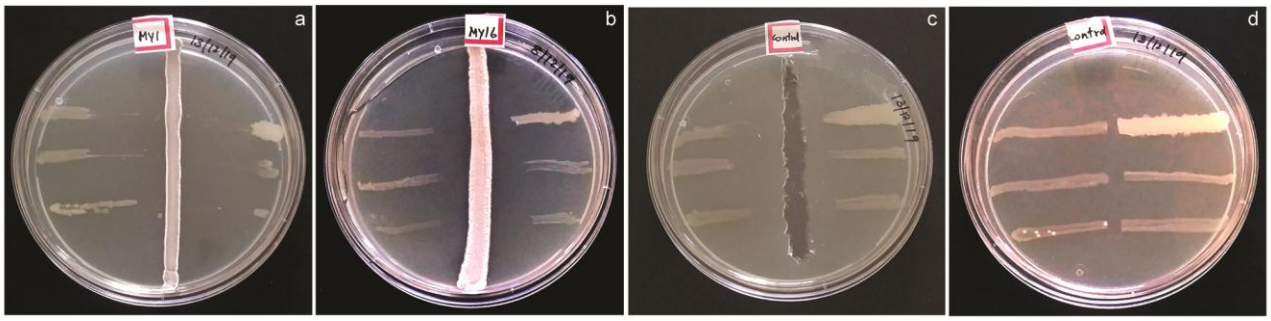

Fig. 6 — Screening of antibacterial activity by cross streak method, a) *Streptomyces minutiscleroticus*, b) *Streptomyces albogriseolus*, c) standard antibiotic streptomycin (0.025 mg/mL) and, d) growth of test organisms.

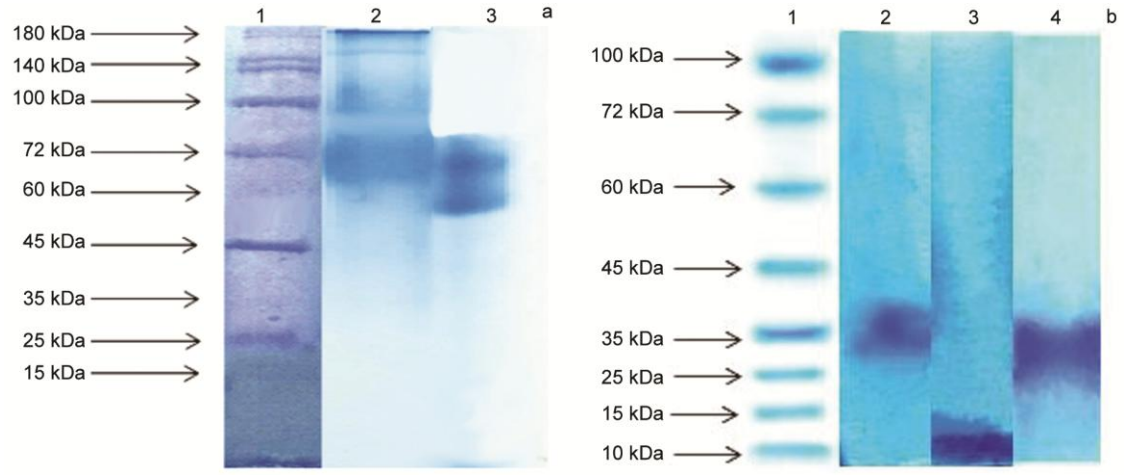

Fig. 7 — a) Electrophoresis separation of the protein extract obtained from *Streptomyces* isolates by SDS-PAGE technique
Lane 1->Molecular weight marker, Lane 2->Peptides separation from *Streptomyces minutiscleroticus*, Lane 3->Peptides separation from *Streptomyces albogriseolus*. b) Protein profile of CM-cellulose peptide fraction. Lane 1- molecular weight marker, Lane 2- *Streptomyces minutiscleroticus* Pk5 peptide separation, Lane 3- *Streptomyces albogriselous* Pk4 peptide separation, Lane 4- *Streptomyces albogriselous* Pk5 peptide separation .

