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Phylogenetic diversity and biological activity of actinomycetes isolated from Gulf of Mannar, Tamil Nadu, India

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ABSTRACT

Marine environmental conditions are extremely different from terrestrial ones, because every class of marine microbes produces a number of bioactive compounds with unique structural features. Of all the marine microbes, the actinomycetes have a special consideration since it produces a novel bioactive compounds. Hence, marine sediments, collected from the coastal areas of Rameshwaram and Dhanushkodi of Tamilnadu state, India, were screened and eighteen isolates were obtained on Starch-Casein agar media by soil dilution technique. However, only seven isolates showed significant antibacterial activity against Escherichia coli, Proteus mirabilis, Bacillus cereus, and Staphylococcous aureus. GC-MS results reveal that two compounds N-Isopropyluredo Acetic Acid and Benzene Propanamine, N-(1, 1-Dimethyl Ethyl) - Alpha Methyl - Gamma Phosphate were present in the isolates. Morphological, cultural, biochemical characterization and Gene sequencing indicated that the isolates belong to Streptomyces genus of Actinomycetes. The Phylogenetic study reveals the species name as Steptomyces globosus, and hence the strain is named as VITR004. Therefore, the isolation, characterization of the rare actinomycetes from the coastal areas of Rameshwaram and Dhanushkodi of Tamilnadu state, India will be useful for the discovery of the novel bioactive metabolites that are effective against wide range of pathogens.

Key words Bioactive compounds, GC-MS analysis, Actinomycetes, starch casein agar.

INTRODUCTION

Microbes have made a major contribution to the health and wellbeing of people throughout the world. In addition of producing primary metabolites, they are capable of producing secondary metabolites. Marine organisms act as a major source for the isolation of new organism with the ability to produce active secondary metabolites [1]. Among such organisms, actinomycetes are of great interest because they are recognized as organisms of academic curiosity produce diverse compounds with a range of biological activities also act as a potential antibiotic producer [2]. They are free living and saprophytic bacteria. About two-thirds of antibiotics have been isolated from actinomycetes [3]. Among actinomycetes, the genus *Streptomyces* act as a richest source of secondary metabolites and continues to act as a major source of new bioactive molecules [4, 5]. About 61% of bioactive substances were isolated from *Streptomyces* [6]. Hence, *Streptomyces* are recognized as primary antibiotic producing organism [7]. This has improved the life standard may be directly or indirectly. The discovery and development of antibiotics to treat life threatening infections by bacteria is perhaps one of the greatest accomplishments of the mid twentieth century. As the frequency of novel bioactive compounds discovered from terrestrial actinomycetes decreases with time, much attention has been focused on screening of actinomycetes from marine environment for their ability to produce new secondary metabolites [8]. The isolated compounds from marine actinomycetes has a wide spectrum of biological

activities such as cytotoxic, antifungal, neurotoxic, antiviral, antineoplastic, antibiotic, antimitotic and antineoplastic activities [9]. With this view, the present study was initiated for bioprospecting of actinobacteria from an unexplored source - Dhanushkodi and Rameshvaram (Gulf of Mannar) for actinobacteria with special reference to antibacterial activity. Further, the identified antagonistic actinomycetes were characterized based on morphological, biochemical, cultural and physiological characteristics. The main objective of this study is to isolate Marine-Actinomycetes from Rameshwaram soil sample and to screen for antimicrobial activity and to obtain the crude extract of the antimicrobial isolate through fermentation and then to characterize the lead compound responsible for the anti-microbial activity.

MATERIALS AND METHODS

Collection and Pre-Treatment of Marine Soil

The marine soil samples were collected from various places from Dhanushkodi and Rameshvaram with atleast 200 metres away from each place just beneath the upper surface (i.e. 15 cm below surface) to avoid the contamination. The soil samples were collected in the sterile small polythene plastic bags. The collected soil samples were brought to the laboratory and dried in a hot air oven for 60-65°C for 1 hour to reduce the non-spore forming microbes.

