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Antimicrobial potential of *Actinomycetes* species isolated from marine environment

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ABSTRACT

Objective: To evaluate the antimicrobial activity of *Actinomycetes* species isolated from marine environment. **Methods:** Twenty one strains of *Actinomycetes* were isolated from samples of Royapuram, Muttukadu, Mahabalipuram sea shores and Adyar estuary. Preliminary screening was done using cross-streak method against two gram-positive and eight gram-negative bacteria. The most potent strains C11 and C12 were selected from which antibacterial substances were extracted. The antibacterial activities of the extracts were performed using Kirby-Bauer disc diffusion method. Molecular identification of those isolates was done. **Results:** All those twenty one isolates were active against at least one of the test organisms. Morphological characters were recorded. C11 showed activity against *Staphylococcus* species (13.0±0.5 mm), *Vibrio harveyi* (11.0±0.2 mm), *Pseudomonas* species (12.0±0.3 mm). C12 showed activity against *Staphylococcus* species (16.0±0.4 mm), *Bacillus subtilis* (11.0±0.2 mm), *Vibrio harveyi* (9.0±0.1 mm), *Pseudomonas* species (10.0±0.2 mm). 16S rRNA pattern strongly suggested that C11 and C12 strains were *Streptomyces* species. **Conclusions:** 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*.

1. 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[1].

Actinomycetes have the capacity to synthesize many different biologically active secondary metabolites such as cosmetics, vitamins, nutritional materials, herbicides, antibiotics, pesticides, anti-parasitic and enzymes like cellulose and xylanase used in waste treatment[2]. They are free living, saprophytic bacteria, and a major source for the production of antibiotics[3].

As the frequency of novel bioactive compounds discovered from terrestrial *Actinomycetes* decreases with time, much attention has been focused on screening of *Actinomycetes* from diverse environments for their ability to produce new secondary metabolites. Studies have shown that *Actinomycetes* isolated from the marine environment are metabolically active and have adapted to life in the sea. *Streptomyces* are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites[4].

More than 70% of our planet's surface is covered by oceans and life on Earth originated from the sea. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than that in the tropical rainforests. As marine environmental conditions are extremely different from terrestrial ones, it is summarized that marine *Actinomycetes* have different characteristics from those of terrestrial counterparts and therefore might produce different types of bioactive compounds[5].

Around 23 000 bioactive secondary metabolites produced

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by microorganisms have been reported and over 10 000 of these compounds are produced by *Actinomycetes*, representing 45% of all bioactive microbial metabolites discovered[6]. Among *Actinomycetes*, around 7 600 compounds are produced by *Streptomyces* species. Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry[7,8].

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*.

2. Materials and methods

2.1. Sample collection and processing

Soil samples were collected from Royapuram, Muttukadu, Mahabalipuram sea shores and Adyar estuary. Using sterile techniques, samples were collected which were serially named as S1, S2, S3, S4 and then they were transported to the laboratory and stored at 4 ℃. The collected soil samples were mixed thoroughly and passed through a 2 mm sieve to remove gravel and debris. Then each sample was dried overnight at 27 ℃ to eliminate the bacterial growth.

2.2. Isolation of Actinomycetes and maintenance

From each dried soil sample (S1, S2, S3, S4), 1 g was taken and mixed separately with 9 mL of sterile distilled water. *Actinomycetes* isolation agar was prepared for each sample using sterilized seawater (100 mL) and autoclaved. A volume of 5 mL of the above suspension was added to 100 mL of *Actinomycetes* isolation agar and to that, 50 μL of amphotericin B was added and mixed well. About 20 mL of media was poured without air bubbles. Then the plates were incubated at 30 ℃ for 14 days. Powdered colonies were observed on the fourth day. Pure cultures of these were obtained by selection and were named as C1, C2 C3, C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, and C21 and stored at 4 ℃. For longer storage, it was grown on nutrient broth for seven days and glycerol was added to make the final concentration of 15% and stored at -20 ℃[9].

2.3. Preliminary screening of Actinomycetes for antibacterial activity by cross-streak method

Isolated strains C1 to C21 were inoculated onto nutrient agar plates by single streak in the center. The plates were incubated at 30 ℃ for 3 days. Ten bacteria *i.e. Staphylococcus* species ATCC 1026, *Bacillus subtilis* (*B. subtilis*) ATCC 11774, *Proteus mirabilis* (*P. mirabilis*) ATCC 12453, *Escherichia coli* (*E. coli*) ATCC 11229, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 10031, *Pseudomonas* species ATCC 10145, *Proteus vulgaris* (*P. vulgaris*) ATCC 49132, *Vibrio fischeri* (*V. fischeri*) ATCC 700601, *Vibrio harveyi* (*V. harveyi*) ATCC 1116 and *Salmonella typhi* (*S. typhi*) ATCC 700931 were used as test organisms. The test organisms were inoculated perpendicular to the antagonist on the agar medium. The plates were incubated at 37 ℃ for 24 h. The microbial inhibitions were observed by determining the diameter of the inhibition zones[10]. Of all the isolates, two best antagonistic *Actinomycetes* isolates were selected, identified macroscopically and microscopically by Gram's staining and used for further studies.

2.4. Extraction of antimicrobial compounds

The selected antagonistic actinomycete isolates were inoculated into actinomycete isolation broth separately and incubated at 30 ℃ in a shaker at (200-250 rpm) for seven days. After incubation the broths were filtered through Whatman No. 1 filter paper. Then the filtrates were centrifuged separately at 5 000 rpm for 10 min to extract the antimicrobial compounds. The supernatant was transferred aseptically into a screw capped bottles and stored at 4 ℃ for further assay[11].

