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Description and Assessment of an Antimicrobial Substance from Marine Actinobacteria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Streptomyces is thought to be the primary source of bacteria for the synthesis of bioactive substances, such as naturally occurring antibiotics. Finding and identifying the actinomycetes exhibiting antagonistic activity was the primary goal of the current investigation. A strain of actinomycetes that was isolated from marine sand samples that were collected off the Indian coast of Vedharanyam exhibited antimicrobial activity against a number of microbial pathogens. The nutritional requirements and cultural conditions for maximal growth and yield of bioactive compounds have been optimized under shake flask condition. Analyze the compound characterization of the sample such as UV, FT-IR, GC-MS and sequencing 16S rRNA for species

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identification. The actinomycetes isolates which are isolated from sediment sample can be further used as antimicrobial activity agents against bacterial and fungal pathogens and their compound characterization.

Keywords: Actinomycetes; antimicrobial activity; sequencing; compound characterization.

1. INTRODUCTION

Since “Actinomycetes” is a combination of the Greek words “atkis” (a ray) and “mykes” (a fungus), it possesses characteristics of both bacteria and fungi, which are both types of microorganisms. High quantities of cytosine and guanine (57-75%) can be found in the genomes of actinomycetes, which are gram positive, aerobic, spore forming bacteria. The ability to grow on a substrate and produce aerial mycelium order Actinomycetales.They have real aerial hyphae and are filamentous like fungi. Actinomycetes are the most useful microbes in terms of biotechnology and economics. [1-6]. Actinomycetes are widely known for producing a large variety of secondary metabolites with a wide range of medical applications, including antibiotics, antifungals, antivirals, antiprotozoal, antiviral, anticholesterol, anticancer, and immunosuppressants. They are crucial to agriculture as well as the pharmaceutical and pharmaceutical industries [7-12]. Numerous plant diseases may be prevented from growing by actinomycetes. Antibiotics, vitamins, amino acids, and other biologically useful compounds are produced by a large number of Actinomycetes [13-19]. Actinomycetes, among other naturally occurring compounds produced by microorganisms, are a good source of antibiotics. Gram-positive, slow-growing bacteria known as actinomycetes are recognized by the growth of aerial mycelium [20-24]. Spores that anchor the substrate are used to create mycelium. Unique bioactive metabolites produced by actinomycetes include antibiotics, enzymes, and plant growth regulators. Actinomycetes have produced a number of significant bioactive chemicals with great commercial value, and they are being regularly tested for new bioactive compounds. Actinomycetes produce secondary metabolites with a variety of biological functions [24-30].

2. MATERIALS AND METHODS

2.1 Location of Soil Sample

The samples were collected from various location of Vedharanyam and Kodiyakkarai marine soil sample (Lat.10.33, Long.79.84`) from Nagapattinam district, Tamilnadu, India. The samples were taken at a depth of 5 to 25 cm. The soil samples were transferred to sterile polythene zip lock bags. Sterile polythene bags were transported aseptically to the laboratory.

2.2 Isolation of *Actinomycetes* from Marine Soil Sample

At room temperature for a week, the soil samples were allowed to air dry by the spread plate and serial dilution method techniques, actinomycetes were isolated and counted [30-35]. One gram of soil was suspended in nine milliliters of sterile distilled water. Upto 10-5 dilutions were used in the process. On the actinomycetes isolation agar plate, 10-2, 10-3, 10-4, and 10-5 were spread out. Based on the morphology of the colonies, the actinomycetes cultures were chosen and purified from mixed organism plate. On the appropriate medium plates, the pure cultures were streaked.

2.3 Culture Conditions

For four to ten days, the pure cultures plates were incubated at 28 ± 2°C. Brown and white colonies in the probable colony structures isolates underwent purification.

2.4 Culture Characteristics

The structure of the spore chain with the actinomycetes morphologies and the morphology of hyphae that bear spores with the entire spore chain, as described by Buchnan and Gibbons [35-47]. Were studied using purified isolates of actinomycetes. To do this, the cover slip method was used to transfer individual colonies to the base of cover slips that were submerged in starch casein agar media.

3. IDENTIFYING COLONIES OF ACTINOMYCETES SP.

3.1 Characterization Morphologically

Actinomycete strains that had been cultivated for three to fourteen days on starch casein agar plates were subjected to morphological characterisation using a magnifying device. Aerial color, aerial mycelium, size, colony nature, reverse side color, and pigmentation were all

noted in the morphology of the colonies, and the isolates were examined under a microscope.

