

PRODUCTION OF ANTIFUNGAL COMPOUNDS FROM ACTINOBACTERIA

Submitted in partial fulfillment of the requirements for the
award of Bachelor of Technology degree in biotechnology

By

Joevathy J (39230021)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

**JEPPIAAR NAGAR, RAJIV GANDHISALAI,
CHENNAI – 600119**

MAY - 2023



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

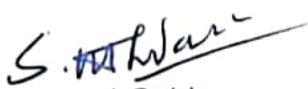
Accredited "A" Grade by NAAC | 12B Status by UGC | Approved by AICTE

www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of JOEVATHY J (230021) who carried out Project entitled "PRODUCTION OF ANTIFUNGAL COMPOUNDS FROM ACTINOBACTERIA" under my supervision from December 2022 to March 2023.


Internal Guide

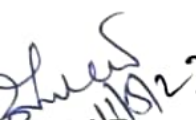
Dr. S. USHA NANDHINI, Ph.D.,



Head of the Department

Dr. V. RAMESH KUMAR, Ph.D.,

Submitted for Viva voice Examination held on 11-05-2023.


Internal Examiner


External Examiner

DECLARATION

I, JOEVATHY J (Reg. No- 39230021), hereby declare that the Project Phase-I Report entitled "PRODUCTION OF ANTIFUNGAL COMPOUNDS FROM ACTINOBACTERIA" done by me under the guidance of Dr. S. USHA NANDHINI, Ph.D., is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology in Biotechnology.

DATE: 11 - 05 - 2023

PLACE: Chennai


SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **Board of Management of SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. RAMESH KUMAR, Ph.D.**, Head of the Department of biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr. S. USHA NANDHINI, Ph.D.**, for her valuable guidance, suggestions and constant encouragement paved way for the successful completion of my phase-1 project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the **Department of Biotechnology** who were helpful in many ways for the completion of the project.

ABSTRACT

Streptomyces are considered the most important bacterial source for natural antibiotics, so this study aims to isolate actinomycetes from the soil samples and focus on their antifungal compounds production and characterization. Isolation of actinomycetes is done by serial dilution method-spread plate method. Subculture is done using Actinomycetes isolation (AI) medium and Primary screening of actinobacteria is performed with fungi by cross streak method. Extraction of secondary metabolite from active strain by solvent extraction method. Agar well diffusion using crude compound for testing antifungal activity. Pure extraction showed inhibition against fungi species aspergillus niger, athelia, fusarium oxysporum, Trichoderma viride. Purification of active crude compounds by TLC and Again agar well diffusion for purified compound for antifungal study. Characterization of active purified compound by GC-MS. Identification of active strain by 16s RNA gene sequencing were performed. Based on morphological characteristics and 16S rRNA analysis , the isolate were identified as *streptomyces griseoincarnatus*

TABLE OF CONTENTS

s.no	contents	page no
i	Abstract	v
ii	List of tables	viii
iii	list of figure	9
1	Chapter 1	10
1.1	Introduction	10
2	chapter 2 - literature survey	12
2.1	Inference from literature	12
2.1.1	Taxonomy	12
2.1.2	Habitat	12
2.1.3	Secondary metabolism of streptomyces spp.	12-13
2.1.4	Antibiotics produced by streptomyces spp.	13-14
2.1.5	The life cycle of streptomyces	15
2.1.6	Techniques used to characterize bioactive compounds produced by actinomycetes	15
2.1.7	High performance liquid chromatography	16
2.1.8	Gas chromatography mass spectrometry	16-17
2.1.9	Nuclear magnetic resonance spectroscopy	17-18
2.1.10	Two-dimensional nuclear magnetic resonance spectroscopy	18
2.1.11	Antifungal compounds from actinomycetes	19
3	Chapter 3	20
3.1	Aim and scope	20
4	Chapter 4	21
4.1	Requirements analysis	21
4.2	Methodology	22-33
4.2.1	Isolation and identification of actinobacteria from soil samples	22
4.2.2	Identification of bacterial isolates	23
I	Colony characteristics	23

