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DIVERSITY AND SCREENING OF POTENT ACTINOMYCETES FROM THE MARINE ENVIRONMENT

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Abstract

To evaluate the antimicrobial activity of Actinomycetes species isolated from marine environment. Five fifty nine strains of Actinomycetes were isolated from the samples collected from Marine environment Rajakkamangalam, Thengapatinam, Manakudy, Tirechendur and Manapad. Both soil sediments and associated actinomycetes were isolated. Preliminary screening was done using cross-streak method against gram-positive and gram-negative bacteria. The most potent strains MA7 were selected based on the antibacterial screening and antognostic effect against *Pesudomonas* sp,. Molecular identification of MA7 as *Streptomyces purvas* was deposited to GenBank sequence database with the following accession number: OR921611. All those twenty one isolates were active against at least one of the test organisms. Morphological characters were recorded. The results of the present investigation reveal that the marine Actinomycetes from coastal environment are the potent source of novel antibiotics. Isolation, characterization and study of Actinomycetes can be useful in discovery of novel species of Actinomycetes

Introduction

Actinomycetes are the most economically and biotechnologically valuable prokaryotes able to produce wide range of bioactive secondary metabolites, such as antibiotics, antitumor agents, immunosuppressive agents and enzymes. These metabolites are known to posses antibacterial, antifungal, neuritogenic, anticancer, antialgal, antimalarial and anti-inflammatory activities (Ravikumar et al.,2011).

The intertidal region of tropical and sub-tropical ecosystem made by more saline content, having excellent reservoir of rich bioactive metabolites (Arumugam et al., 2017). It is a unique environment for the survival of microorganisms and its depends on the microbial diversity based on the supplied nutrient. This unique environment having punch of secondary metabolites that exhibited excellent biological properties against various infections. For synthesis of available

bioactive metabolites, various factors are involved and contributed in the metabolic pathways that led to the biosynthesis of unique bioactive compounds (Dandan *et al*., 2018). Among the microbial community, bacteria is the most dominant microbes and available 90% and less than 1% of the marine microbes only identified and used for bio-metabolites identification (Kafilzadeh and Dehdari, 2015). Compared to terrestrial, less than 5% of the marine microbes are identified and reported till-date.

Among the microbial community, actinomycetes are a superior bacteria and it is a power of complex secondary metabolites. It is an aerobic, fungus like bacteria, rich GC content of genetic materials, aerobic, branched and unicellular (Hozzein et al., 2019). Among the actinomycetes, various genus are available with excellent biomedical characteristic nature including Streptomyces, Nocardiopsis, micromonospora, Saccharopolyspora, Amycolatopsis, Actinomadura and Actinoplanes (Behera et al., 2017; Hames-Kocabas and Uzel, 2012). In particular, the genus Streptomyces is most reported actinomycetes having excellent bioactivity molecules producing ability compared with others. In the present investigation, an effort was made to screen different marine sediments which is a large unscreened and diverse ecosystem for the isolation of potent antibiotic producing Actinomycetes.

Materials & Methods

Isolation of actinomycetes from Marine environment

The soil sediments and associated actinomycetes from marine organisms were collected at five different places of Kanyakumari and Tirunelveli District, Southeast Coast of Tamil Nadu, India. The clear soil of one gram was suspended into nine mL sterile saline water, and spins the mixture for 2 min. Then, the supernatant of the sample was diluted serially with 10^{-4} –10^{-7}, and it spread on Starch casein agar plate and allowed to maintain until 7 days for proper growth. After 7 days incubation, the original, well matured, spore forming ability of the actinomycetes were screened. All the emerged strains were picked and separately streaked on Starch casein agar slant.

Seaweeds of both drifted and alive were collected, washed with seawater followed by freshwater to remove the debris. Carried to the laboratory with polythene bags, one gram of seaweed was crushed in mortar and pestle, the extract was suspended in nine ml sterile saline and vortex for 2 min. Then, the supernatant of the sample was diluted serially with 10^{-4} –10^{-7}, and it spread on Starch casein agar plate and allowed to maintain until 7 days for proper growth. After 7 days incubation, the original, well matured, spore forming ability of the actinomycetes were screened.

