Hygeia :: journal for drugs and medicines
October 2013 – March 2014
OPEN ACCESS
A half yearly scientific, international, open access journal for drugs and medicines
Research article section: Pharmaceutical Biotechnology
Article ID- Hygeia.J.D.Med/108 /13

16S rDNA Phylogenetic Analysis of Actinomycetes Isolated from Marine Environment Associated with Antimicrobial Activities

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Article history: Received: 2 July 2013, revised: 20 July 2013, accepted: 11 August 2013, Available online: 10 October 2013

ABSTRACT

Plan: *Isolate and characterize the antimicrobial actinomycetes from sediments of salt pan region of Vedharanyam, located in the Nagapattinam District, Tamil Nadu, India.*
Methodology: *The salt pan soil samples were collected The Physico - chemical parameters of soil sample were analyzed. Totally 16 actinomycete strains were isolated. The isolated strains were identified based on the morphological, biochemical, and physiological characteristics. All the 16 actinomycetes were selected for antimicrobial activity. Totally 16 actinomycetes strains were isolated. Antifungal activity was determined against two fungal pathogenic such as Aspergillus niger, Fusarium moniliforme. Streptomyces griseoflavus SDAP101 showed maximum level of inhibition against Aspergillus niger followed by Fusarium moniliforme. Molecular characterization of Streptomyces griseoflavus* SDAP101*were evaluated by PCR amplification of 16SrRNA gene. The genomic DNA and amplified products were separated in agarose gel and the16SrDNA gene of Streptomyces griseoflavus*SDAP101*species isolated from soil was partially sequenced using specific 16SrDNA sequence primer. The phylogenetic analysis revealed that its closest neighbor was EU827474.1. The16SrDNA secondary structure and the restriction sites of SDAP101 were predicted using Genebee online software respectively.*
Outcome: *The isolation, characterization of the rare actinomycetes from the saltpan region will be useful for the discovery of the novel bioactive metabolites that are effective against wide range of pathogens*
Key words*: Streptomyces sp. SDAP101, antibacterial activity, molecular taxonomy, phylogenetic analysis.*

1. INTRODUCTION

Actinomycetes, the filamentous bacteria, are primarily saprophytic microorganisms of the soil, where they contribute significantly to the turnover of complex biopolymers, such as lignocellulose, hemicellulose, pectin, keratin, and chitin[1]. The actinomycetes have provided many important bioactive compounds of high commercial value and are being routinely screened for new bioactive substances. These searches have been remarkably successful, and approximately two-thirds of naturally occurring antibiotics, have been isolated from actinomycetes [2.] About 61% of all the bioactive microbial metabolites were isolated from actinomycetes especially from streptomycetes and also from some rare actinomycetes (non streptomycetes) [3].

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Hygeia.J.D.Med. Vol.5 (2), October 2013
Hygeia journal for drugs and medicines,. 2229 3590, 0975 6221

It has been emphasized that actinomycetes from marine sediments may be valuable for the isolation of novel strains of actinomycetes, which could potentially yield useful new products.

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 possess antibacterial, antifungal, anticancer, antialgal, antimalarial and anti-inflammatory activities [4.] Actinomycete shave 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 [5]. They are free living, saprophytic bacteria, and a major source for production of antibiotics [6.]

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 [7.] 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 Actinomycete shave different compound [8.]

2. MATERIALS AND METHODS

2.1. Soil sample collection

Marine sediment samples were collected from various locations, Chola lighthouse, Old lighthouse and New lighthouse [near Point Calimere, Nagapattinam District, Lat. 10_ 18' N and Long. 79_ 51' E (seashore), Vedaranyam [(Lat. 10_ 22' N and Long. 79_ 51' E (saltpan)], representing three different environments found in Palk Strait region of South East Coast of India. Physical and chemical properties of sediments were analysed and recorded. Samples were collected at random from the top layer of each location and brought to the laboratory and stored for further study. Samples were also taken from each site for analysing physico-chemical parameters such as pH, electrical conductivity, nitrogen, phosphorus, potassium, soil texture, lime status, ferrous, manganese, zinc and copper. [9-11].The correlation co-efficient analysis between physico-chemical parameters of soils and actinomycetes populations was also made using SPSS package.