II	Gram's stain	24
4.2.5	Primary screening	24
4.2.6	Mass cultivation	24
4.2.7	Extraction of active pure compound	25
4.2.8	Secondary screening	26
4.2.9	Characterization of antimicrobial metabolites	27
i	Gas chromatography - mass spectroscopy	27-29
ii	16S rRNA gene sequencing	29-33
5	Chapter 5	34
5.1	Workflow	34
6	Chapter 6	35
6.1	Results and discussion	35-39
7	Chapter 7	40
7.1	Summary and conclusion	40
8	References	41-47

LIST OF TABLES

S.no	Table	Page no
1	Table 2.1	14
2	Table 4.1	21
3	Table 4.2	22
4	Table 4.3	31
5	Table 4.4	32

LIST OF FIGURES

fig.no	Figure	Page no
4.2.1	Soil sample, serial dilution	22
4.2.2	Spread plate method	23
4.2.6	Mass production	25
4.2.7	Pure extract	26
4.2.8	Inhibition zone against aspergillus	26
6.1.1.1	Isolation of actinomycetes	35
6.1.1.2	Subculture	35
6.1.2.1	Antifungal activity 1	36
6.1.2.2	Antifungal activity 2	36
6.1.2.3	Antifungal activity 3	36
6.2.4	Phylogenetic tree	38

CHAPTER 1

1.1 INTRODUCTION

This study aims to isolate actinomycetes from soil samples and focus on their antifungal compounds production and characterization. According to Waksman and Henrici's inaugural description of the Actinobacteria phylum in 1943, the *Streptomyces* genus is a member of this group. These gram-positive bacteria have an odd morphology, are widely dispersed in soil, and can thrive on a variety of substrates, allowing them to colonize practically all habitats. *Streptomyces* also has a lengthy life cycle and an advanced secondary metabolism. Due to their capacity to create a variety of bioactive chemicals with antibacterial, antifungal, anti-inflammatory, and anti-tumor actions, they attracted a lot of interest from the scientific community. More than 65% of the antibiotics and antifungals currently utilized in agriculture and medicine come from this family, with *Streptomyces* producing more than 10,000 of these bioactive chemicals. The *Streptomyces* genus is also well known for its capacity to create VOCs, which are low molecular weight molecules with the ability to diffuse through the soil and environment at normal temperature and pressure [1]. Actinomycetes, especially *Streptomyces* species, have drawn a lot of attention as biocontrol agents. Numerous studies have been done on the effectiveness of antagonistic *Streptomyces* spp. in the biological control of plant pathogenic fungi such *Pyrenochaeta lycopersici*, *Sclerotium rolfsii*, and *Fusarium oxysporum*. The potential of *Streptomyces* species to produce antibiotics and volatile organic compounds (VOCs) is well documented. Low-molecular-weight chemicals called VOCs rapidly evaporate at room temperature and pressure, giving them the property of diffusion through the soil and environment. According to reports, the emission of VOCs by soil bacteria promotes plant growth, exhibits nematicidal activity, and causes systemic resistance in crops in addition to inhibiting the growth of fungal diseases [2]. *Streptomyces* are frequently employed in the commercial drug business to make antibiotics and are also recognized as a scarce source to make essential chemicals for agriculture and medicine. Secondary metabolites can be extracted using a variety of solvents, and different techniques, including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR), can be used to

determine the active metabolites and their structures. Today, LC-MS and GC-MS are frequently used in conjunction for screening various chemicals. For complicated biological mixtures, the effectiveness of GC-MS for compound separation and identification is very high. This technique is primarily used to create volatile chemical fingerprints that make analysis tedious. Lower molecular mass volatile or semi-volatile substances can be separated using GC-MS. Another technique that combines liquid chromatography and mass spectrometry is called LC-MS. spectrometry in mass It has the ability to recognize and analyze a broad variety of chemicals, many of which are found in trace amounts and have molecular masses ranging from 10 to 300,000.