Characterization of actinomycetes

All the morphological, biochemical and phonotypical characterization of the isolated actinomycetes colonies were identified by light microscope and noted based on the observation (Venkata Raghava Rao and Raghava Rao, 2013). The smear of the selected actinomycetes strains

were carried out with using Gram staining kit and then, reverse side pigment, melanoid pigment aerial, substrate mycelium, and spores formation was observed at 40xmagnification.

Screening the isolates against the pathogens

Screening of actinomycetes was performed on the Mueller–Hinton agar medium employing the perpendicular streak method. In the sterile agar medium, the pure isolate of actinomycetes was streaked along the diameter of the plate. The plate was incubated at 28°C for 7 days. Pure colony of test bacteria *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa,* Klebsiella pneumoniae, *Clostridium perfingens, Staphylococcus pyogenes,* and *Salmonella typhi, Bacillus subtilis* was transferred into fresh nutrient broth and incubated at 37°C for 24 hours until the visible turbidity. After adjusting the turbidity equal to that of 0.5 McFarland with the cell count of 1.5×10^8 , the test organisms were streaked perpendicular to the isolate. The plates were further incubated at 37°C for 24 hours, and the antimicrobial activity was estimated from the zone of inhibition of test organism (Rai *et al*.,2016). . Of all the isolates, one best antagonistic Actinomycetes isolates were selected, identified macroscopically and microscopically by Gram's staining and used for further studies

DNA extraction, 16S rRNA sequencing

The method described by Ausubel et al.,1994 was slightly modified and used for genomic DNA isolation. The 72 h cultures grown on Actinomycetes isolation agar were scraped and suspended in 1.5 mL 1xTE buffer. Cells were pelleted by centrifugation for 5 min at 8 000 rpm. Supernatant was discarded and pellet was resuspended in 567 µL of 1xTE buffer. Afterwards 30 µL of 10% sodium dodecyl sulfate (SDS) and 3 µL of 20 mg/mL proteinase K were added. The eppendorf tubes were mixed thoroughly and the samples were incubated for 1 h at 37°C. Then 100 µL of NaCl solution was added and mixed thoroughly and the samples were then incubated for 10 min at 65°C. Chloroform extraction was performed twice using one equal volume of chloroform/isoamyl alcohol (24:1). First one equal volume of chloroform/isoamyl alcohol was added and the samples were centrifuged for 5 min at 10 000 rpm. The aqueous phase was transferred into a new eppendorf tube and chloroform extraction was repeated. The aqueous phase was transferred into a clean eppendorf tube. DNA wool was obtained by the addition of 0.6 volume of isopropanol. The DNA wool was transferred into a new eppendorf tube containing 500 µL ethanol (70%) and washed. When DNA was not visible after the isopropanol addition, these samples were centrifuged for 10 min at 10,000 rpm to pellet genomic DNA. After discarding the isopropanol, genomic DNA was washed with 500 µL 70% ethanol. DNA was pelleted, dried (10 min at 37°C) and dissolved in 200 µL 1xTE using alternating heat/cold shocks (10 min at 80°C, 20 min at -20°C twice). Afterwards phenol/chloroform extraction was performed in order to purify DNA. One and a half volume of phenol was added and mixed slowly. After that 1.5 volume of chloroform/ isoamyl alcohol was added, mixed and centrifuged for 2 min at 8 000 rpm. The aqueous phase was transferred into a new eppendorf tube and 300 µL of chloroform/isoamyl alcohol was added and mixed. It was centrifuged for 2 min at 8 000 rpm and upper phase was

transferred into a new tube. DNA was precipitated by adding 1/10 sample volume of 6 M NaCl. The sample was mixed well.

Two volume of 99% ethanol was then added and mixed thoroughly. The samples were then centrifuged for 15 min at 8 000 rpm. The liquid phase was removed and the pellet was washed with 300 µL of 70% ethanol. After centrifugation for 5 min at 8 000 rpm, ethanol was removed without disturbing the pellets. The samples were centrifuged for 20 sec at 8000 rpm. Excess ethanol was removed and the pellets were dried for 10 min at 37°C. Finally according to the pellet size, appropriate amount of 1xTE (50, 100, 150 and 200 µL) was added and DNA was dissolved by alternating cold-heat shock for 10 min at 80°C and 20 min at -20°C. Dissolved genomic DNA samples were stored at -20°C (Lemons *et al*., 1985).