Isolation of Actinomycetes

Starch casein agar medium was used for the isolation of actinomycetes. 1 gram of marine soil was suspended in 9 ml sterile double distilled water. Then the samples were diluted serially for upto 10^{-5}. From each dilution, 0.1 ml was spread with an sterile L- shaped rod evenly over the Starch casein agar plates. The plates were incubated at 30°C for 7 days. After incubation, colonies were purified using streak plate technique and the pure cultures were sustained on SCA slants at 4°C for further use [10, 11]

Primary Screening

The purified isolates were screened during the primary screening against the selected strains by perpendicular streaking method. In this method, Muller Hinton agar plates was used and each plate was streaked with individual isolates at the centre of the plate and incubated at 37°C for 7 days. Later 24 hr fresh sub cultured test bacteria were prepared and streaked perpendicular to the isolates and incubated at 37°C for 13 hours [12]

Characterization of Active Isolate

Gram Staining

The suspected isolate were made smeared on the clean slide and allowed to dry and then heat fixed.

The smear was Gram stained and observed under the microscope [13]

Morphological Characterization

According to the recommendations of International *Streptomyces* Project (ISP) potent antagonistic actinomycete isolates were further characterized based on morphological, biochemical, cultural and physiological features. Microscopic characterization was carried out by cover slip culture method and formation of aerial and substrate mycelium, and arrangement of spores on mycelium were observed under high power objective of light microscope [14]

Biochemical Characterization

Melanin Production

The production of melanin pigment on agar slants of peptone – yeast extract Iron was determined and further tested the production in tryptone–yeast extract broth. The inoculated tubes were observed after 2 days and 4 days. Appearance of deep brown, greenish brown, greenish black or black colours were recorded as melanin positive. Absence of brown to black colour, or total absence of diffusible pigment, was considered as negative for melanin production [10]

Indole Production Test

To determine the ability of an organism to produce indole. The organism is inoculated in tryptone broth and incubated at 37ºC for 24 hours. Formation of red ring at the top of liquid layer on the addition of Kovac's reagent is considered as positive test result **[**13**]**

Methyl Red and Voges Proskauer Test
To find out whether an organism is MR/VP positive or not. The organism is inoculated in MR/VP broth and incubated at 37 ºC for atleast 48 hours [13]

Citrate Utilisation Test
A drop of 4-6 hour old isolate was inoculated onto Simmons citrate agar slants and incubated for 24 hours at 37 °C. A change in colour from green to deep prussian blue formation was considered to be positive test result and no colour change was considered to be negative test result [13]

Urease Production Test
The isolates were inoculated onto Christensen's urease agar slants and incubated at 37 °C for 18-24 hours. When the colour of the medium changed to pink, it was considered positive test result [13].

Triple Sugar Iron Agar (TSI) Test
The isolates were inoculated onto triple sugar iron agar test and incubated at 37 °C for 18-24 hours. When the colour of the medium changes to yellow or pink, it is considered to be positive [13]

Sequencing Analysis 16s rRNA
DNA Extraction
Bacterial Genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit. An isolated bacterial colony was picked up and suspended in 1ml of sterile water in a microfuge tube. Centrifuge it for 1 minute at 10,000–12,000 rpm to remove the supernatant. Add 200 µl of Insta Gene matrix to the pellet and incubate it at 56 °C for 15 minutes. Vortex at high speed for 10 seconds and place the tube in a 100 °C in boiling water bath for 8 minutes. Finally, vortex the content at high speed for 10 seconds and Spin at 10,000–12,000 rpm for 2 minutes. As a result, 20µl of the supernatant was used per 50 µl of PCR reaction [15].

PCR Protocol
Using below 16S rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler [16]

Primer Details	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Add 1µL of template DNA in 20 µL of PCR reaction solution. Use 27F/1492R primers used for bacteria, and then PCR reaction performed with below conditions: Initial Denaturation 94℃ for 2 min and then 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. Final Extension at 72℃ for 10 min. DNA fragments are amplified about 1,400bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

Purification of PCR products
Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol
Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequencing Primer Details

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

Bioinformatics Protocol

The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering.

Secondary Metabolite Analysis

Gas Chromatography (GC)

It is a joint type of chromatography castoff in analytic chemistry for separating and analysing complexes that can be vaporised starved of decomposition Typical uses of GC include testing the transparency of a particular substance, or unscrambling the different components of a mix. In gas chromatography, movable phase is carrier gas, usually an inert gas. Stationary chapter is a glass or metal tubing called a column(Fig.1) The gaseous compounds being scrutinized interact with the walls of the column, which is coated with different stationary phases. This causes each composite to elute at a different time, acknowledged as the retention period of the compound. The judgment of retention times is what gives GC its diagnostic usefulness. A number of indicators are used in gas chromatography. The most public are the flame ionization detector (FID) and the current conductivity detector (TCD). Both are penetrating to a wide range of gears. TCD are essentially common and can be used to detect any factor other than the carrier gas.