3.2 Light Microscopy

A cover slip The culturing method was used for investigations using light microscopy. Five to six sterile cover slips were positioned at a 45° angle on an actinomycetes culture plate, which had been prepared. At the junction of the medium and the coverslip, the actinomycetes culture was gradually liberated. For 4 to 8 days, at 28°C, the plates were incubated. The cover slips were then taken off and examined with a high power microscope. We saw and noted the physical traits of substrate mycelium, sporangia, spores, and aerial mycelium.

4. TEST ORGANISM

The actinomycetes potential as antibacterial agents was examined. Aspergillus brasiliensis and Candida albicans both exhibit antifungal activity. Antibacterial activity against *Enterococcus sp, Streptococcus pyogenes, E.coli, Klebsiella pneumonia.*

5. ACTINOMYCETES ARE SCREENED FOR ANTIFUNGAL ACTIVITY

The above-mentioned test organisms were used to perform the initial screening of the isolates for well diffusion method. In well diffusion method Anti-fungi assay agar plates were used and inoculated with the fungal pathogens *Candida albicans, Aspergillus brasiliensis* by cross streak metod and put a well on petriplate and inject the potential marine actinomycetes broth in various concentration then kept at 28°C for 2 to 5 days to incubate. Every test organisms activity was recorded. Around the colonies, the diameter The inhibitory zone measured in millimeters.

6. ACTINOMYCETES ARE SCREENED FOR ANTIBACTERIAL ACTIVITY

Actinomycetes In order to check isolates shown strong antibacterial activity using the diffusion technique. The bacterial pathogens were cross streaked on Nutrient Agar medium by swab and after 10 minutes put a well on agar plate and inject the potential marine *actinomycetes* broth in various concentration and incubated at 37°C for 24 to 48 hours. The activity against each test organism was recorded. The colonies inhibitory zone diameter, measured in millimeters, was noted.

7. EXTRACTION OF BIOACTIVE COMPOUNDS

After being infused into actinomycetes isolation broth, the isolates were cultivated at 28ºC for seven days and 120 rpm in a rotary shaker for fifteen minutes, the supernatant was centrifuged at 4000 rpm in order to extract the bioactive compound. an equivalent volume of every solvent, diluted 1:1 (that is, methanol, ethanol, ethyl acetate, acetone, hexane, and chloroform). For additional analysis, the supernatant was aseptically transferred into a sterile container and stored at 4ºC. Against the test pathogens, the activity of the molecules generated from each solvent was assessed.

8. RESULTS AND DISCUSSION

A total of ten actinomycetes were isolated from the sampling sites. Primary screening indicated that all the isolates showed antibacterial activity. Among ten isolates VAC 4 exhibited broad-spectrum activity against pathogenic bacteria *E. coli, S.pyogenes* and pathogenic fungi *Aspergillus brasiliensis, Candida albicans.* It stained Gram positive and the spore chain morphology was simple rectus. The spore surface was smooth as identified by SEM (Fig. 3). The serial dilution approach was used to separate microbes from soil then spreading was used to create a mixed culture, as seen below.

8.1 Gram Staining

In a spotless glass slide, a small amount of culture was gently cooked over a flame. The smear was gently rinsed in slow-flowing tap water after being coated for one minute in a thin film of crystal violet. After flooding Gram's iodine solution was applied to the smear for one minute, the area was rinsed with tap water. It was a smear decolored using alcohol until the violet colour stopped evaporating. After washing the slide with water and flooding the smear with counterstain safranin for two minutes, the slip was drained, allowed to air dry, and then examined under a microscope. The culture that had retained its violet hue was a Gram-positive bacterium [37-44].