CHAPTER 2

LITERATURE SURVEY

2.1 Inference from literature survey

2.1.1 Taxonomy

The Genus *Streptomyces* belongs to Actinobacteria. Actinobacteria have about 18 main lineage which are now notarized inside the bacterial area with many turns among the soil category. Actinomycetes is a member of the order Actinomycetales, which divided into eight different families and 63 genera depending on Berge's manual for classification (Holt *et al.*, 1994; Olano *et al.*, 2009). A taxonomic classification is obtainable from the Global Biodiversity Information Facility (Anderson and Wellington, 2001)

kingdom:	Bacteria:
Phylum:	Actinobacteria
Class:	Actinobacteria
Order:	Actinomycetes
Family:	Streptomycetaceae
Genus:	<i>Streptomyces</i>

2.1.2 Habitat

The *Streptomyces* are highly distributed in nature. Soil is the primary habitat, they are also found in a variety of habitats like plants, deserts, ice in the South Pole, sea and insects (Bull *et al.*, 2011). *Streptomyces* have an important role in biodegradation by decomposition of many compounds like lignin, therefore, they have been focused in microbial research and development for decades (Chen *et al.*, 2012).

2.1.3 Secondary Metabolism of *Streptomyces* spp.

The biochemistry of *Streptomyces* is highly notable, as they produce half of all known antibiotics. Secondary metabolites of *Streptomyces* have benefits in human

medicine, may work as antibacterial, antifungal and antitumor, also in agriculture, may work as plant protection agents, growth promoters, herbicides and antiparasitic (Berdy *et al.*, 1964; Hopwood, 1999). The antibiotic production of cultured *Streptomyces* on agar usually corresponds with the early stages of morphological differentiation. *Streptomyces coelicolor* is the most known and understood species among *Streptomyces*. It has a single linear chromosome instead of the circular one found in bacteria, and the complete sequence showed DNA length about 8,667,507bp containing 7,825, by which it became the largest bacterial genome, nearly twice the size of *Escherichia coli*, *Bacillus subtilis* and *Mycobacterium tuberculosis*. Genes number are more than that in *Saccharomyces cerevisiae*, which has 6,183 genes. These findings give *Streptomyces coelicolor* a complex life cycle in an environment which must adapt to it continuously (Blattner *et al.*, 1997; Kunst *et al.*, 1997; Bentley *et al.*, 2002). The species *S. coelicolor* has 4 different secondary metabolites with antibiotic activity: The Calcium Dependent Antibiotic (CDA), visually detected actinorhodin (Act) which is the best example of all *Streptomyces* antibiotics with color dependent on environmental pH (blue in neutral and alkaline solutions and red in acidic), methylenomycin (Mmy) and undecylprodigiosin (Red) (Hopwood, 1999; Liu *et al.*, 2013). The Actinomycetes have a high ability to produce antibiotics. The metabolic variety of the Actinomycetes is due to encoding hundreds of transcriptional factors in their genome permitting them to control gene expression in response to specific needs. They are widely distributed in nature, living in cold and tropical areas, in addition to their ability to live in a very hard condition like that in desert. *Streptomyces* strains are also found to occur in fresh water and marine environment (Weyland, 1981; Diab and Zaidan 1986; Goshi *et al.*, 2002).

2.1.4 Antibiotics produced by *Streptomyces* spp.

The Streptomycetes are capable of producing secondary metabolites such as antibiotics, anthelmintic enzymes, herbicides, anti- cancer drugs, growth factors like vitamin B12 and immune- modulators (Hasani *et al.*, 2014). Streptothricin is the first antibiotic obtained from *Streptomyces* spp. and discovered in 1942, then the discovery of Streptomycin in 1943, after that the scientists focused upon other antibiotics within this genus (Hasani *et al.*, 2014). Rachel Brown extracted the first antibiotic in 1949 for treatment of fungal disease called Nystatin from

Streptomyces noursei (Orna, 2001). About 80% of the antibiotics are isolated from *Streptomyces* spp. Over twothirds of the clinically useful antibiotics are related to this genus (Kieser *et al.*,2000). Some of antibiotics that produced by *Streptomyces* spp.are listed in table (2.1). Although the success in antibiotics discovery,infectious diseases still the second causes of death in the world due to microbial resistance, so researchers try to discover new antibiotics that are more stable to inactivating enzyme produced by resistant bacteria to change them to be more effective agents (Nikaido,2009).Discovery of actinomycin, the first antibiotic from an Actinomycete, many anti-tumor agents and bioactive compounds have been produced from actinomycetes.Pathogenic species of Actinomycetes to human may be obligate infections acquired from exogenous species at natural wastes, particularly those under the genera *Streptomyces*, *Tetinomadara* and *Nocardia*. The optimum temperature for Actinomycetes growth ranges from 25°C to 30°C. Most members of Actinomycetes are mesophilic, but the thermophilic are found and play a critical role in the degradation of various organic residues in compost (Nolan and Cross 1988).