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2.2. Isolation of actinomycetes from soil samples

2.2.1. Media and Culture Conditions

Starch casein agar medium [g/l: starch - 10, casein - 0.3, KNO_3 - 2, NaCl - 2, K_2HPO_4- 2, $MgSO_4$. $7H_2O$ - 0.05, $CaCO_3$ - 0.02, $FeSO_4$ $7H_2O$ - 0.01 and agar 18; and supplemented with Griseofulvin and Cycloheximide (Himedia, Mumbai) 25 and 10 mg/ml] was used for the isolation of actinomycetes and enumeration [12].

The diluted sediment samples (0.1 ml) were spread over the medium with a sterilized bent (L) rod and plate spinner. The inoculated plates were incubated at 30°C for seven to ten days. After incubation, colonies were purified using streak plate technique and maintained for further investigation.

2.2.2. Identification of isolated cultures

Purified isolates of actinomycetes were identified using morphological and cultural characteristics by the methods as described in the International Streptomyces Project (ISP) [13.] The morphology of the spore bearing hyphae with the entire spore chain, the structure and arrangement of the spore chain with the substrate and aerial mycelium of the actinomycetes were examined using slide culture technique and identified [14]. After growth, the slide cultures were examined under light microscope. [15]

2.2.3. Microbial pathogens

Among bacterial pathogens, *Staphylococcus aureus,* (ATCC 25923), *Klebsiella pneumoniae,* (ATCC 10273), were used as a target organisms. Among fungal pathogens *Aspergillus niger,* (MTCC 281), *Fusarium moniliforme* were used as target organisms.

2.3. Antibacterial activity of Actinomycetes [16]

Nutrient agar medium was prepared and sterilized and poured into the sterile petriplates. The test organisms were spread over on the surface of nutrient agar medium. After solidification using sterile Cork borer wells (5mm) were made on the medium and inoculated the cell free filtrates separately in the wells and incubated at 37°C for 24 hours. After incubation the diameter of the zone of inhibition around the wells was measured.

2.4. Antifungal activity of Actinomycetes[28]

Potato Dextrose Agar medium was prepared and sterilized and poured into the sterile petriplates. The test organisms were spread over the surface of cultivated medium, after solidification using sterile cork borer, wells (5mm) were made on the medium and inoculated in the cell free filtrate separately and placed on the medium and incubated at 37°C for 3 – 4 days. After incubation the diameter of the zone of inhibition around the well, was measured to evaluate the antimicrobial activity of actinomycetes isolates.

2.5. Molecular Taxonomy

The full length 16SrDNA fragment was amplified by PCR from the above isolated genomic DNA. A single discrete PCR amplicon band of 1.5 Kb was observed when resolved on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out using BDT v 3.1 cycle sequencing kit on ABI 3730xl genetic analyzer. Consensus sequence of 1434bp of 16SrDNA gene was generated from forward and reverse sequence data using aligner software. The 16SrDNA sequence was used to carried out BLAST with the NRdata base of NCBI gene bank data base.

2.6. Nucleotide Sequence accession

The 16SrDNA sequences for the *Streptomyces* sp. (SDAP101) have been deposited in Gene Bank *http://www.ncbi.nlm.nib.gov/genebank.*

2.6.1. Phylogenetic Tree

The reference sequences required for comparison were down loaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/genebank. All the sequences were aligned using the multiple sequence alignment programs CLUSTAL W development by (Higgins *et al.,* 1992).

2.6.2. 16SrDNA secondary structure prediction of Streptomyces sp.

The secondary structure of *Streptomyces* sp. (SDAP101) NCBI were predicted using the Bioinformatics tools available in online www.genebee.msu.su/services/rna2-reduced-html.

7. RESULTS

The physico-chemical parameters of the soil samples from saltpan region of Vedharanyam, Nagapattinam District, Tamilnadu, were analysed. The sample showed pH 8.1, electrical conductivity – 6.44 Ms, Salinity – 5 ppt, turbidity – 73 NTU, Total Dissolved solids – 4.20 ppt, Dissolved oxygen – 40.70 mg/litre, temperature – 33.3°C.A total of sixteen different actinomycetes isolates were recovered from saltpan soil samples. Based on the cultural and microscopic characterization of actinomycetes. Statistical correlations were made by using Physico-chemical parameters of soil sample and Total Actinomycetes Populations. (TAP).