The purity of DNA solutions was checked spectrophotometrically at 260 and 280 nm, and the quantities of DNA were measured between 260 and 280 nm. The 16S ribosomal RNA gene was amplified by using the PCR method with Taq DNA polymerase and primers F (5'AGAGTTTGA TCCTGGCTCAG 3') and R (5'ACGGCTACCTTGTTACGACTT 3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 5 min followed by 30 cycles at 94°C for 45 sec, primer annealing at 42°C for 1 min and primer elongation at 72°C for 40 sec. At the end of the cycling, the reaction mixture was held at 72°C for 10 min (Weisburg et al.,1991). PCR amplification was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer. The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http://www. ncbinlm-nih.gov/.

Results

Description of the study area

The soil sediments were collected from the marine environment such as estuary, coastline and marine organism such as seaweed, barnacles and sponges Map.1.

Sampling Site I: Rjakkamangalam (Map-1) geographically located in the lattitude 8°6'50"N and longitude 77°22'43"E of Kanyakumari district. The Rajakkamangalam estuary contains a coastal ecosystem with a mangroves habitat, sand dunes. This estuary is considered one of the breeding and feeding grounds for many birds like painted stork, cormorant, spot billed pelicans, purple swaphen, dabchick, garganey, purple heron, open bill stork, black winged stilt, etc.

Sampling Site II: Thengapattinam (Map-1) in Kanyakumari district is a less-explored, serene sandy beach fringed by coconut groves and palms. It is noted for its estuary where Thamirabarani river merges with the Arabian Sea.

Sampling Site III : Manakudy estuary (Map-1) is a fishing hamlet facing the Arabian Sea on the west coast of Kanyakumari. 8km from Kanyakumari, The Manakudy estuary is the confluence of river Pazhayar and has an area of about 150ha. It is a sand built estuary connected to the Sea during the rainy season. Kanyakumari District is located on the southern extremity of the Indian Peninsula between lat 8 0 2 ’ and 80 4 ’N and between long 770 26’ and 770 30’E. Pazhayar is one of the main river systems in the District and this river takes its origin at the Western ghats. From the place of its origin it traverses 23.1km before entering the Arabian Sea through the Manakudy estuary.

Map 1: Study area

Sampling Site IV: Tiruchendur (Map-1) is located on the shoreline overlooking the Bay of Bengal in the south-eastern part of Tamil Nadu. Thiruchendur or Thiruchendur Alaivai is a holy place near Tirunelveli, in Tamil Nadu. It is situated on the coast of Manner. It is 40 Kms from Tuticorin. Geographically it is located between 9’00 to 7’30’N latitude and 78 to 30’E longitude. The physiographic feature of the coast is raised beaches with sand bars parallel to the present coastline.

Sampling Site V: Manapad (Map-1) is a coastal village in far-southern Tamil Nadu, India. It is situated in the Gulf of Mannar Biosphere Reserve and is 11 kilometres from Tuticorin and 12

kilometres south of Tiruchendur (GOMBR). Near Manapad, there are a lot of coastal communities where fishing is the primary industry.

Table 1. Total number of isolates from the respective sample station with different Marine sampling sources

Place	Source	Species name	count (CFU/cm^4)
Arabian Sea **-** 368 X10^4			
Rajakkamangalam 122 X10^4	Seaweed (RSA) 7X10^4	1.*Gracilaria edulis* 2.*Caulerpa Scalpeliformis*	2X10^4 5X10^4
	Barnacles (RBA)	*Balanus amphitrite*	11X10^4
	Sponges (RSpA)		15X10^4
	Soil sediment (RES)	Estuary soil sediment	11X10^4
	Soil sediment (RCS)	Coastal shore soil	47X10^4
	Mangrove sediment (RMS)	Mangrove root soil sediment	31X10^4
Thengapatinnam 128 X10^4	Seaweed (TSA) 36X10^4	1.*Sargassum sp* 2.*Caulerpa Scalpeliformis* 3. *Padina* sp	11X10^4 12X10^4 11X10^4
	Barnacles (TBA)	*Balanus amphitrite*	9X10^4
	Sponges (TSpA)		10X10^4
	Soil sediment (TES)	Estuary soil sediment	23X10^4
	Soil sediment (TCS)	Coastal shore soil	24X10^4
	Mangrove sediment (RMS)	Mangrove root soil sediment	28X10^4
Manakudy 118X10^4	Seaweed (MSA) 46 X10^4	1.*Gracilaria edulis* 2.*Caulerpa Scalpeliformis* 3. *Ulva lactuca*	16X10^4 10X10^4 15X10^4