Table 2.1 - Some antibiotics produced by *Streptomyces* spp.(Hasani *et al.*,2014)

Streptomyces spp	Antibiotic	Streptomyces spp	Antibiotic
<i>S. nodosus</i>	Amphotricin B	<i>S. aureofaciens</i>	Chlortetracycline, Dimethylchlor Tetracycline
<i>S. orchidaccus</i>	Cycloserin	<i>S. vensuella</i>	Chloramphenicol
<i>S.erythraeus</i>	Erythromycin S	<i>S.viridifaciens</i>	Tetracycline
<i>S.noursei</i>	Nistatin	<i>S.vinaceus</i>	Viomycin
<i>S.mediterranei</i>	Rifampin	<i>S.ambofaciens</i>	Spiramycin
<i>S.griseus</i>	Streptomycin	<i>S.avermitilis</i>	Avermicin
<i>S.knanamyceticus</i>	Kanamycin	<i>S.garyphalus</i>	Cycloserine
<i>S.tenebrarius</i>	Tobramycin	<i>S.ribosidificus</i>	Ribostamycin
<i>S.spectabilis</i>	Spectinomycin	<i>S.roseosporus</i>	Daptomycin
<i>S.fradiae</i>	Neomycin, Actinomycin, Fosfomycin, Dekamycin	<i>S.lincolensis</i>	Lincomycin, Clindamycin

2.1.5 The Life Cycle of Streptomyces

The life cycle of *Streptomyces* starts with a single spore germination, producing multi nucleoid filaments, then these filaments form a vegetative mycelium by branching on the surface and into the culture medium(Hardisson *et al.*,1978).The hyphae expand and grow exponentially forming a complex filamentous network which continues to penetrate the medium and utilizes the organic molecules by extracellular hydrolytic enzymes (Chater, 1993).The motility of *Streptomyces* vegetative form gives a good advantage to other bacteria which have less motility during their colonization in solid soil. The substrate mycelium will break the surface barrier to form aerial hyphae during exhaustion of nutrient supplies in the surrounding environment. Aerial growth coincides with the beginning of the secondary metabolism in cultured solid media (Chater, 1989). The continuous growing of aerial mycelium is occurred by the utilization of the vegetative mycelium. During un suitable conditions, the aerial mycelium growth stopping, then multigenomic tips undergo multiple septation and forming unigenomic perispore compartments. A characteristic grey pigment formed by holding nearly 50 mature spores together in chains. The exospores of *streptomyces* unlike the endospores of other Gram-positive bacteria, such as *Clostridium* and *Bacillus*, by their sensitivity to extreme pH and heat, however they resist desiccation (Schwedock, *et al* 1997; Ryding, *etal.*,1998).

2.1.6 Techniques used to characterize Bioactive Compounds Produced by Actinomycetes:

Many techniques such as elemental analysis, mass spectrometry (MS), Fourier transform-infrared spectrum (FT-IR), nuclear magnetic resonance (1H-NMR), high performance liquid chromatography and gas chromatography–mass spectrometry (GC–MS) analyses are used to analyze and characterize the active compounds from micro-organisms. Thin-layer chromatography (TLC) have characteristic chromatographic profiles and reactivity to secondary metabolites from microorganisms which help in the separation and identification of compounds in mixtures (El-Ahmady *et al.*, 2017).