The culture filtrate of *Saccharopolyspora* sp., *Nocardia* sp., *Jonesia* sp., *Actinobispora* sp., *Streptoverticillium* sp., *Micromonospora* sp., *Streptomyces exfoliatus, Streptomyces griseoflavus, Streptoverticillium baldaccii, Dactylosporangium* sp., *Streptomyces cyaneus, Nocardioides* sp., *Streptomyces albus, Terrabacter* sp. *Saccharomonospora* sp., *Actinobispora yunnanesis* was determined against two pathogenic bacteria. The culture filtrate showed significant antibacterial activity. The results indicate that, all the sixteen actinomycetes were highly active against *Staphylococcus aureus, Klebsiella pneumoniae, Jonesia* sp. (7mm), *Streptomyces griseoflavus,* (8mm), *Streptomyces cyaneus* (2mm), *Streptomyces exfoliatus,* (5mm), *Streptomyces albus* (2mm). (Fig:1)

Saccharopolyspora sp., *Nocardia* sp., *Jonesia* sp., *Actinobispora* sp., *Streptoverticillium* sp., *Micromonospora* sp., *Streptomyces griseoflavus, Streptoverticillium baldaccii, Dactylosporangium* sp., *Streptomyce exfoliatus, Streptomyces cyaneus, Nocardioides* sp., *Streptomyces albus, Tetrrabacter* sp. *Saccharomonospora* sp., *Actinobispora yunnanesis* was determined against two pathogenic fungi. The culture filtrate were failed to inhibit the pathogenic fungi. Only *Streptomyces griseoflavus,* (0.5mm), *Streptomyces cyaneus* (0.1mm), *Streptomyces exfoliatus* (0.3mm), *Streptomyces albus* (0.1mm) showed maximum level of inhibition zone towards the *Aspergillus niger* , where as remaining species showed negative result.(Fig:2).

The 16SrDNA genes of *Streptomyces* sp. (SDAP101) isolated from salt pan soil was partially sequenced using specific 16SrDNA sequence primer for forward and reverse primer 8F: (5' AGA GTT TGA TCC TGG CTC AG 3') 1492R: (5' ACG GCT ACC TTG TTA CGA CTT 3'). The sequences of *Streptomyces* sp. (SDAP101)16SrDNA were deposited in NCBl to get the accession number. The accession number of *Streptomyces* sp. is (GQ408915).

The sequences in the EMBL database, the phylogenetic analysis revealed that 1434 bp sequences of the saltpan isolate *Streptomyces sp.* (SDAP101). Fig: 3

The secondary structure of 16SrDNA of *Streptomyces sp.* (SDAP101) showed 26 stems in their structure. However, similar in energy thresh bold, cluster factor, conserved – factor, compensated factor, conservatively, part of sequence, greedy parameters and treated sequences as indicated by genebee software www.genebee.msu.su/services/rna2-reduced.html. Fig: 4