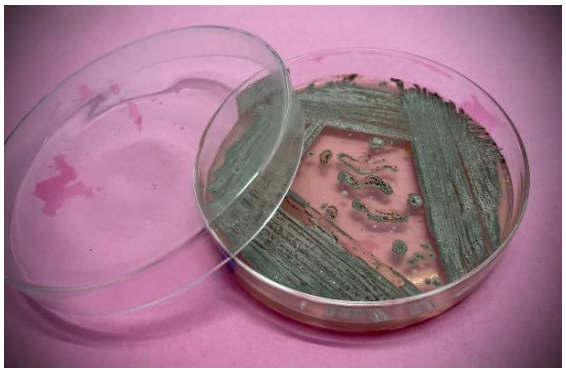


Fig. 1. Streak plate method of Actinomycetes (VAC 4)

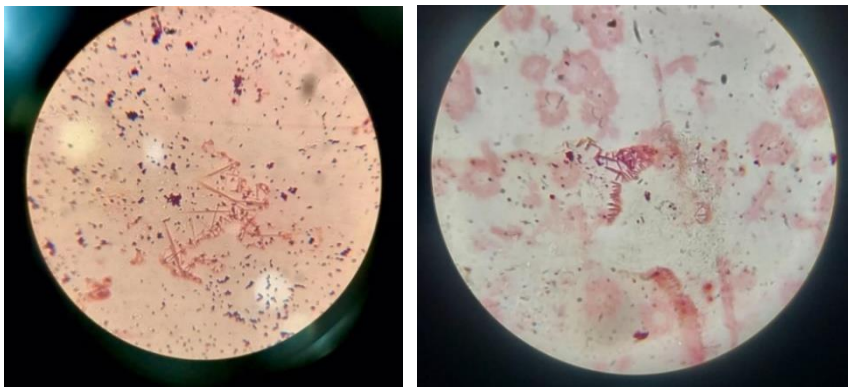
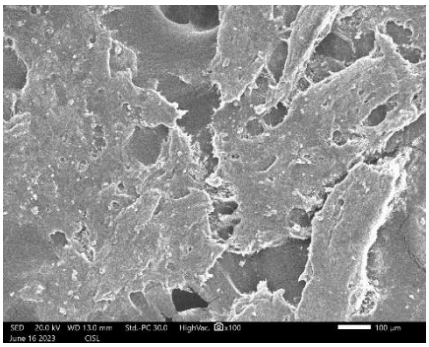
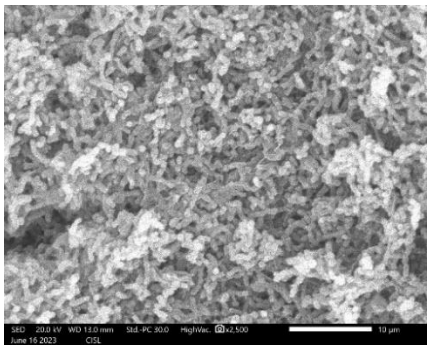


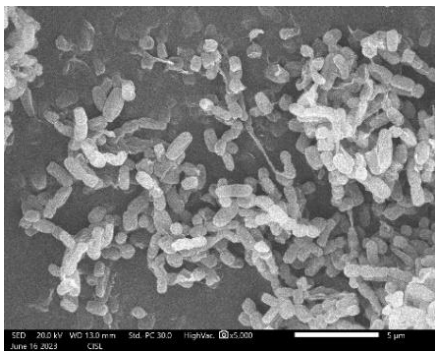
Fig. 2. Gram staining of *Streptomyces smyrnaeus*



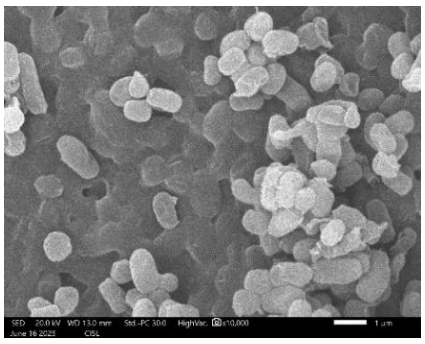
100 Magnification



2500 Magnification



5000 Magnification



10000 Magnification

Fig. 3. SEM analysis of morphology of *Streptomyces smyrnaeus*

8.2 Scanning Electron Microscope (SEM)

Scanning electron microscope examination. The microscope's specimen chamber was filled with the metal stubs and coated coverslips that were attached to them. Low magnification was used to scan the field until the line of growth was found. Then, regions with distinct, intact sporing structures were chosen for closer inspection. Ilford H.P. 3 film was used to capture appropriate subjects.

A beam of electrons is used in this apparatus to scan the specimen, and as the electrons ionise the specimen's atoms, they produce secondary electrons. The screen image is created by collecting some of these, together with some reflected primary electrons, which have enough energy to escape into the vacuum. The specimen has a "shadowed" look that draws attention to surface details because the secondary electron collector was positioned at an angle to the initial beam [45-54].