2.5. Antimicrobial activity

The antimicrobial activities of those extracts were tested against different test organisms. The procedure applied in doing the sensitivity tests was in accordance with those prescribed by WHO for the modified Kirby-Bauer technique. With the use of a sliding caliper, the zones of inhibition were measured after 16-18 h of inhibition and recorded.

2.6. DNA extraction, 16S rRNA sequencing

The method described by Ausubel *et al* 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 ℃. Then 100 μL of NaCl solution was added and mixed thoroughly and the samples were then incubated for 10 min at 65 ℃. 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 ℃) and dissolved in 200 μL 1xTE using alternating heat/cold shocks (10 min at 80 ℃, 20 min at −20 ℃ 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 8000 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 8000 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 8000 rpm. The liquid phase was removed and the pellet was washed with 300 μL of 70% ethanol. After centrifugation for 5 min at 8000 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 ℃. 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 ℃ and 20 min at −20 ℃. Dissolved genomic DNA samples were stored at −20 ℃[10]. 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 ℃ for 5 min followed by 30 cycles at 94 ℃ for 45 sec, primer annealing at 42 ℃ for 1 min and primer elongation at 72 ℃ for 40 sec. At the end of the cycling, the reaction mixture was held at 72 ℃ for 10 min[13].

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/.

3. Results

Table 1
Isolation of *Actinomycetes* from soil samples.

S.No.	Geographical loctions	Sampling spots	Types of samples	Number of isolates
1	Royapuram	Sea shore soil	Soil sample with water	8
2	Muttukadu	Sea shore soil	Soil sample	6
3	Mahabalipuram	Sea shore soil	Soil sample with water	4
4	Adyar	Estuary	Soil sample with water	3

Table 2
Preliminary screening of *Actinomycetes* for antimicrobial activity by cross-streak method.

Isolates	*V. harveyi*	*Staphylococcus* species	*V. fischeri*	*Pseudomonas* species	*B. subtilius*	*P. mirabilis*	*E. coli*	*S. typhi*	*P. vulgaris*	*K. pneumonia*
C1	−	+	−	−	+	−	−	−	−	−
C2	−	−	−	−	−	−	−	−	+	−
C3	+	−	+	−	−	−	−	−	−	−
C4	−	−	−	−	+	−	−	−	+	−
C5	−	+	−	−	+	−	−	−	−	−
C6	−	−	−	−	−	−	−	−	−	+
C7	−	+	−	−	−	−	−	−	+	−
C8	+	−	+	−	−	−	−	−	−	−
C9	−	−	−	−	−	−	−	−	+	−
C10	−	+	−	−	+	−	−	−	−	−
C11	+	+	−	+	−	−	−	−	−	−
C12	+	+	−	+	+	−	−	−	−	−
C13	−	−	−	−	−	−	−	−	−	+
C14	−	−	−	−	+	−	−	−	−	−
C15	+	−	+	−	−	−	−	−	−	−
C16	−	−	−	−	−	−	+	−	−	−
C17	−	+	−	−	+	−	−	−	−	−
C18	−	−	+	−	−	+	−	−	−	−
C19	−	+	−	−	+	−	−	−	−	−
C20	+	−	+	−	−	−	−	−	−	−
C21	−	−	−	−	−	−	−	−	+	−

A total of 21 isolates were isolated from the soil samples. The numbers of samples and isolates in each sample were presented in Table 1. Out of the 21 isolates, two cultures *i.e.* C11 and C12 were the two strains selected for further analysis, since they showed significant antibacterial activity against test organisms. The results were shown in Table 2. The two cultures C11 and C12 were identified and confirmed by microscopic and macroscopic examination. C11 strain is a gram positive, cocci in nature, long spore chain, and filamentous bacteria. C12 strain is a gram positive, cocci with spiral spore chain bacteria. The macroscopic appearance of the isolate C11 showed leathery, white powdery colonies in *Actinomycetes* isolation agar whereas C12 showed creamy, pin point, powder colonies. The isolates also produced antimicrobial compounds. Two selected isolates *i.e.* C11 and C12 were tested for the antimicrobial activity. The highest inhibition was shown by the cultures C11 and C12 against *Staphylococcus* species. Culture C11 showed activity against *Staphylococcus* species (13.0±0.5 mm), *Pseudomonas* species (12.0±0.3 mm) and *V. harveyi* (11.0±0.2 mm). Culture C12 showed activity against *Staphylococcus* species (16.0±0.4 mm), *B. subtilis* (11.0±0.2 mm), *Pseudomonas* species (10.0±0.2 mm) and *V. harveyi* (9.0±0.1 mm).

Genomic DNA of the strains was isolated and the PCR amplified 16S rRNA was cloned into *E. coli* and sequenced. By using six primers (three forward and three reverse), 1 461 bp of the 16S rRNA was sequenced. The 16S rRNA sequence was aligned with the known 16S rRNA sequences of other bacteria. It was found from 16S rRNA sequence that the strains C11 and C12 were *Streptomyces* species.

4. 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[14]. 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[15]. Production of antibiotic substance is sea water dependent[15]. 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[16]. 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[17]. 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[18]. 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*[19]. 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*, than gram negative *Pseudomonas* species and *V. harveyi*. 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 *Vibrio cholera*[20]. Dhanasekaran *et al* found that estuarine *Actinomycetes*, which remained largely, ignored, showed promising antibacterial activities[21,22]. 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[23]. Boudemagh *et al* 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, C11 and C12 were identified as *Streptomyces* species 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

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. Recent advances of molecular genetics in this genus have enabled us to elucidate not only the organization of biosynthetic genes for their secondary metabolites but also regulatory mechanisms closely linked to the cellular differentiation processes. Although such information has so far not been successful in contributing to practical strain improvement, rational approach of combinatorial biosynthesis is expected to be useful in generating new compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

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