	Barnacles (MBA)	*Balanus amphitrite*	$13X10^4$
	Sponges (MSpA)		$10X10^4$
	Soil sediment (MES)	Estuary soil sediment	$15X10^4$
	Soil sediment (MCS)	Coastal shore soil	$19X10^4$
	Mangrove sediment (RMS)	Mangrove root soil sediment	$20X10^4$
***Bay of Bengal* -**$181 X10^4$			
Tiruchendur $96X10^4$	Seaweed (BSA) $23 X10^4$	1.*Gracilaria edulis* 2.*Caulerpa Scalpeliformis* 3.*Padina tetrastromatica*	$6X10^4$ $5X10^4$ $12X10^4$
	Barnacles (BBA)	*Balanus amphitrite*	$11X10^4$
	Sponges (BSpA)		$5X10^4$
	Soil sediment (BES)	Estuary soil sediment	$21X10^4$
	Soil sediment (BCS)	Coastal shore soil	$19X10^4$
	Mangrove sediment (RMS)	Mangrove root soil sediment	$17X10^4$
Manapad $85X10^4$	Seaweed (BMSA)	1.*Gracilaria edulis* 2.*Caulerpa Scalpeliformis* 3. *Ulva lactuca*	$9X10^4$ $5X10^4$ $7X10^4$
	Barnacles (BMBA)	*Balanus amphitrite*	$11X10^4$
	Sponges (BMSpA)		$5X10^4$
	Soil sediment (BMES)	Estuary soil sediment	$11X10^4$

	Soil sediment (BMCS)	Coastal shore soil	$19X10^4$
	Mangrove sediment (RMS)	Mangrove root soil sediment	$18X10^4$

A total of 559 isolates were isolated from the soil sediments and associated organism. The numbers of samples and isolates in each sample were presented in Table 1. Out of the 559 isolates, active strains 20 were screened and then identified as *Streptomyces spp*. (12 isolates), *Nocardia spp*. (3 isolates), and *Micro monospora* spp. (7 isolates) on the basis of microscopic observation and biochemical tests, as shown in Figure 1 and Table 2. Most isolates gave different pigmentation, as shown in Figure 2.

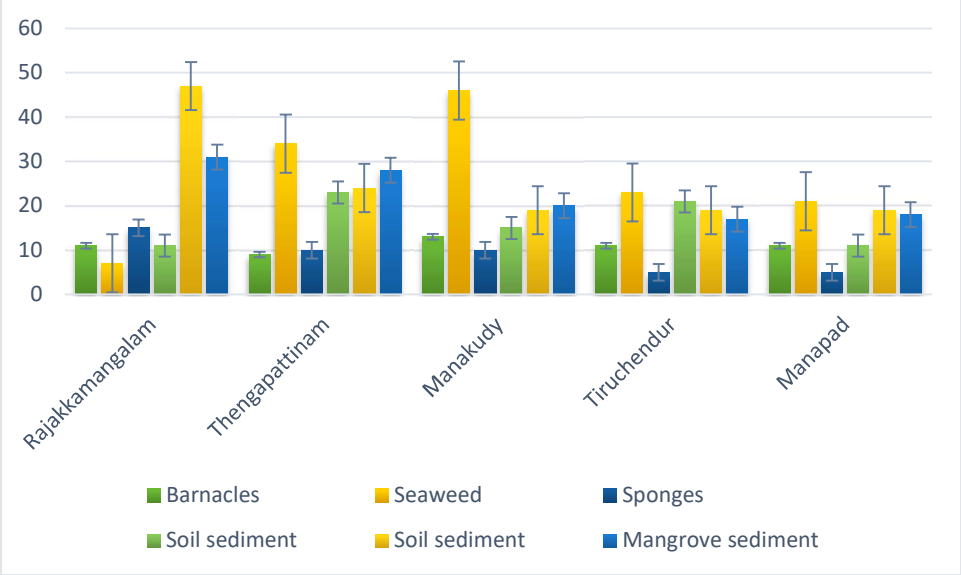


Fig. 2 Stationwise actenomycetes population from various marine environment

Totally 559 strains were isolated from the study area among them Arabian sea study location was loaded with $368X10^4$ and comparatively low population 181 $X10^4$ was recorded in Bay of bengal. (Fig.3). Rajakkamangalam and Manakudy was recorded high actinomycetes load when compared with other sample sites (Fig.2).