8.3 Antifungal Activity of *Streptomyces smyrnaeus*

Actinomycetes, notably those of the genus Streptomyces, are known to produce a diverse spectrum of antifungal chemicals that hinder the growth of numerous fungal diseases. These findings emphasize the potential of actinomycetes, particularly Streptomyces species, as a rich source of antifungal compounds that can be used to generate novel antifungal medications to battle diverse fungal infections affecting humans, animals, and plants. Although actinomycetes have the potential to be biocontrol agents of phytopathogenic fungi, few studies have been conducted on actinomycetes from marine, saline, and wetland environments, which have equal or greater potential as biocontrol agents than actinomycetes isolated from terrestrial environments.

Back side view

Front side view

Fig. 4. *Aspergillus brasiliensis*

Back side view

Front side view

Fig. 5. *Candida albicans*

Table 1. Measurement of antifungal activity in mm

Zone of Inhibition (mm)					
Pathogens	**25µl**	**50µl**	**75µl**	**100µl**	**+Ve (100 µl)**
Candida albicans	18 Mm	21 Mm	23 Mm	25 Mm	32 Mm
Aspergillus brasiliensis	20 Mm	23 Mm	26 Mm	30 Mm	35 Mm

+ve – Fluconazole

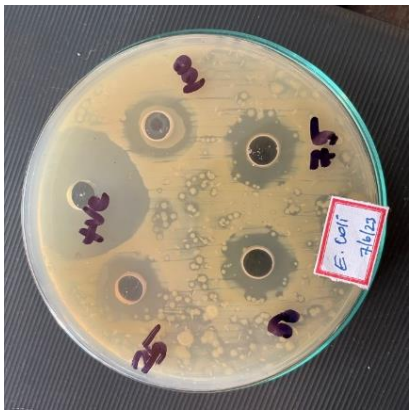


Fig. 6. *E.coli*

Fig. 7. *Streptococcus pyogenes*

Table 2. Measurement of antibacterial activity in mm

Zone of Inhibition (mm)					
Pathogens	**25µl**	**50 µl**	**75 µl**	**100 µl**	**+ve (100 µl)**
E.coli	12mm	15 mm	18 mm	20 mm	30 mm
S.pyogenes	8mm	10 mm	12 mm	15 mm	25 mm

+ve – Chloramphenicol

8.4 Antibacterial Activity of *Streptomyces Smyrnaeus*

Actinomycetes antibacterial activity is mostly due to the formation of secondary metabolites, which include a wide range of antibiotics. These chemicals frequently target specific bacterial activities, resulting in cell death or growth suppression. For example, the actinomycete isolates from the coasal islands revealed a high proportion of antibacterial activity, especially against Gram-positive bacteria, which are normally more susceptible due to their cell wall structure. In conclusion, actinomycetes are an important resource in the continuous hunt for novel antibacterial drugs. Their ability to synthesize a wide range of bioactive chemicals puts them as critical participants in combatting antibiotic resistance and addressing the global health threat posed by resistant bacteria. Continued investigation of their potential in uncharted situations may result in important advances in antibiotic development.

Two pathogens have zone of inhibition from four bacterial pathoens. These two pathogens show inhibition. Results are mentioned the table.

Aspergillus brasiliensis, Candida albicans, and *Streptococcus pyogenes, E. coli,* were all hostile to actinomycetal isolates respectively. This observation led to the conclusion that the actinomycetal isolates produced an antibiotic and antifungal chemical, which was extracted in ethyl acetate. It was also noted that the most promising actinomycete for generating compounds was the actinomycetal isolate. The location of antimicrobial marine actinomycetes in Vedharanyam's saltpan sediments coast and the antimicrobial activity of marine actinomycetal isolates that hinder the growth of diverse bacteria are shown in Tables 1 and 2.

8.5 Characterization of *Streptomyces Smyrnaeus* by FT-IR Spectroscopy

Transform of Fourier an analytical method for identifying organic, polymeric, and occasionally inorganic materials is infrared spectroscopy, sometimes referred to as FT-IR analysis or FT-IR spectroscopy. Infrared light is scanned across test samples using the FT-IR analysis method to observe chemical characteristics. Using a Perkin Elmer 2000 FT-IR spectrophotometer, the actinomycete isolate's crude ethyl acetate extract's infrared (IR) spectra were recorded between 400 and 4000 cm-1 (Arulappan et al., 2012). Based on the peak values, the functional groups and vibration kinds were evaluated [55-60].

