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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF ACTINOBACTERIA ANTAGONISTIC TO CLINICAL PATHOGENS FROM SOUTHEASTERN ARABIAN SEA MARINE SEDIMENT

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ABSTRACT
Streptomyces are common soil bacteria that produce secondary metabolites, including several antibiotics; however, the characteristics of marine *Streptomyces* are largely unknown. Hence marine sediments, collected from the coastal areas from off Cochin fort (Kerala) latitude and longitude (9°57'52.8" N, 76°14'05.1" E). Southeastern Arabian Sea, India. Totally twenty isolates were obtained among this isolatesone isolate namely INR1 showed significant antibacterial activity against both clinical and fish pathogens. Morphological, cultural and biochemical characterization indicated that the isolate belong to *Streptomyces* genus.Molecular characterization of INR1 was evaluated by PCR amplification of 16SrRNA gene. Phylogenetic analysis of16S rRNA sequencing studies revealed that the strain is closely related to *Streptomyces mutabilis*. Further investigations may yield greater understanding of these organisms in this and other marine environments and may be a reservoir of novel microorganisms and secondary metabolites.

KEYWORDS: *Streptomyces mutabilis* INR1, antimicrobial activity, 16S rRNA sequences, Microbial pathogens, phylogenetic analysis. Sediment samples were taken from 3 sites in the Laguna Madre to isolate marine *Streptomyces*. Diverse characteristics with regards to their biochemical profiles, carbon source utilization and inhibition toward other organisms.

INTRODUCTION
A large number of pathogenic bacteria increase common use of antibiotics, its leads to the development of antibiotic resistant bacteria. This antibacterial resistance is presently an urgent focus of research and new antibiotics are necessary to combat these pathogens. Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms because of their ability to produce many array of product such as antibiotics, anti-parasitic agents antitumor agents, enzymes cosmetics, secondary metabolites, nutritional materials immunosuppressive agents, vitamins, pesticides and herbicides (Williams et al., 1983; Valli et al., 2012). Indeed, the Gram-positive *Streptomyces* species produce about 75% of commercially and medically useful antibiotics (Miyadoh, 1993). In the course of screening for new antibiotics, several research studies are currently oriented towards isolation of new *Streptomyces* species from different soil and water samples. The genus *Streptomyces*, filamentous soil bacteria, has been described as the greatest source of the commercially available antibiotics (Marinelli, 2009; Bull et al., 2005). *Streptomyces* are also reported to produce other valuable bioactive secondary metabolites acting as antitumor agents, immunosuppressive agents and enzymes (Berdy, 2005; Tanaka et al., 2013). Moreover, the microbial production of secondary metabolites including antibiotics is affected greatly by nutritional condition (Stanbury et al., 1995).

Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites (Baskaran et al., 2011). Among the microorganisms, Actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities (Bredholt et al., 2008). The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Now a day's considerable progress is being continuing within the fields of chemical synthesis and in the field of engineered biosynthesis of Antibacterial compounds. So, the nature still remains the richest and the most versatile source for new antibiotics. (Kpehn and Carter, 2005; Baltz, 2006; Pelaez, 2006). The development of resistance to multiple drugs is a major problem in the

treatment of infectious diseases caused by pathogenic microorganism this multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistance pathogens. The present study is aimed at characterization of the actinobacteria isolated from sediments soil having antimicrobial activities against gram positive and gram negative bacteria.

MATERIALS AND METHODS

Sample collection and strain isolation

The sediment samples were collected from off Cochin fort (Kerala) latitude and longitude (9°57'52.8" N, 76°14'05.1" E). Southeastern Arabian Sea, India. 5g of soil was taken and mixed with calcium carbonate and air dried at 20°C for 3 days. Then soil sample was serial dilution agar plating method using a Starch Casein Nitrate (SCN) agar Medium consisted of the following; 10g soluble starch, 0.3g casein, 2g potassium Nitrate, 2g NaCl, 2g KH_2PO_4, 0.05g$MgSO_47H_2O$, 0.02g CaCO3, 0.01g $FeSO_4$ $7H_2O$ and 15g agar, per liter of distilled water. The medium was supplemented with cycloheximide (50 mg/L) to inhibit the growth of undesirable bacteria and fungi, respectively. The plates were incubated at 30°C for 3-4 days. After incubation, predominant colonies were isolated and randomly collected from agar plates by repeated streaking on nutrient agar plates, the pure colonies were collected and stored in growth medium supplemented with 20% glycerol at –20ºC for further studies.