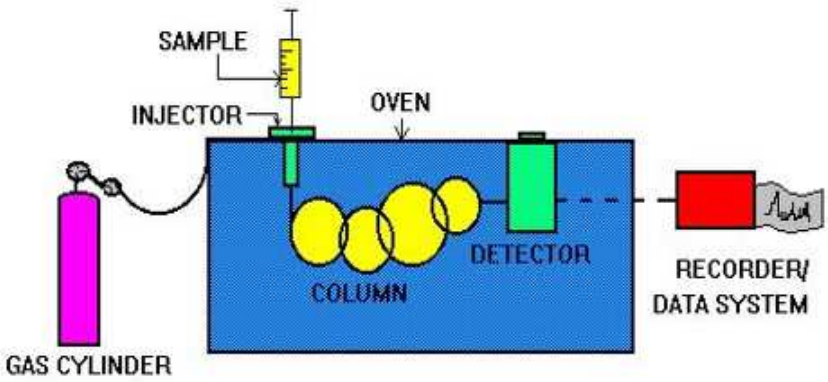

Fig.1 Illustration of gas chromatography

Mass Spectroscopy Analyzer

The physics overdue mass spectrometry is that a charged unit passing through a magnetic field is ricocheted along a circular path on a ambit that is relational to the mass to charge ratio, m/z. The three essential parts of the MS analyser remain the ionisation source, the analyser, the sensor. Find a way to “charge” an atom or bit (ionization source). Place charged atom or fragment in a magnetic field / electric field and quantity its speed or radius of warp relative to its mass-to-charge ratio (mass analyzer). Detect ions using microchannel plate or photomultiplier tube (Detector) (Fig.2). The three essential parts of the MS analyser remain the ionisation source, the analyser, the sensor. Find a way to “charge” an atom or bit (ionization source). Of the total sea surface, only 7–8% is sea area and the rest is deep sea, of which again 60% is roofed by water of more than 2000 m deep.

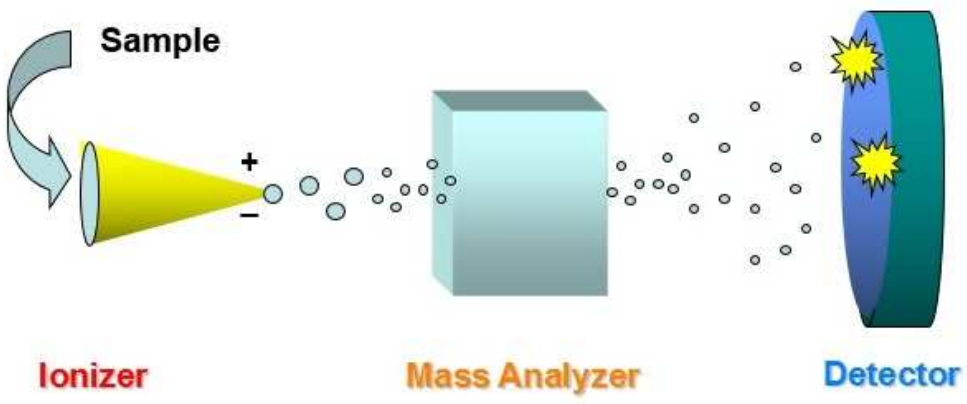

Fig. 2 Illustration of mass spec analyzer

Determination of Anti-Bacterial Activity

Agar diffusion methods using bacteria, as test organism is one of the most widely used assay methods. This method is used to find antibacterial activity in culture broth of actinomycetes. This assay is based on the technique of allowing an antibiotic to diffuse through an agar gel, which has been previously seeded with test organisms. This is the most widely used and accepted method employing the diffusion technique. This assay is done in two ways.

Agar well method - In which wells of definite volume are cut on agar plates seeded with test microbes. Culture filtrate is added to the wells in fixed quantity using micropipette and incubated.