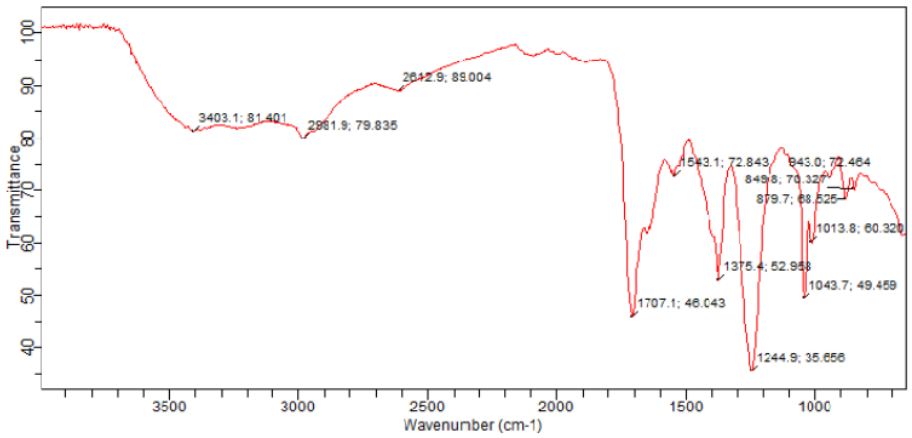


Fig. 8. FT-IR analysis of actinomycetes

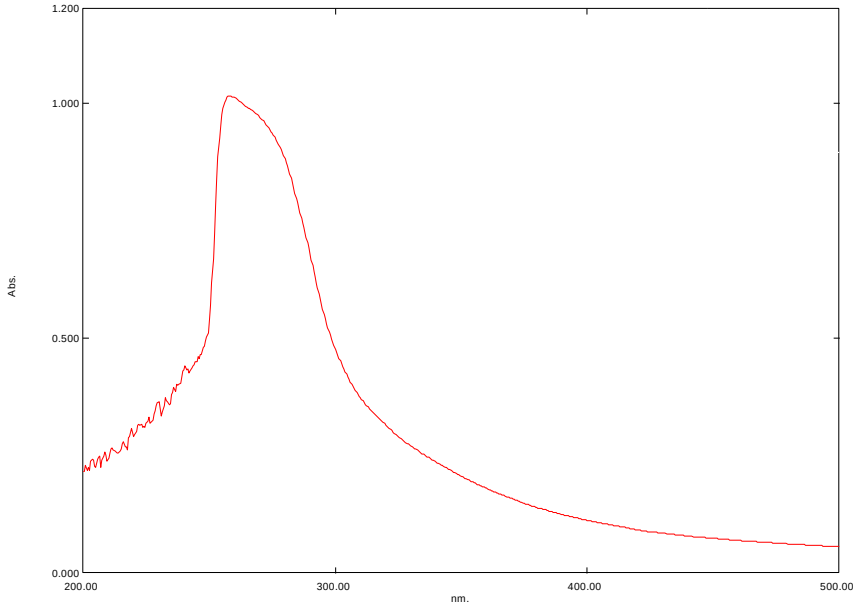


Fig. 9. UV-visible spectrophotometer analysis of actinomycetes

8.6 UV-Visible Spectrophotometer Analysis

The UV spectra of "*Streptomyces smyrnaeus*" acquired from this investigation were compared to the range of wave length and the general pattern maximal absorbance peaks. The active extract's UV range, which is 200–500 nm, was measured. The term "ultraviolet-visible spectrophotometry" (UV/Vis) describes spectroscopy used to measure absorbance or reflectance in the ultraviolet-visible spectrum. Analytical techniques, involving ultraviolet-visible (UV-VIS) spectroscopy can quantify the amount of analyte based on how much light it absorbs.

8.7 Gas Chromatography Mass Spectrometry Analysis (GC-MS)

GC-MS was used to evaluate the crude extracts from within cells. One isolate had a total of 30 peaks found in the GC-MS study. By using GC-MS, the compound's structure was further clarified. The compound's mass spectrum was discovered to be identical. to that of N,N'-Dimethylpiperazine, and the GC-MS library also provided confirmation of the compounds structure.