Morphological and biochemical characteristics

Colony characterization

The isolates were streaked on to yeast extract malt extract agar (ISP 2), glycerol asparagine agar (ISP5), starch casein agar and Colony morphology was recorded with respect to colours of mature sporulating aerial mycelium, substrate mycelium, macro morphology, diffusible pigment, colony reverse colour, colony texture etc. were recorded after observing the plates under stereomicroscope.

Coverslip culture technique

Spore chain morphology was studied by inoculating the test organisms on starch casein nitrate agar. Sterile cover slips were placed on the agar media at 60° angle which was inoculated with the tested organisms. The cover slips were removed after 5 days of incubation and were observed under oil immersion objective. Morphology of aerial mycelium and substrate mycelium of the selected actinomycetes were observed using cultures developed on the following agar plates Bennett's agar.

Biochemical characterization

Nitrate reduction and citrate utilization was tested in 0.1% potassium nitrate broth and Simmon's citrate agar medium respectively. Red colouration on adding the nitrate reagents were noted as positive. Change in the colour of medium from green to prussian blue in the case of citrate utilization test was recorded positive. Hydrogen sulphide production was detected using 0.5% lead acetate strips inserted into the nitrate broth. Blackening of lead acetate strips was recorded as positive. Hydrolysis of urea (2%), esculin (0.1%) and lysozyme resistance (0.05%) of the isolates were determined. A change of colour in the medium from yellow to pink was noted as urea hydrolysis, brownish black colouration in the medium as esculin hydrolysis and growth in the presence of lysozyme as lysozyme resistance.

Utilization of nitrogen sources

Ammonium chloride, ammonium sulfate, L-asparagine, cysteine, phenylalanine, potassium nitrate, sodium nitrate and sodium nitrite were added separately to the Bennett's medium at the final concentration of 0.1% (W/V). The utilization of these nitrogen sources by the test actinomycete was determined after 5 days of incubation. (Williams et al, 1983).

Utilization of carbon source

The ability of actinomycetes in utilizingvarious carbon sources is analyzed. viz., arabinose, xylose,inositol, mannitol, fructose, rhamnose, sucrose and raffinoseas sources of energy were studied based on the method recommended by ISP. These carbon molecules were sterilized by ether sterilization(Shirling and Gottlieb).

Antibacterial activity of Actinomycetes

The antimicrobial activity of the sediment isolates was analyzed by agar streak method against clinical pathogens and fish pathogens such as *Staphylococcus epidermitis*(MTCC 9040), *Escherichia coli*(MTCC1696), *Shigellaflexneri*(MTCC1457), *Pseudomonas aeroginosa* (MTCC2453), *Bacillus megaterium* (MTCC2763), *klebsiellapneumonae*(MTCC4031), *Salmonella typhi*(MTCC3224), *Vibrio cholera* (MTCC3906), *Providencia rettgeri* JX136696, *Aeromonas* sp. JX136697, *Aeromonas* sp. JX136698, and *Aeromonas enteropelogenes* JX136699.The actinomycetes isolate was streaked as a straight line and incubated at 27 °C for 4days. After 4 days pathogenic strains were cross streakon the same platebut not touching each other, and then incubated at 37 °C for 24 h. The plates were examined for the zone of inhibition.

DNA preparation, PCR amplification and sequence analysis

The isolated strain was grown at 30 °C for 2 days in a rotary shake 250 ml Erlenmeyer flask containing 50 ml of Luria Bertani broth medium. 1.5 ml of culture was centrifuged at 10,000 rpm for 10 minutes. Collect the pellet and transferred in to sterile porcelain dish and crushed with tissue grinders. The crushed mycelium was transferred into fresh tube containing 210 µl of TE buffer supplemented with lysozyme (10µl). Add 30µl of 10% SDS (w/v) and 20 µl of proteinase K into the tube and incubated at 55°C for 30 minutes. The lysate was cooled down and extracted once with equal volume of phenol: chloroform solution (v/v, 1:1) at 10,000rpm for 5 minutes. The aqueous phase was transferred carefully to

a fresh tube and DNA was precipitated by adding 70-90% ethanol and keeping at -20°C for 30 minutes. The pellet was formed by centrifuging at 10,000 rpm for 10 minutes. The pellet was washed twice with 90% ethanol and dissolved the pellet in TE buffer. To obtain RNA free DNA added 20µl of RNase solution (20 µg/ml) and then incubated at 37°C for 1 hr. The sample was once again extracted with equal volume of phenol: chloroform and precipitated as above.