from *S. minutiscleroticus*, the peptide fractions were eluted based on salt gradient and the fraction eluted at 1.0 M NaCl showed the maximum absorption peak at 280 nm exhibited significant antibacterial activity shown in Fig. 9 and estimated their zone of inhibition of about 10.0 mm against all the test pathogens. Reference control chloramphenicol 50 µL (1 µg/mL) measured 20.0 mm growth inhibition. This significantly proved that the peptides from *S. albogriseolus* and *S. minutiscleroticus* may be considered antimicrobial peptides with low molecular weight as shown in separation profile Fig. 7b. Antimicrobial peptides obtained from *S. albogriseolus and S. minutiscleroticus* were checked for minimum

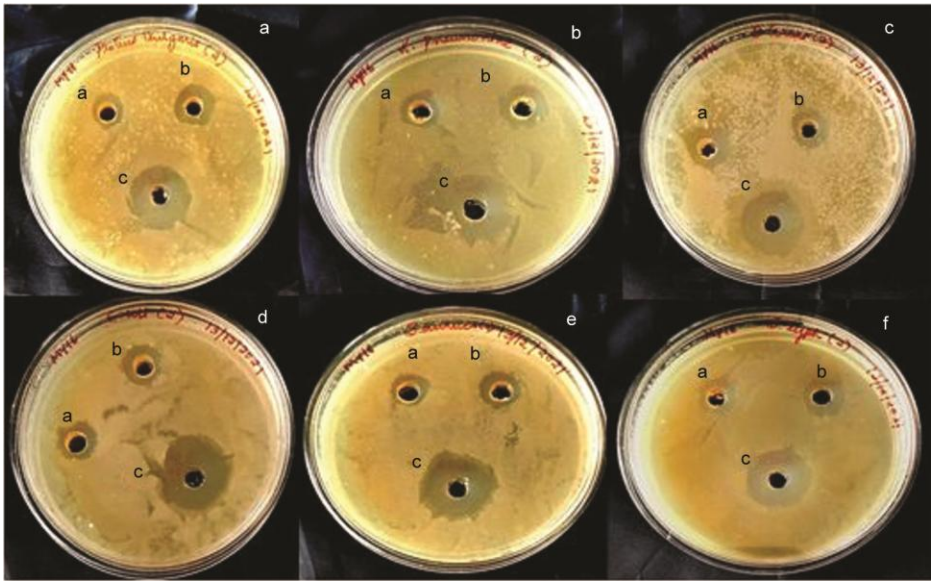

Fig. 8 — Antibacterial activity of *Streptomyces albogriseolus* peptide fractions Pk4 (a) and Pk5 (b) of the CM-Cellulose chromatography in comparison with standard antibiotic using chloramphenicol (1 µg/µL) (c). a) *Proteus vulgaris*, b) *Klebsiella pneumoniae*, c) *Bacillus cereus*, d) *Escherichia Coli*, e) *Staphylococcus aureus*, f) *Salmonella typhimurium*.