2.1.7 High performance liquid chromatography

Chromatography is a method to separate mixture of materials into their components on the basis of their molecular structure and molecular texture, by pumping a pressurized liquid which is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) containing the sample mixture (also called a mobile phase) through a tube contained a solid adsorbent material (stationary phase). The composition and temperature have a role in the separation process by affecting the physical interactions between sample components and adsorbent. Each sample's compartment interacts with the adsorbent slightly and differently, causing their separation according to different flow rates. The speed of separation depends, on the column's nature, the chemical nature of the component and on the mobile phase composition (Lindsay, 1992). The characteristic of a given analyte is identified under certain conditions, and the time needed by a specific analyte to elute from the column is known the "retention time". Columns are filled with adsorbents, varying in the surface chemistry and in particle size (Gerber *et al.*, 2004). The strong interactions of the sample components with the stationary phase make the movement out of the column more slowly than weaker interactions. This difference in rates causes the decisiveness of various components, separations in chromatography can be done by assortment of stationary phases, including immobilized paper (paper chromatography), silica on glass plates (thin-layer chromatography), liquids (liquid chromatography) and volatile gases (gas chromatography) (Czaplick, 2013).

2.1.8 Gas Chromatography Mass Spectrometry (GC/MS)

It is an apparatus that used to identify the components in a mixture like hydrocarbons, essential oils and solvents. The electron capture detector and a flame ionization detector can quantitatively determine the materials even very low concentrations. It is widely used especially in biochemistry because of its simplicity, sensitivity, and effectiveness in separating components of mixtures quantitatively and qualitatively to fix thermo chemical standards as heats of solution and vaporization, vapor pressure, activity coefficients and for purification of compounds (Androw, 2007;

Kadhim *et al.*, 2016). Many studies have been increasingly applied to analyze medicinal plants which proved to be an excellent method for the analysis of non-polar compounds and volatile essential oil, alkaloids, lipids and fatty acids. The GC-MS is important in medicinal chemistry researches, pharmaceutical analysis, pharmacognosy, pharmaceutical biotechnology and pharmaceutical process control. (Abeer *et al.*, 2017). The GC-MS consists of two components: the gas chromatograph and the mass spectrometer. The work of Gas chromatography depends on the length, diameter, film thickness of the capillary column, in addition to the phase properties (e.g. 5% phenyl polysiloxane). The separation of the different molecules within a mixture depends on the chemical properties between these molecules and on their relative affinity for the stationary phase of the column during the pass of the sample along the column. The molecules are retained and eluted from the column at different times called the “retention time”, then these molecules are captured, ionized, accelerated and deflected by mass spectrometer. The ionized molecules are separated by breaking each one into ionized fragments then these molecules are detected by mass to charge ratio (Sparkman, *et al.*, 2011)

2.1.9 Nuclear magnetic resonance spectroscopy (NMR):

Nuclear magnetic resonance spectroscopy is also called magnetic resonance spectroscopy (MRS). Analytical chemistry is used for determining the content, purity and molecular structure of a sample. By NMR, the mixtures containing known compounds are analyzed quantitatively, determine the molecular conformation in solution, while the unknown compounds are detected through matching against spectral libraries or by estimating the basic structure directly. The basis of NMR is that nuclei have spin and are electrically charged. The signal is produced by the nuclei excitation with radio waves (the radio waves are the source of energy in NMR, which have more than 107 nm long wavelengths, so low energy is produced by the interaction of low wavelengths with a molecule) into NMR, then a sensitive radio receiver detects the signal. External magnetic field is applied to the sample, then the energy transfer from base energy to higher energy forming a single energy gap (John, 1965; Schweiger and Geschke, 2001; Blackburn, 2003). NMR spectroscopy identifies complex molecules, giving good details on molecule like structure, reaction state, chemical environment and dynamics. NMR is used to any kind of sample that

has nuclei possessing spin, like ^1H NMR which determine H atoms type and number in a molecule and ^{13}C NMR which determine carbon atoms type in a molecule (John, 1965; Schweiger and Geschke, 2001; Blackburn, 2003). The disadvantage of NMR is the need for a large amount of purified material (2– 50 mg), which resolves through a workup. To allow well and equal resolved spectra, the sample should be dissolved in a solvent, because the solids NMR analysis requires a dedicated magic angle spinning machine. The time needed by NMR analysis is relatively long, so it is not suitable for the fast phenomena. NMR is exactly not very sensitive, in spite of higher frequencies and higher sensitivity, a large amount of impurities may appear in NMR spectrum (Paudler, 1974).