PCR amplification of the 16S rRNA of the strain was performed using the primers :Universal primers The amplification was performed on a Thermal cycler (Eppendorf) under the following condition: an initial denaturation 94 C for 5 min, followed by followed by 30 amplification cycles of 94 C for 1 min, 55 C for 30 sec, and 72 C for 2 min and a final extension stepof 72 C for 15 min. The PCR product was detected by agarosegel electrophoresis and was visualized by ultraviolet fluorescenceafter ethidium bromide staining. The sequencing reaction was performed by Shrimpex Multidisciplinary Biotech Privet limited The chromatograms were compared with the available nucleotide sequence of Actinomycetes species in the National Center for Biotechnology Information (NCBI) database. The gene sequences of isolated strain was identified and subsequently submitted to NCBI and accession numbers were obtained.

RESULTS AND DISCUSSION

Actinomycetes are most important microorganisms which capable for synthesis of bioactive compounds. Most of the 70% commercial antibiotic obtained from soil actinobacteria (Williams 2008).In order to exploit the actinomycets crucial to isolate and characterize.The present study was designed to investigate the novel actinomycetes and their antimicrobial properties. A total of twenty strains were obtained from the sediment sample were screened based on morphological and best antibacterial activity against the target pathogens. Among the isolates INR1 strain exhibiting notable antimicrobial activity against target pathogens (Fig 1) was selected for further probiotic characterization, no antagonistic activity was observed in the remaining nineteen strains tested against the target pathogens.

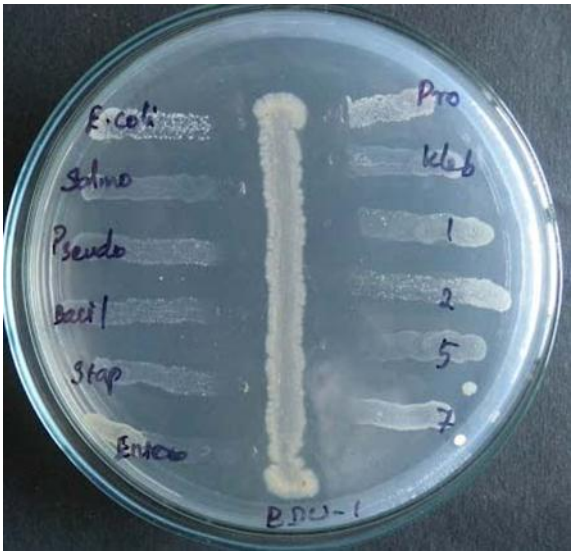

Figure 1: Antimicrobial activity of actinomycetes.

Isolate produced aerial mycelium which was white in colour but lacked powdery spore mass. The isolated Actinomycetes were identified based on the colony morphology and Gram staining (Holt et al. 1994). INR1 exhibited positive response to catalaseproduction and citrate utilization, nitrate reduction, utilization of carbon and nitrogen source but negative for urease, hydrogen sulphide production, starchhydrolysis, gelatine liquefaction, indole, methyl red andvogues-proskauer tests. The details of morphological, physiological and biochemical characteristics of the isolateare given in Table 1. The sequences were submittedto the Genbank database and accession number was obtained, *Streptomyces mutabilis* INR1 KT887962. Using Fig Tree v1.3.1 software, the phylogenetic tree was constructed with strainsexhibiting 99% similarity such as *Streptomyces mutabilis*173900.1, *Streptomyces olivaceus* 200342.1, *Streptomyces djakartensis* 862830.1, *Streptomyces geysiriensis* 112459.1 etc. (Fig. 2).The 16S rDNA sequence data supported the assignment of this isolate INR1 to the genus *Streptomyces*. Based on the morphological, cultural, biochemical and molecular characteristics the strain hasbeen included under the genus *Streptomyces* and deposited at NCBI genbank with an accession numberKT887962.