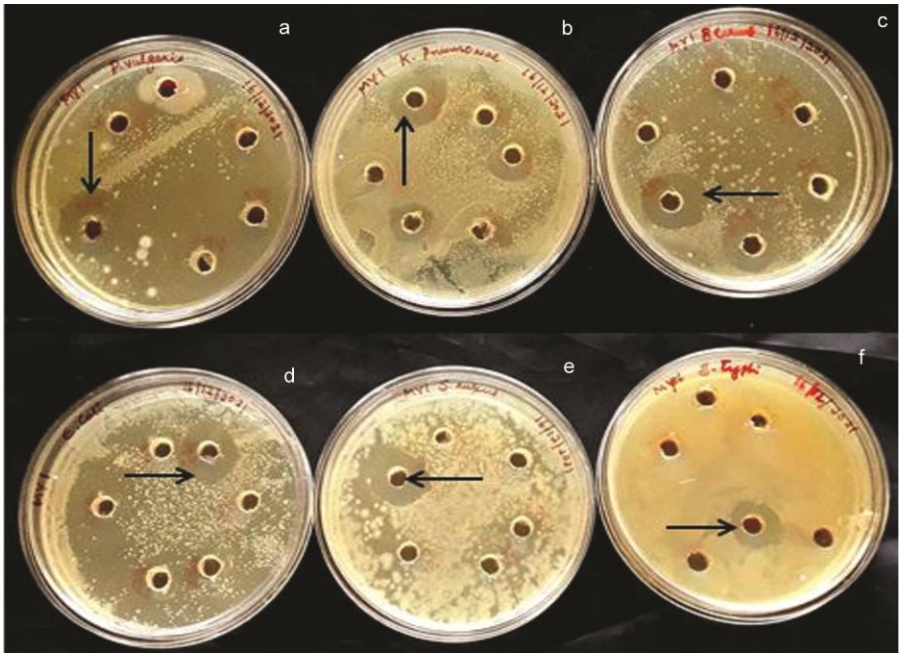

Fig. 9 — Arrow indicating theantibacterial activity of *Streptomyces minutiscleroticus* peptide fractions Pk5 eluted from 1M NaCl of the CM-Cellulose chromatography. a) *Proteus vulgaris*, b) *Klebsiella pneumoniae*, c) *Bacillus cereus*, d) *Escherichia Colli*, e) *Staphylococcus aureus*, f) *Salmonella typhimurium*.

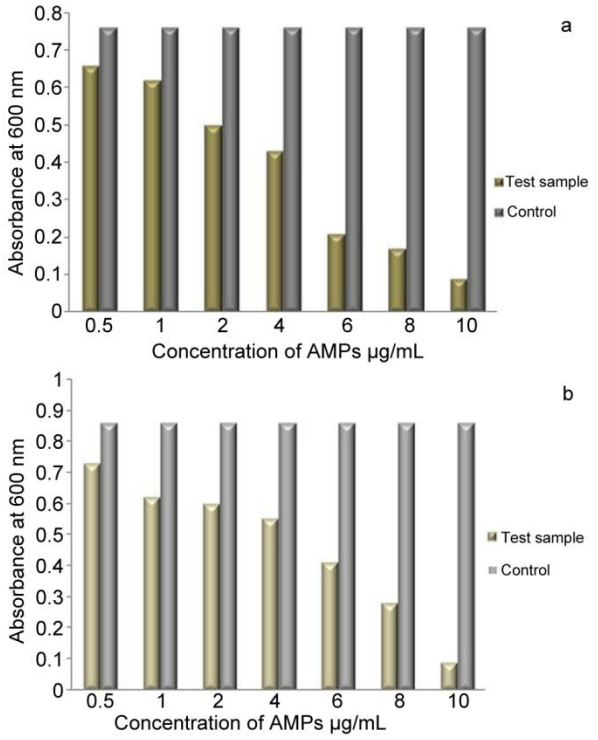

Fig. 10 — a) MIC values of *Streptomyces minutiscleroticus* AMP's against *E. coli*, b) MIC values of *Streptomyces albogriseolus* AMP's against *B. cereus*.