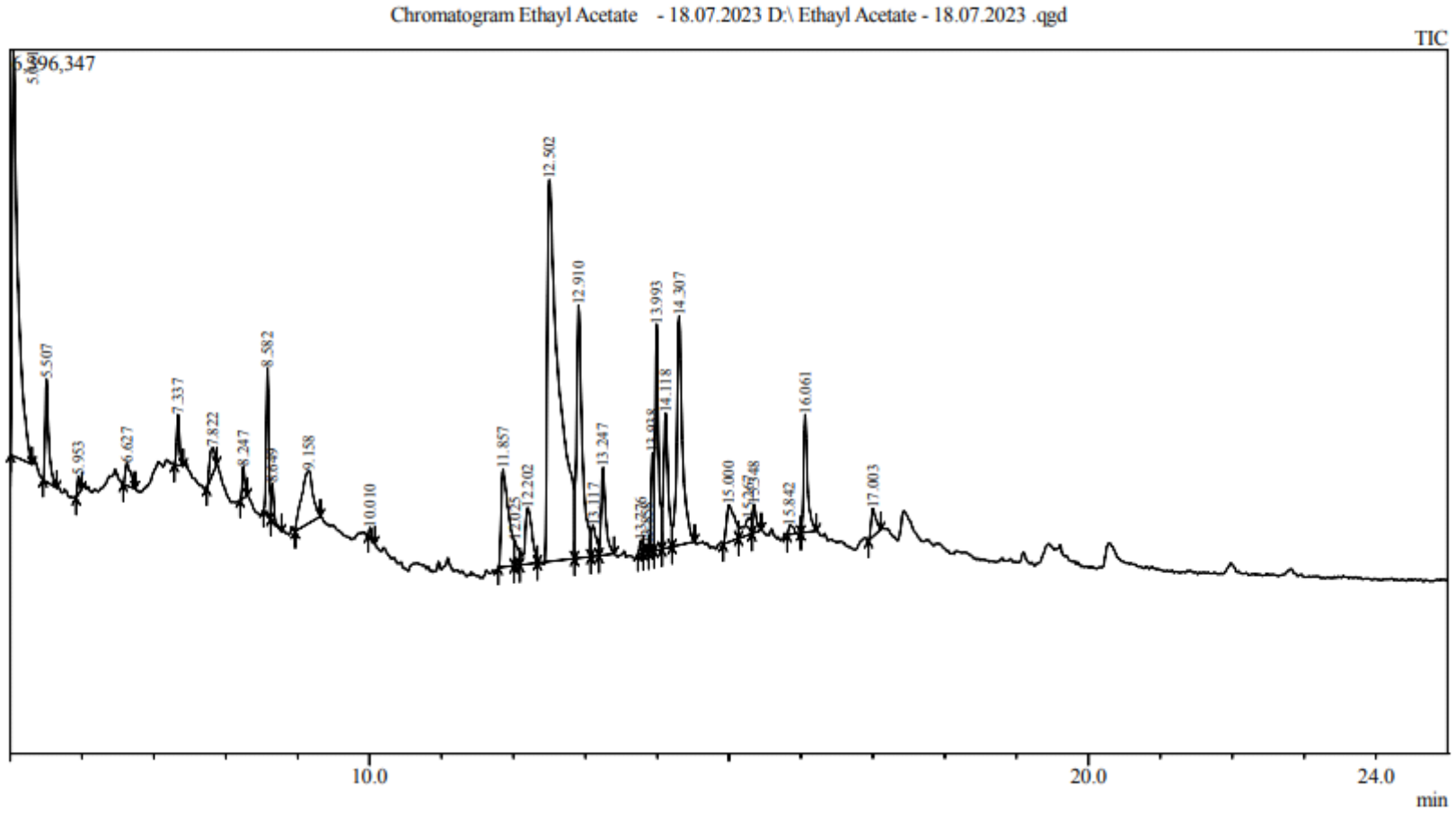


Fig. 10. GC-MS analysis of intracellular extract of *Streptomyces smyrnaeus*

Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	5.051	20790707	14.56	3692317	14.62	5.63	N,N'-Dimethylpiperazine
2	5.507	3158991	2.21	932066	3.69	3.39	Glutaric acid, trimethylsilyl ester
3	5.953	393057	0.28	159804	0.63	2.46	Ethanol, 2,2'-oxybis-, diacetate
4	6.627	1026685	0.72	204216	0.81	5.03	ACETIC ACID 4-OXO-CYCLOHEXYL EST
5	7.337	1229309	0.86	459653	1.82	2.67	1-Naphthalenamine, 4-nitro-
6	7.822	1246490	0.87	232448	0.92	5.36	2(Rs)-ethyl thiazolidine-4(R)-carboxylic acid
7	8.247	726257	0.51	286695	1.14	2.53	1-(2-Phenylethyl)-pyrrol
8	8.582	3370288	2.36	1335499	5.29	2.52	Quinoline, 1-acetyl-1,2-dihydro-8-methoxy-2,
9	8.649	772877	0.54	336412	1.33	2.30	4-Quinolinecarboxamide, N-hydroxy-
10	9.158	5318646	3.72	475039	1.88	11.20	1-[(2-Hydroxyethyl)(2-hydroxypropyl)amino]-
11	10.010	179125	0.13	94015	0.37	1.91	Diethyl Phthalate
12	11.857	6254570	4.38	885321	3.51	7.06	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-di
13	12.025	835816	0.59	225989	0.90	3.70	(3S,6S)-3-Butyl-6-methylpiperazine-2,5-dione
14	12.202	3489809	2.44	510946	2.02	6.83	2-Furanmethanamine, tetrahydro-N-[(tetrahydr
15	12.502	39615119	27.74	3469901	13.74	11.42	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