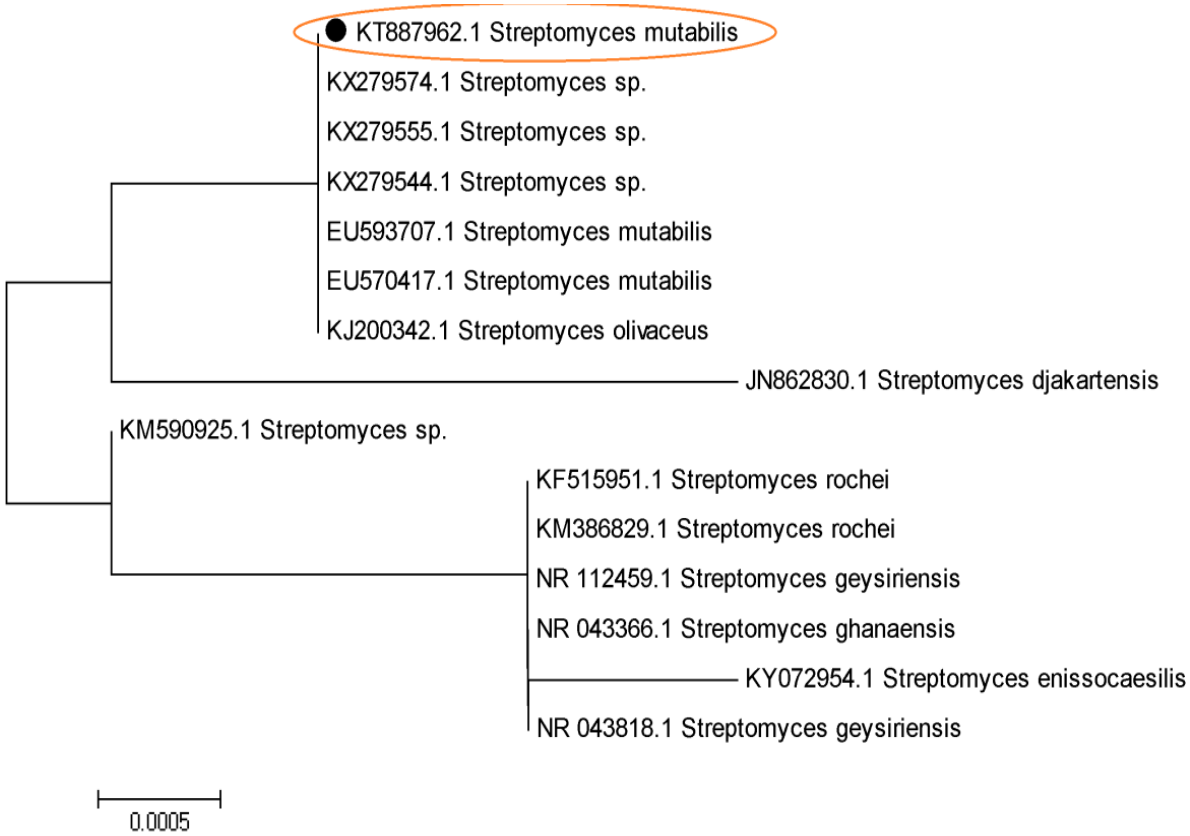

Figure 2: Phylogenetic tree constructed by the neighbour joining method showing species relatedness of the isolates.

Table 1: Morphological and Biochemical characterization of actinomycetes.

Characteristics Strain INR1	
Colour of aerial mycelium	White
Melanoid pigment	-
Sporechain morphology	Spiral
Nitrate	+
Citrate	+
Catalase	+
Urease	-
Indole	-
MR-VP	-
Arabinose	+
Xylose	+
Inositol	+
Mannitol	+
Fructose	+
Sucrose	+
Rhamnose	+
Riffinose	+

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CONCLUSION

The present study was an attempt to identify strains of *Streptomyces* from sediments that display activity against microbial clinical and fish pathogens. Such attempts need to be sustained so as to screen more isolates for novel therapeutics. However, the study only provided a basis for antimicrobial potential of the isolates. There is need to carry out bioassay guided fractionation to isolate the actual bioactive compounds.

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