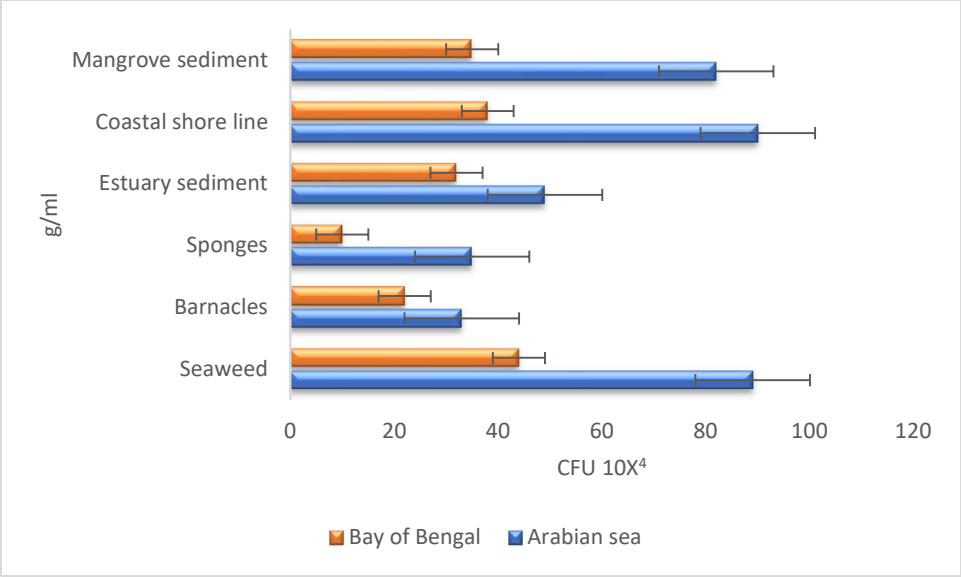


Fig.3 Actinomycetes density in two different coast

Table 2: Enzymatic characteristics of the isolates.

Sample	Cat	Oxi	Gel	Amy	Lip	Cas
MA1	-		+	+	-	-
MA2	+	+	-	-	-	-
MA3	+	-	+	+	+	+
MA4	-	-	+	+	-	+
MA5	+	-	+	+	-	+
MA6	-	+	+	+	+	+
MA7	+	+	+	+	+	+
MA8	+	+	+	+	+	+
MA9	-	+	+	+	+	+
MA10	-	-	+	+	+	+

MA1

MA2

MA3

MA4

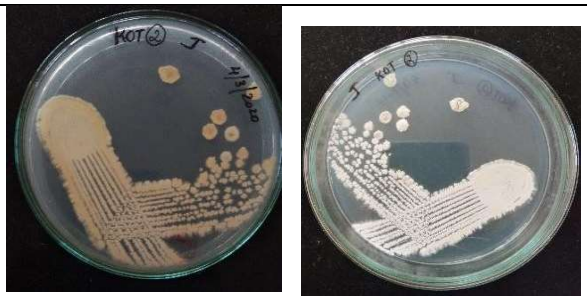
MA5

MA6

MA7

MA8

MA9



MA10

Plate 1: Actinomycetes Isolates from marine ecosystem

The color of colonies varied accordingly from yellow (48%), brown (10%), gray (13%), and grayish white (6%) and 3% had, blackish grey, , light pink, and greyish black to white pigments. Plate.1 shows the production of pigment by MA5, MA6, MA7, and MA8 isolates.