16	12.910	12676819	8.88	2297209	9.10	5.52	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
17	13.117	1335718	0.94	266936	1.06	5.00	1,2-Benzenedicarboxylic acid, bis(2-methylpro
18	13.247	3439869	2.41	797345	3.16	4.31	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
19	13.776	317350	0.22	119616	0.47	2.65	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-dien
20	13.858	226580	0.16	65883	0.26	3.44	EICOSAMETHYLCYCLODECASILOXANE
21	13.938	2581600	1.81	891335	3.53	2.90	prolylleucyl anhydride
22	13.993	6587105	4.61	2054317	8.14	3.21	l-(+)-Ascorbic acid 2,6-dihexadecanoate
23	14.118	5423143	3.80	1229125	4.87	4.41	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
24	14.307	11429743	8.00	2081168	8.24	5.49	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipy
25	15.000	2586746	1.81	338061	1.34	7.65	N,N'-Isopropyl-N''-4-[N-aziridyl]butylguanidi
26	15.267	1283055	0.90	148398	0.59	8.65	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
27	15.348	926702	0.65	256776	1.02	3.61	TETRACOSAMETHYLCYCLODODECASI
28	15.842	354482	0.25	79129	0.31	4.48	OCTADEC-9-ENOIC ACID
29	16.061	4032375	2.82	1070629	4.24	3.77	Octadecanoic acid
30	17.003	1214593	0.85	253763	1.01	4.79	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-
		142823626	100.00	25250011	100.00		

Fig. 11. Bioactive compounds of intracellular extract of *Streptomyces smyrnaeus*