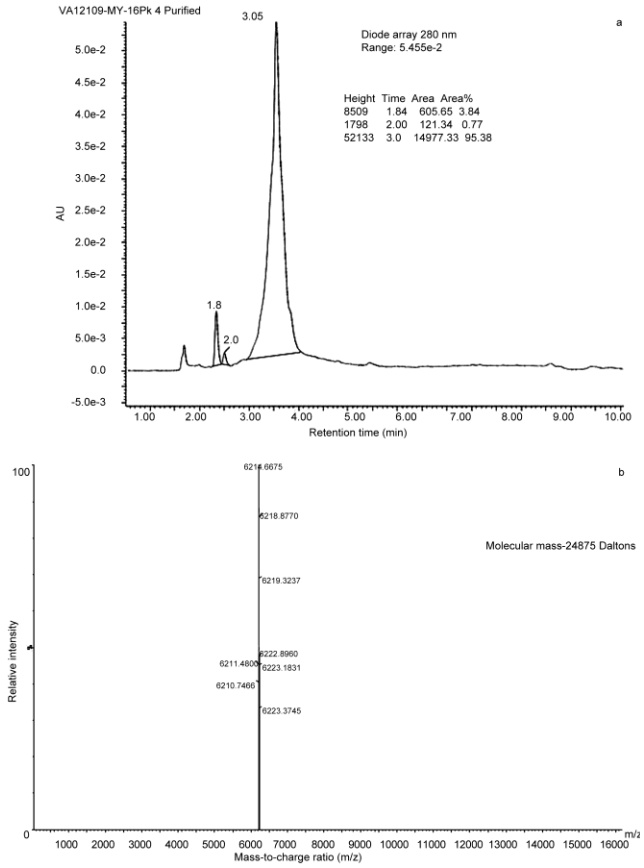

Fig. 11 — a) Maximum elution profile of *Streptomyces albogriseolus* Pk4 in liquid chromatography mass spectrometry at 3.05 min. b) Ultra performance liquid chromatography ionization/ quadrupole time of flight mass spectrometry (UPLC-ESI/QTOF-MS) analysis of *Streptomyces albogriseolus* Pk4.

inhibitory concentration (0.5, 1, 2, 4, 6, 8, 10 µg) against six test strains. Absorbance at OD_{600nm} was measured and compared with the growth of bacterial culture with no peptides. MIC values of both the isolates revealed significant inhibition against the bacterial growth represented in Fig. 10a and b. Further the *Streptomyces* antimicrobial peptides were subjected to LC-MS revealed the presence of peptides showing a single predominant peak of monomeric subunits with a mass charge ratio of Pk4 about 6000 m/z 24.87 kDa shown in Fig. 11a and b, and Pk5 about 5580 m/z 28.61 kDa shown in Fig. 12a and b from *S. albogriseolus*. From *S. minutiscleroticus* antimicrobial peptide Pk5 of 10000 m/z31.27 kDa were detected and depicted in Fig. 13a and b.

Discussion

The present investigation reveals, among the actinomycetes group of microorganisms the *Streptomyces spp.* isolated from mangrove soil in Mangalore coastal region have been characterized based on their microscopic, biochemical, and phylogenetic features. The strains of *Streptomyces spp.* showed distinct characteristics and thus identified as *S. albogriseolus* and *S. minutiscleroticus.* The earlier investigation reported that actinomycetes isolated from estuaries of the marine environment showed similar studies for colony morphology such as aerial mass and substrate mycelium with varied colour, powdery texture, and leathery appearance for most of the isolates[29].

In our study, the observation of surface morphology by coverslip culture method as performed has shown simple rectus and branched filaments. This coverslip technique was justified further by subjecting the pure cultures of *Streptomyces* spp. by scanning electron microscopy image analysis through gold particles staining. This gave more insight into information on the spore morphology and arrangement that aided in the identification of the two species of actinomycetes belonging to the *Streptomyces* genus. For species-level identification, phylogenetic analysis of the 16s rRNA gene sequence was carried out to confirm the identification of *S. albogriseolus* and *S.*