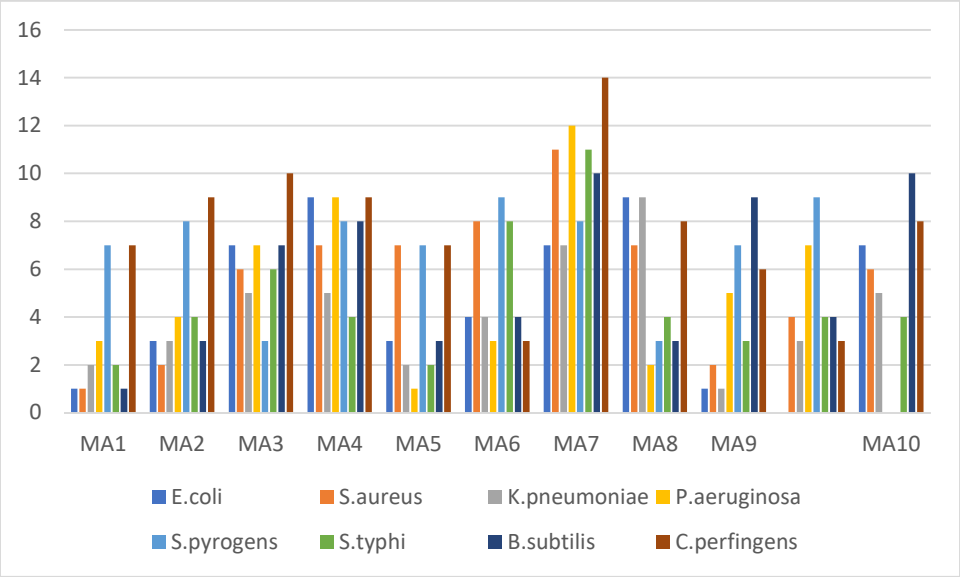


Fig.4. Screening the isolates against Human pathogens

The identified isolates were then subjected to primary screening as presented in Table 1. one culture i.e. MS7 were the only strains selected for further analysis, since they showed significant antibacterial activity against test organisms. The results were shown in Table 3. The cultures MS7 were identified and confirmed by microscopic and macroscopic examination. MS7 strain is a gram positive, long spore chain, and filamentous bacteria. The macroscopic appearance of the isolate MS7 showed leathery, white powdery colonies in Actinomycetes isolation agar. The highest inhibition was shown by the cultures ma7 against *S.aureus, S.typhi, c. perfringens* and *B.subtilis*

Molecular identification

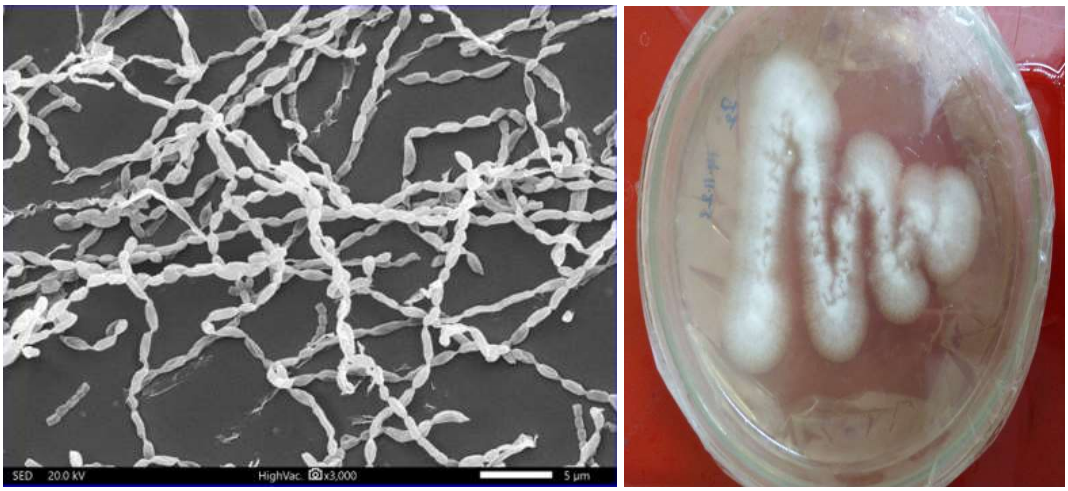


Fig.5. Spore chain morphology of marine actinobacterium *Streptomyces parvus*- MA7

The obtained sequence of the 16S rRNA gene indicated the promising actinomycete isolate from the present study (isolate MA7 from Mangrove sediment in Rajakkamangalam, Arabian sea), which was similar to *S. parvus* with a maximum identity of 98%. The nucleotide sequence was deposited to GenBank sequence database with the following accession number: OR921611.