The 1D ^1H NMR spectroscopy is the most common technique used for studying metabolites. The 1D ^1H NMR techniques advantages include measuring the metabolite concentrations directly using a single internal standard and the spectral acquisition is rapid. There is a limitation in proton chemical shifts and a high resonance number from various metabolites, so severe overlapping of spectral peaks is encountered by 1D NMR spectra of biological materials. This limitation demonstrates a considerable challenge for unique identification and quantification of metabolites. Using 2D ^1H NMR is a good solution for spectral overcrowding because it provides more accurate specificities and more molecular information about the metabolite. However, most of 2D NMR applications are limited due to their long acquisition time (Huang *et al.*, 2015).

2.1.10 Two-dimensional nuclear magnetic resonance spectroscopy (2DNMR):

There are many methods in two-dimensional NMR which give data in an area defined by two frequency axes, in addition to providing more information than one-dimensional NMR spectra, especially for determining the structure of a molecule, particularly for too complicated molecules to work with one dimensional NMR. In two-dimensional NMR, the emission is settled around a single frequency, and related resonances are observed, so good observation of the neighboring substituents of the functional group that allowed a good identification of the resonances (Martin and Zekter, 1988; Mateescu and Valeriu, 1993).

2.1.11 ANTIFUNGAL COMPOUNDS FROM ACTINOMYCETES

Antifungal compounds produced from actinomycetes are *4-methoxystyrene*, *2-pentylfuran*, *anisole*, ergotamine, amicomacin, fungichromin, rapamycin, and N-Acetyl-D, L-phenylalanine, Bafilomycin B1, Bafilomycin C1, Maltophilin, *eicosane* ($C_{20} H_{42}$) and *dibutyl phthalate* ($C_{16} H_{22} O_4$), Nonadecene, geosmin, l-linalool, 2-mercaptoethanol, and heneicosane, (3E, 5E, 7E, 9E, 11E, 13E, 15E)-16-(pyrrolidin-1-yl) hexadeca-3, 5, 7, 9, 11, 13, 15-heptaen-2-one, Triadimenol, propamocarb, 2-phenylethanol (2-PE), b-caryophyllene (b-CA), and methyl 2-methylbutyrate (M2M), dimethyl trisulfide, benzenamine, dimethyl disulfide, 2-(chloromethyl)-2-cyclopropyloxirane, 2, 4-ditert-butylphenol, and 1-ethylthio-3-methyl-1, 3-butadiene, Phenol, 2,4-bis(1,1-dimethylethyl)- Hexanoic acid, pentyl ester, Eicosanoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl] ethyl ester, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), and Octahydro-2H-pyrido(1,2-a) pyrimidin-2-one, Pyrrolopyrazine, Benzeneacetic acid, naphthalene, tetradecane, hexadecane, pentadecane, cyclohexadecane, bis(2-methylpropyl) ester of 1,2-benzenedicarboxylic acid, 1-nonadecene, 1-octadecanol, 9-hexacosene, and mono(2-ethylhexyl) ester, 3-carene 2,5-dione, beta-cubebene, phenol, 2-(1,1-dimethylethyl)-6-methyl, were found based on GC-MS and LC-MS/MS analyses[1,2,3,4,5,6,7,.....,20].

CHAPTER 3

AIM AND SCOPE

- Isolation and identification actinomycetes from soil sample and finding its antifungal activity.
- Characterization of active purified compounds by GC-MS, FT-IR & spectrophotometer.
- Identification of active strain by 16sr RNA gene sequencing.

Only small no. of antifungal drug types exist, so resistance can severely limit treatment options. Some types of fungi like candida auris, can become resistant to all the antifungal drugs so its very important to find more antifungal compounds. objective of the project is to find the antifungal compounds which can treat skin infections

CHAPTER 4

4.1 REQUIREMENT ANALYSIS

Table 4.1-Equipment and apparatus required

Equipment and Apparatus
Autoclave
Filter tips
GC-MS
Incubator
Light Microscope
Laminar flow
Micropipettes
Millipore filters (0.22 µm)
Millipore filters (0.45 µm)
Petriplates
Inoculation loop
Oven
Conical flask
Refrigerator
Sensitive balance
Test tubes
Bunsen burner
Separating funnel