Fig: 1 Anti bacterial activity of Actinomycetes

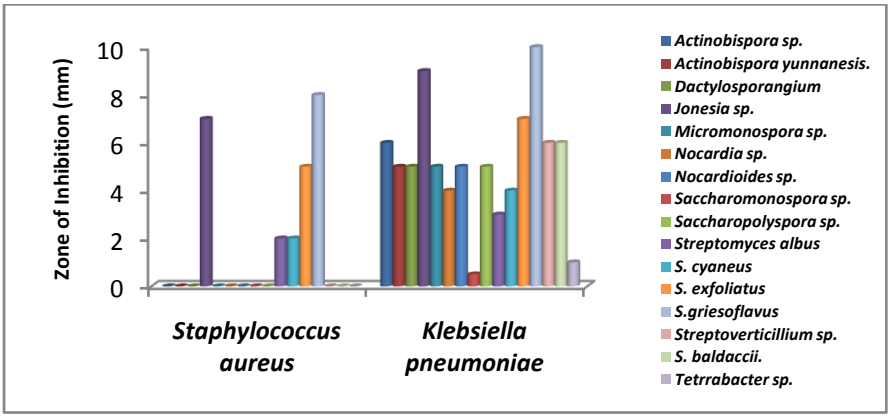


Fig: 2 Anti fungal activity of Actinomycetes

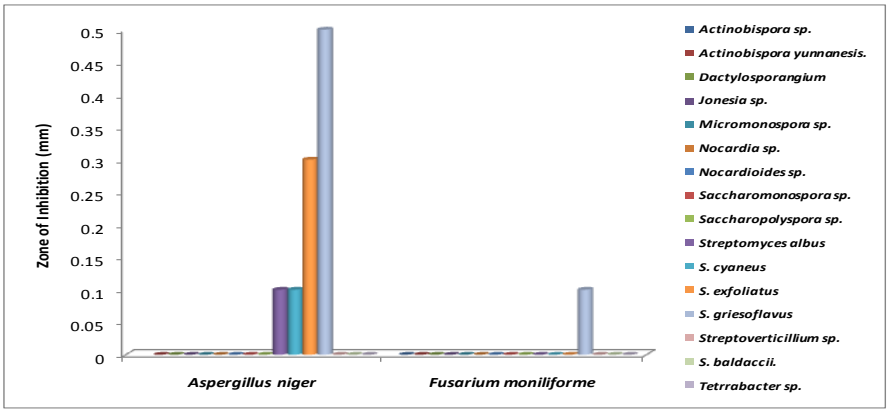
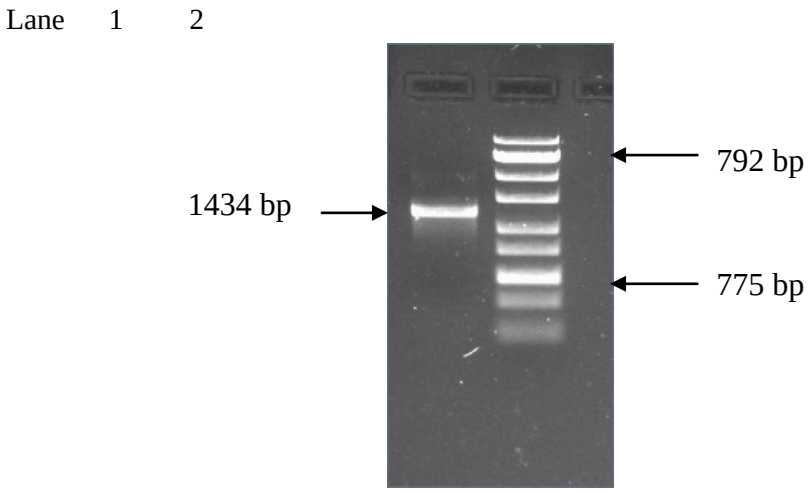


Fig: 3 Gel Image of 16SrDNAamplicon



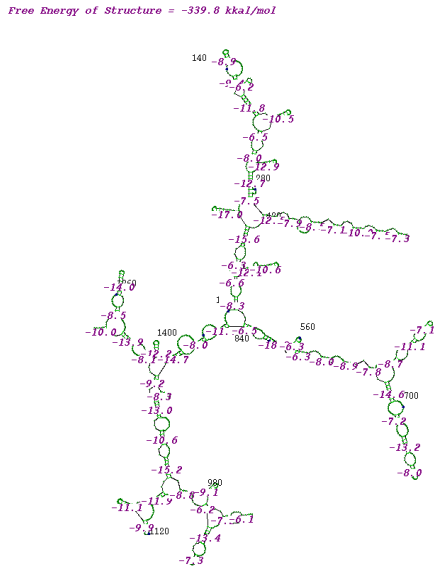


Fig:4 Secondary structures 16SrDNAof Streptomyces sp. (SDAP101).

8. DISCUSSION

The first report on marine actinomycetes was made by [16] from the salt mud Actinomycetes especially streptomycetes have been reported from the marine sub habitats such as marine soil. [17-20]. A total of 40 actinomycetes strains, isolated from Antarctica were tested for antagonistic activity against 7 Gram positive and gram negative bacteria and yeasts. During the initial screening 60% of the strains showed inhibition potential against test microorganisms. [21]. Analysis of the 16SrDNA beings by isolating DNA [22] and amplifying the gene coding for 16SrDNA using the Polymerase Chain Reaction [23].

The purified DNA fragments are directly sequenced. The reactions are performed using DNA sequences in order to determine the order in which the bases are arranged with in the length of sample. [24] and a computer is then used for studying the sequences for identification using phylogenetic analysis procedures.

CONCLUSION

Microorganisms inhabiting the marine ecosystem are more diverse and unique with the ability to produce unique chemical entities. These ecosystem need to be extensively studied to gain a complex knowledge and its unexhausted reserve of secondary metabolites. Our searches of actinomycets lead to the identification of *Streptomycetes* sp. (SDAP101) with fairly moderate antibacterial and antifungal activity. However further studies are need with respect to the structural characterization and the biological activity of the secondary metabolites.

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S.Deepa, K.Kanimozhi and A.Panneerselvam. 16S rDNA Phylogenetic Analysis of Actinomycetes Isolated from Marine Environment Associated with Antimicrobial Activities. Hygeia.J.D.Med.* **2013**; 5(2): page.43.-50. Available from http://www.hygeiajournal.com / Article ID- Hygeia.J.D.Med/108/13.