Kirby-baur disc method — Sterile paper discs impregnated with culture filtrates are placed over seeded agar plates with test microbes

RESULTS AND DISCUSSION

The first report on marine actinomycetes was made by [17] from the salt mud. Actinomycetes especially *Streptomyces* have been reported from the marine sediments [18]. The regular isolation of actinomycetes from littoral sediments does not indicate that these organisms are indigenous to the marine and brackish water areas. In the present study, 18 isolates were isolated from the coastal areas of Rameshwaram and Dhanushkodi of Tamilnadu state.

Isolation

Actinomycetes was isolated from soil sample by spread plate technique. The actinomycetes are further confirmed by morphological and biochemical test. The image obtained under microscope is shown in figure.3

Characteristics	Results
Morphology	
Gram staining	Gram positive, filamentous bacteria
Acid fast staining	Non acid fast
Substrate mycelium	Yellowish brown
Aerial mycelium	Grey
Spore mass colour	Greyish white
Spore chain	Linear
Biochemicals	
Melanin	Positive
Indole	Negative
MR	Positive
VP	Negative
Citrate	Negative
Urease	Negative
TSI	Alkaline slant/Alkaline Butt. Positive gas production

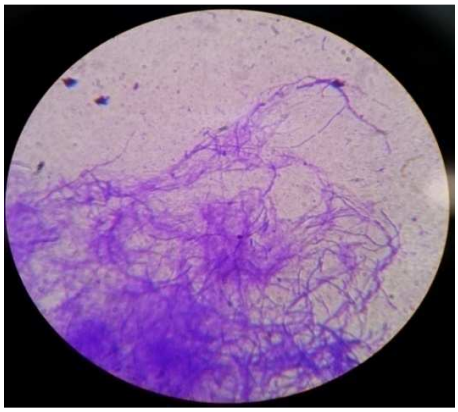

Fig. 3. Image obtained under microscope

Primary Screening

Bioactive isolates were identified by differentiating the zone of inhibition from the plates in which actinomycetes are isolated. (Fig.4)

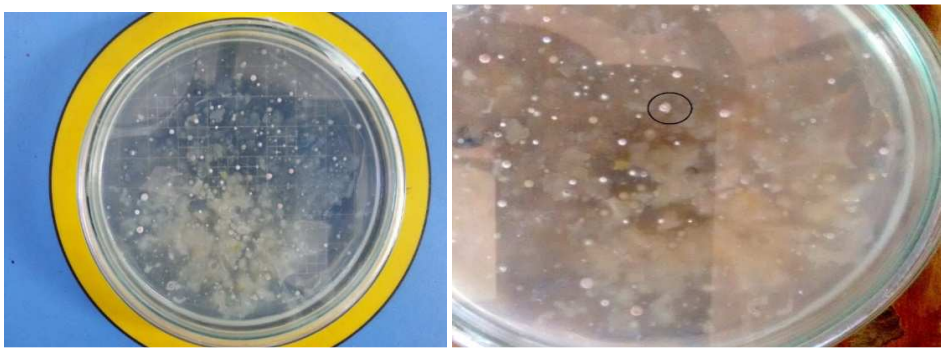

Fig. 4 Identification of active isolate by Zone of inhibition

Screening of Antimicrobial Activity by Cross Streak Method

This study was done by the Cross streak method against four pathogenic strains of bacteria namely *E.coli, Bacillus cereus, Proteus mirabilis, Staphylococcus aureus.* The result obtained from cross streaking method gave us our strain of interest (Fig.5). Out of 18 isolates, the 4th sample gave us the best result in forming zone of inhibition, by showing a good anti-bacterial activity against the pathogens.

Fig. 5 Cross streaking

Sequencing Analysis – [16s r RNA]

The gene was sequenced and the DNA is sequenced. The Species was identified as *Steptomyces globosus*.(Fig.6 and 7).