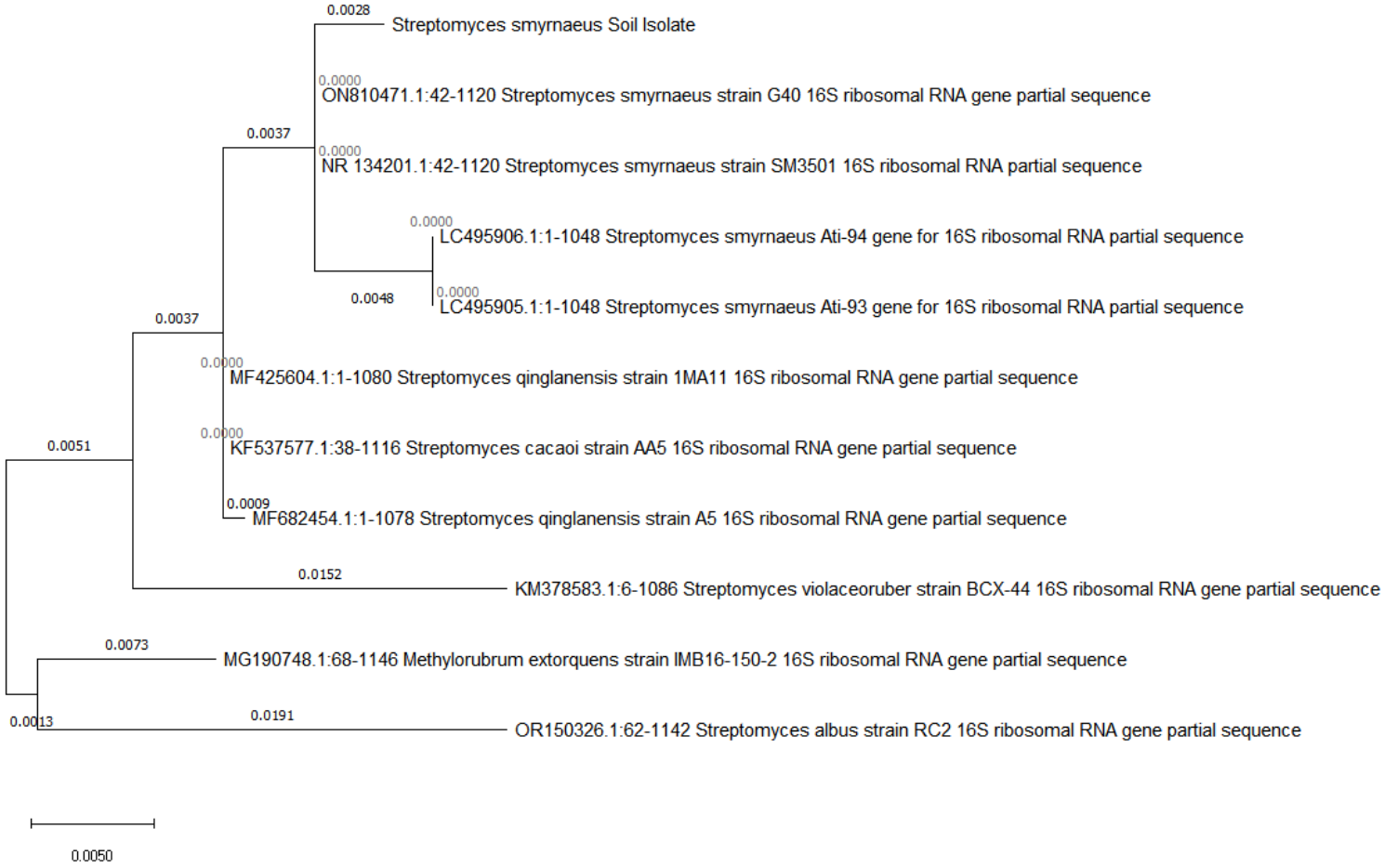


Fig. 12. 16S rRNA gene sequencing phylogenetic tree of *Streptomyces smyrnaeus*

8.8 16S rRNA Gene Sequencing and Phylogenetic Analysis

The Biobee Spin EX pure Microbial DNA isolation kit was used to isolate DNA from microbial sources. The 16S rRNA gene sequencing produced several findings. The 16S rRNA sequence was blast-similarity searched using the NCBI blast similarity search tool. Multiple sequence alignment was carried out after the phylogenetic analysis of the query sequence with the closely related sequence of the blast findings. The NCBI Nucleotide BLAST analysis shown the isolated bacteria from marine soil shown 100% of query coverage and 98.33% of sequence identity to "*Streptomyces smyrnaeus"*.

NCBI Accession Number: OR554236

Streptomyces is thought to be the primary source of bacteria for the synthesis of bioactive substances, such as naturally occurring antibiotics.

9. CONCLUSION

The distinct Streptomyces strains are significant as possible sources of strong, broad-spectrum antibacterial drugs, as indicated by the results of the current investigation. Purification and structural evaluation of these strains active ingredients. There are currently ongoing investigations in this area. Actinomycetes are classified as Gram-positive bacteria and it prevalent in both soil and water. Their capacity to generate a broad spectrum of bioactive substances, including as antifungals, antibiotics, and anticancer agents, is well recognized. In addition to being used in the production of enzymes and other industrial products, actinomycetes are also essential for the decomposition of organic matter in soil and are used in the fermentation of foods like soy sauce and cheese as well as other fermented foods. Actinomycetes are considered a useful resource for the discovery of novel natural products because of their distinct sets of enzymes that allow for the synthesis of compounds with a broad range of possible uses.

DISCLAIMER (ARTIFICIAL IN TELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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