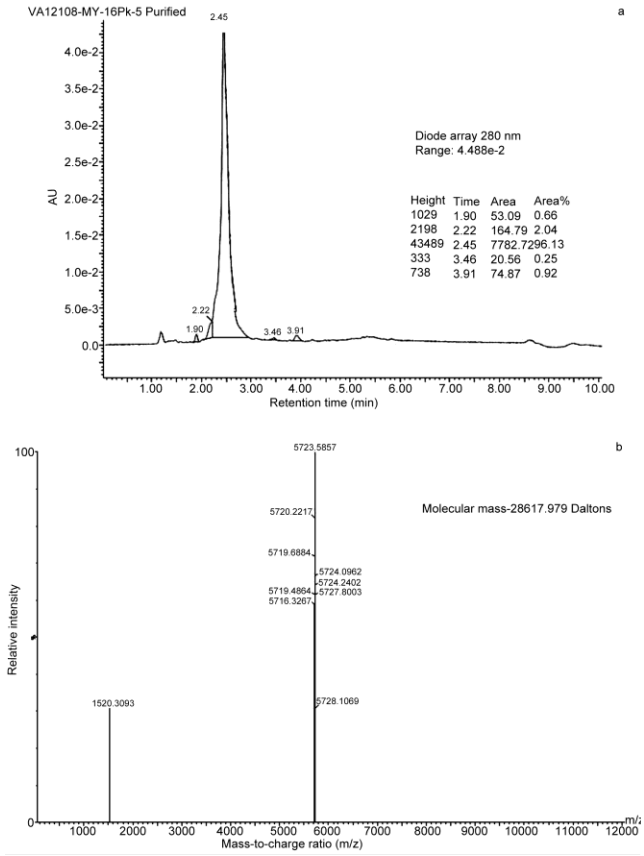

Fig. 12 — a) Maximum elution profile of *Streptomyces albogriselous* Pk5 in liquid chromatography mass spectrometry at 2.45 min. b) Ultra performance liquid chromatography ionization/ quadrupole time of flight mass spectrometry (UPLC-ESI/QTOF-MS) analysis of *Streptomyces albogriseolus* Pk5.

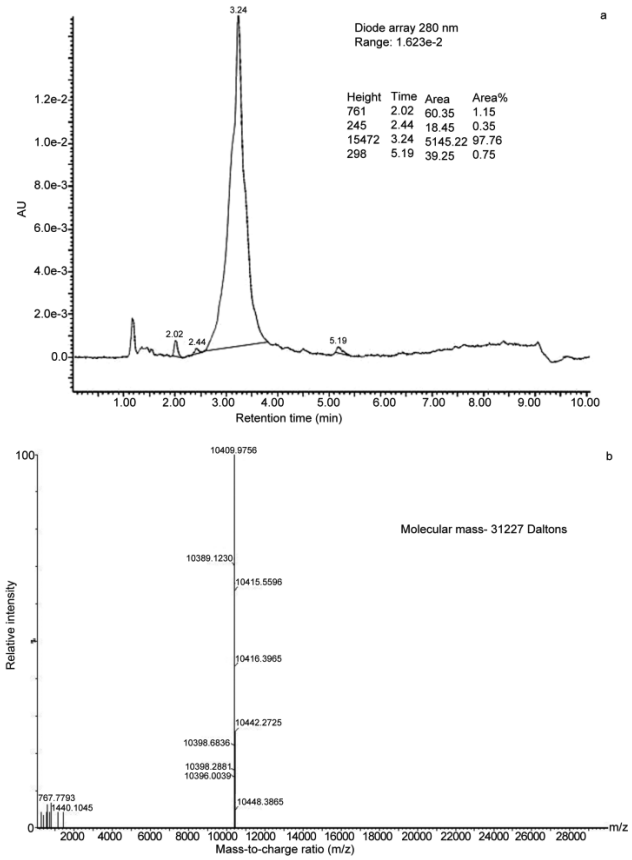

Fig. 13 — a) Maximum elution profile of *S. minutiscleroticus* Pk5 in liquid chromatography mass spectrometry at 3.24 min. b) Ultra performance liquid chromatography ionization/ quadrupole time of flight mass spectrometry (UPLC-ESI/QTOF-MS) analysis of *Streptomyces minutiscleroticuss* Pk5.

minutiscleroticus. The genomic DNA from the isolates were amplified with appropriate PCR conditions using gene-specific forward and reverse primers for 16s rRNA gene amplication. DNA was assessed and 16s rRNA sequence was imported to BLAST using NCBI Databases. The MY1 isolate initially characterized and determined as actinomycetes and belonging to *Streptomyces* genera revealed 92% identity for *S. minutiscleroticus*, with GenBank accession number MW479445. Similarly, the MY16 isolate showed 100% relatedness to *S. albogriseolus* with GenBank accession number MW479446.