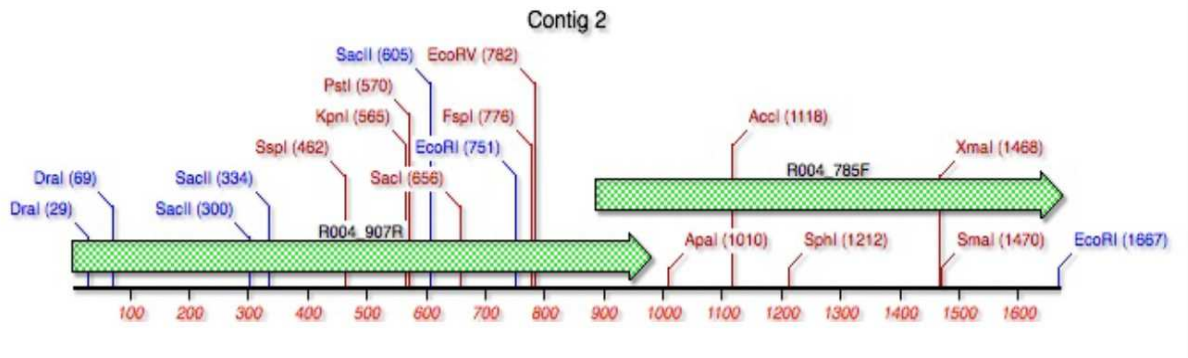

Fig.6 Gene sequencing

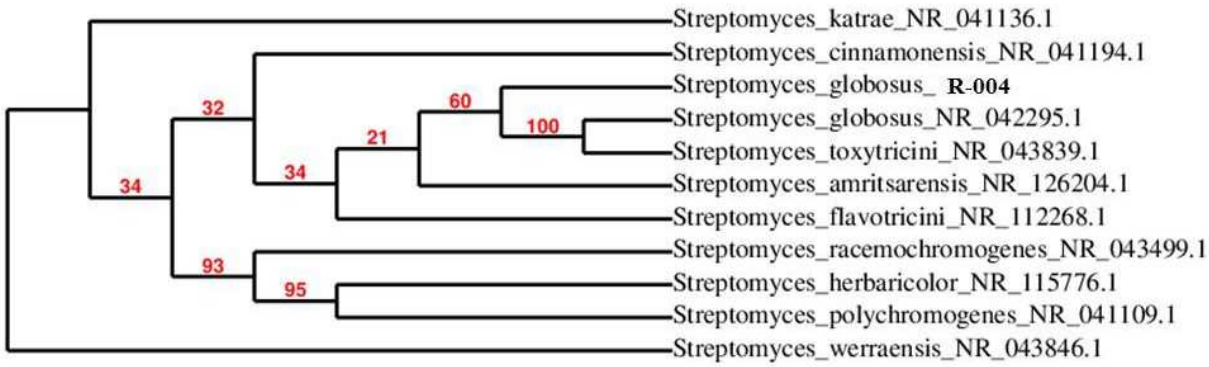

Fig.7 Phylogenetic Tree

Sequence-I [*Steptomyces globosus*] 16S rRNA gene VITR004

ATTTTGGGGTAGTTTGCGGGGGAAATTTTAAAAGTTTAATAAAGTTTTTTTTAAGGGGGGGGGGAATTT
AAAAAGGAAGGGGGGGGGGGGGGGGGGGGGGGGTTTTTAAGTTTTTTTAATCTGCTCAGGACGAA
CGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCTCCTTCGGGAGGGGATTAGTGGCGAA
CGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC
GGATACGACTGCGGAAGGCATCTTCCGCGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCT
ATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCG
GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCA
GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCAGTCACGTC
GGATGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCATTCGATACGGGCTGGCTAGAGTGTGGTAGGGG
AGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG
ATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACG
CATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACC
GGAAACATCCAGAGATGGGTGCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTC
GGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTC
ATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTG
AGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC
GGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC

CGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTCGAA
GGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGGAAACCCCGAAAAGGGGGGGGAAGCATTTTC
CCCCTTGTCCGAATGCCAGTATCCGTTTGCCCCGGTTCCTCCGGAATTCTTC

Secondary Metabolite Analysis

GC-MS

Our sample was processed for GC- MS, 3 compounds were isolated in our sample. The chromatogram was obtained (fig.8) and the individual compound was analysed, after that the molecular weight was identified and the related compounds were taken out with the best match.