Discussion

Actinomycetes comprise 10% of the total bacteria colonizing marine aggregates. Marine habitat has been proven as an outstanding and fascinating resource for innovating new and potent bioactives producing microorganisms. Marine microbes are particularly attractive because they have the high potency required for bioactive compounds to be effective in the marine environment, due to the diluting effect of sea water. Members of the Actinomycetes, which live in marine environment, are poorly understood and only few reports are available. Actinomycetes account 70% of the earth's surface and represent attractive source for isolation of novel microorganisms and production of potent bioactive secondary metabolites (Usha et al., 2011)

The present study was aimed to isolate Actinomycetes from marine environment and screen them for the production of secondary metabolites. In the present study the medium was supplemented with amphotericin B to eliminate the fungal contamination. The same method was previously done by Remya and Vijayakumar (Remya and Vijaykumar, 2008).

Production of antibiotic substance is sea water dependent (Remya and Vijaykumar, 2008). In the present study also, the Actinomycetes isolation agar medium was prepared using sterile sea water. Okazaki and Okami observed that compared to other Actinomycetes, Streptomyces species showed efficient antagonistic activity (Okazaki and Okami,1972).

This was similar to the present investigation which also showed efficient antagonistic activity of Streptomyces species. The isolated Actinomycetes were identified based on the colony

morphology and Gram staining (Holt et al., 1994) . In the present work, we have identified the Actinomycetes by the presence of powdered colonies on the surface of agar plate. Actinomycetes are gram positive and filamentous in nature. Muth et al also stated the filamentous nature of Actinomycetes which are gram positive (Kokare et al.,2004). According to Kokare et al during the screening of the novel secondary metabolites, Actinomycetes isolates are often encountered which showed more active antimicrobial activity against gram positive bacteria than gram negative bacteria. Streptomyces species showed significant antibacterial activity against Staphylococcus aureus and Pseudomonas aeruginosa (Devi et al.,2006).

This was similar to the present findings. In the current study, also the Streptomyces species showed a good antimicrobial activity against Staphylococcus species, B. subtilis, Ethan gram negative Pseudomonas species and E.coli. The present study agreed with the earlier findings of Devi et al in which it has been reported that Streptomyces species showed significant antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa and E.coli (Dhanasekaran etal.,2009).

Dhanasekaran et al.,2009 found that estuarine Actinomycetes, which remained largely, ignored, showed promising antibacterial activities (Rabbani et al.,2007, Reddy et al.,2011). The promising antibiotic producing isolates were identified as Streptomyces species. The present study also showed similar findings. Rabbani et al stated that the accuracy and specificity of polymerase chain reaction amplification suggest more dominant role than culture method. For such a screening, amplification of 16S rRNA and other genotypic approaches are taking over traditional ways.

Boudemagh et al.,2005 also mentioned in his work that molecular approaches for identification are often used due to their speed and efficiency. Among the used methodologies the reaction in chain of the polymerase chain reaction is widely practiced. The gene 16S rRNA is the tool mainly used for molecular identification of bacteria. It is a chromo sonic gene present in all bacteria species (universal gene) whose sequence is specific to each species and whose ends 5′ and 3′ (15 first and 15 last bases) are conserved in all bacteria species. In this study, we amplified 16S rRNA gene, eluted, sequenced and analyzed by BLAST, MA7 were identified as Streptomyces purvas after BLAST. In the present investigation, it has been observed that compared to other Actinomycetes, Streptomyces species showed efficient antagonistic activity. Only very few reports are available on the occurrence and distribution of antagonistic Streptomyces in the marine environment. The marine Streptomyces have not received much attention. Recent investigations indicate that the tremendous potential of marine Actinomycetes, particularly Streptomyces species

Conclusion

The marine Streptomyces have not received much attention. Recent investigations indicate that the tremendous potential of marine Actinomycetes, particularly *Streptomyces purvas* species as a useful and sustainable source of new bioactive natural products. Thus, the results of the present

investigation reveal that the marine Actinomycetes from coastal environment are a potent source of novel antibiotics. It is anticipated that isolation, characterization and study of Actinomycetes can be useful in the discovery of novel species of Actinomycetes. Actinomycetes are the most important resources of these secondary metabolites.

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