In several reports phylogenetic analysis of *Streptomyces* spp. gave a vast phenotypic description to study the species differentiation. Molecular identification of 16s rRNA gene sequence of the isolate AA1 has had shown 100% similarity to *Streptomyces lincolnensis* detected in phylogenetic tree and their necleotide sequence was deposited in the GenBank database using BLAST Tool[30,31]. Similarly, our results on the BLAST tool and nucleotide deposition in the GenBank for the two species of *Streptomyces* with phylogenetic tree comparison are depicted in Fig. 4 and 5. These identified *Streptomyces* strains when tested by primary screening and extraction of peptides from the production media showed promising production of antimicrobial peptides. Studies have shown that the crude protein extract and novel peptides from actinobacteria exhibited antibacterial activity against the *Salmonella typhi* and other Gram-positive bacteria[32,33].

We performed the extraction of peptides followed by ammonium sulfate precipitation and salt removal by membrane dialysis. Thus this procedure allowed to concentrate the microbial peptides in a significant amount that could be determined by the Lowry's method of estimation. Both the *Streptomyces* organisms initially yielded significant peptides with a

comparable molecular weights of 62 and 70 kDa in the dialysates. Results of the present study support that the *Streptomyces* isolates of mangrove sediments have prospective activity in producing novel antimicrobial peptides. In a previous report purification and characterization of actinomycins from *Streptomyces* active against Methicillin resistant *Staphylococcus aureus* were determined[34].

Our work proceeded further in the identification of peptides from *S. albogriseolus* and *S. minutiscleroticus* by subjecting them to CM-Cellulose chromatography. The CM-pooled fractions were analyzed for antibacterial activity. We could demonstrate the antimicrobial activity of these microbial peptides against human pathogenic strains such as *Bacillus cereus* ATCC 10876, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumonia* ATCC9621, *Salmonella typhimurium* ATCC 23564, *Escherichia coli* ATCC 8739. In a recent study, *Bacillus* antimicrobial peptide produced by *Bacillus paralicheniformis* was found to exhibit a bacteriostatic effect on *Salmonella typhi* and *Listeria monocytogenes*[35].

In our study, peptide purification was carried out by ion-exchange chromatography on Carboxymethyl (CM)-cellulose with 0.1 M sodium phosphate buffer pH 7.2. Bound proteins which showed antibacterial activity was eluted by a series of 1 and 2M NaCl gradients were pooled and the SDS-PAGE protein profile showed the presence of a single major peptide band with low molecular weight in the peak fractions. This data was further confirmed by LC-MS analysis determined the mass charge range that showed the single predominant peak at various retention times. The peak fractions obtained from *S. albogriseolus* Pk4 showed 24.87 kDa, Pk5 showed 28.61 kDa and *S. minutiscleroticus* Pk5 showed 31.27 kDa. Previous reports on optimization of antimicrobial peptides extraction and LC-MS protocol were similarly carried out to confirm the quantitative analysis in biological samples[36].

Our study reports the occurrence of *Streptomyces* sp. producing antimicrobial peptides from unique habitats such as mangrove soils on Mangalore Coasts. Confirmation of antimicrobial activity was checked by determining the MIC values of the peptides taken into consideration on Lowry's protein quantification. In both the species of *Streptomyces*, we could observe around 0.01 mg/mL concentration was effective in bactericidal effect by broth dilution method that could inhibit the test organisms growth. Thus, we could demonstrate the active peptides isolated from *S. minutiscleroticus* and *S. albogriseolus* from the mangrove soil of the Mangalore coast has antimicrobial potential. These peptides certainly serve as alternative therapeutic peptides of microbial origin for target-specific mechanisms as antimicrobials against resistant pathogens. Also, the *Streptomyces* peptides could be delineated to identify their role in many cellular mechanisms of deregulated diseases such as cancer and other pharmacologically important pathways.

Conclusion

In the current study peptides from *Streptomyces minuitiscleroticus* and *Streptomyces ablogriseolus* obtained through intracellular extraction can be considered effective antimicrobial peptides. These intracellular bioactive peptides showed promising inhibitory activity against test pathogens and the present investigations can be a source for future research progression towards mechanism-based specific activity against resistant microbes.

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Conflict of interest

All the authors declare no conflicts of interest.

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