Retention time	Molecular weight	Hit obtained
20.9	207.16	N-Isopropyluredo Acetic Acid
28.4	441.27	Unkown
30.55	591.4	Benzene Propanamine, N-(1,1-Dimethyl Ethyl)- Alpha Methyl - Gamma Phosphate

Molecular weight with their retention time and closely matched compounds

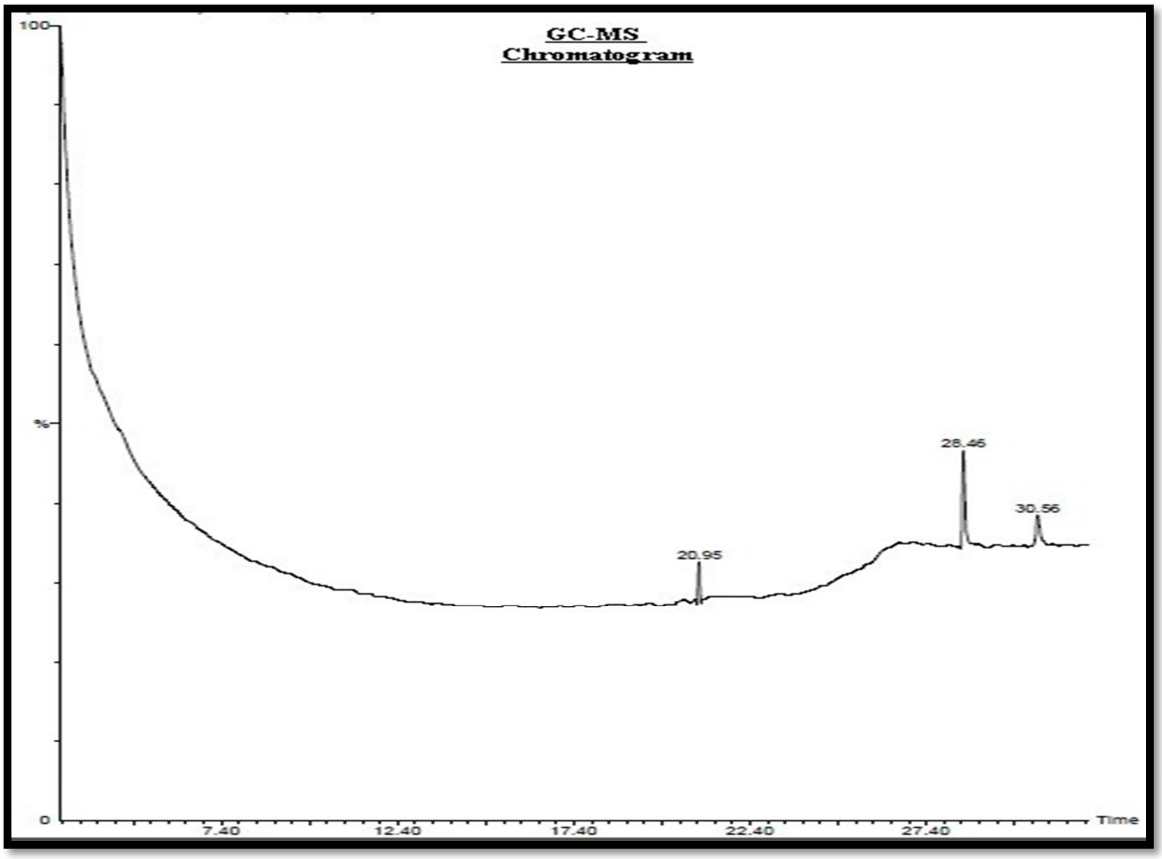

Fig.8 Chromatogram obtained in GC-MS results

Determination of Anti Bacterial Activity

Well Diffusion Method

The pathogens showed zone of inhibition against the crude extract. This was compared with the standard antibiotic.The results showed high inhibition than when compared to that of control.

Disc Diffusion
The pathogens showed zone of inhibition against the crude extract. This was compared with the standard antibiotic. The results showed high inhibition than when compared to that of control.

CONCLUSION

The present study reveals the potential of the marine actinomycetes as a best source of antimicrobial compounds. The starch casein agar was found to be suitable for isolating actinomycetes from marine sediments, which were collected from the coastal areas of Rameshwaram and Dhanushkodi of Tamilnadu state. Of eighteen isolates only six exhibited substantial antibacterial activity. The selected actinomycetes found to be capable of inhibiting the most pathogenic microorganisms such as *E.coli*, *Proteus* sp. Our searches of actinomycets lead to the identification of *Streptomyces globosus* and named our strain as VITR004. Further investigations are needed in order to determine the structural characterization and the biological activity of the secondary metabolites. Thus, there is a definite scope for bioprospecting of antagonistic actinomycetes from coastal areas of Rameshwaram and Dhanushkodi of Tamilnadu state once appropriate further studies are undertaken.

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