Table 4.2 -Biological media used in isolation and screening process

s.no	Media
1	Actinomycetes isolation agar
2	Potato dextrose broth
3	Mueller Hilton agar

4.2 METHODOLOGY:

4.2.1 Isolation and identification of actinobacteria from soil samples

About one gram of dried sediment soil samples was used to make suspension, by adding it to 99 ml of sterile distilled water (stock suspension). Serial dilutions from 10^{-1} to 10^{-5} were made from the stock suspension and left for 10 minutes. After shaking, 0.1 ml of each dilution was pipetted and put on actinomycetes isolation agar, then spread by a sterile L-rod to make a uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7 to 14 days. Based on cultural characteristics, suspected colonies of actinomycetes were selected for being characterized as small, white, and pinpoint, rough, chalky and a clear zone of inhibition around them. The colonies were transferred from the first screening step (mixed culture) into separate agar plates and incubated at $28 \pm 1^\circ\text{C}$ for 7 days to obtain a pure growth of actinomycetes species, the last steps were repeated several times. The pure culture was kept at 4°C for a further study.

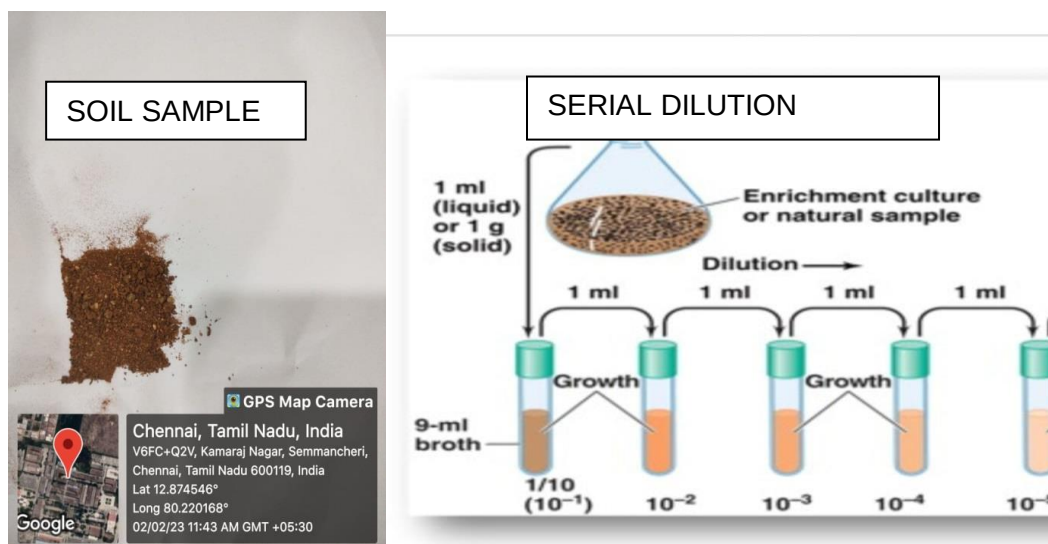


Figure 4.2.1 : soil sample, serial dilution

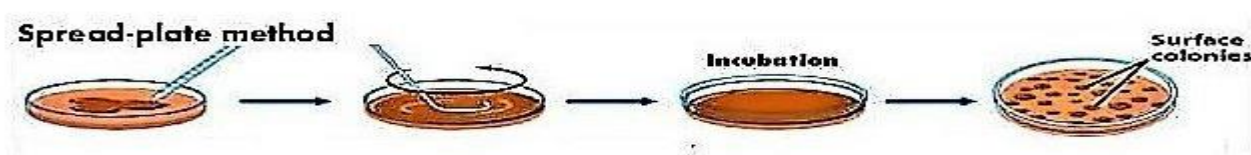


Figure 4.2.2 : spread plate method

4.2.2 Identification of Bacterial Isolates

Suspected *Streptomyces* colonies were identified according to cultural and physiological characteristics as follows:

Morphological characterization:

I. Colony Characteristics (Saadoun et al., 2015)

Suspected *Streptomyces* isolates which grew on ISP 2 and GYE medium were characterized morphologically according to the colony characteristics as follows:

- Mass color or mature, sporulating aerial surface growth.
 - The color of substrate mycelium as viewed from the reverse side.
 - Diffusible soluble pigments other than melanin.
- Mature cultures spore mass surface was observed after 7-14 days of

incubation and the color of aerial mycelium of *Streptomyces* was determined by a code collected by Prauser (1964) for color tabs of Baumann Farbtonkarte Atlas.

ii. Gram's stain:

The hyphae and spore-bearing hyphae morphology were determined by a direct microscopic examination of the culture surface on opened dishes of the crosshatched cultures using 100 x magnification. The *Streptomyces* species were determined according to the sections: straight, rectus, flexible, flexeous, Retinaculum-Apertum and spiral. A single colony was transferred by a loop to a clean glass slide. The smear was stained with crystal violet, treated with iodine, decolorized by the ethanol (95%), and stained with safranin, then examined by a microscope (Aghighi et al., 2004).

4.2.5 Primary screening (Giant Colony/ cross streak technique)

Nutrient agar plates were inoculated with *Streptomyces* isolate by a single streak of inoculum in the middle of plates. After 7 days of incubation at 28°C, the test bacterial isolates were streaked perpendicular to the *Streptomyces* (a single streak at a 90° angle to the *Streptomyces* isolates), then the plates were re-incubated at 37°C for 24 hours. The antibacterial activities were observed by the naked eye in which the tested bacteria failed to grow near the *Streptomyces* line (Madigan et al., 1997; Oskay, 2009).

4.2.6 MASS CULTIVATION:

Mass cultivation of streptomycetes isolates were done using actinomycetes isolation broth in a conical flask for 100 ml. 2.17 g of actinomycetes isolation broth powder is added to 100 ml of distilled water and then autoclaved for 20 min at 121°C. After that using inoculation loop the culture were inoculated. Totally 500 ml of broth solution were prepared and inoculated.



Figure 4.2.6 : mass production

4.2.7 EXTRACTION OF ACTIVE PURE COMPOUND:

Mass cultivation of actinomycetes were shaken well rigorously and centrifuged. After Centrifugation supernatant is collected and using separating funnel. Pure crude compound is extracted.

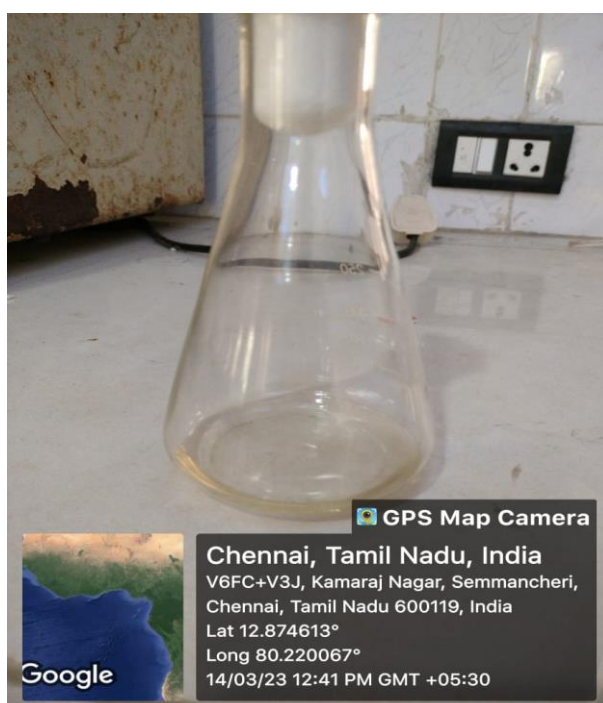


Figure 4.2.7 : pure extract

4.2.9 characterization of antimicrobial metabolites.

i. Gas chromatography – mass spectroscopy (GC – MS) analysis of bioactive-metabolite

The GC-mass chromatography analysis was performed to identify the active antifungal compounds in the active purified compound extract.

Method

[AOC-20i]

# of Rinses with Presolvent	:4
# of Rinses with Solvent(post)	:5
# of Rinses with Sample	:0
Plunger Speed(Suction)	:High
Viscosity Comp. Time	:0.2 sec
Plunger Speed(Injection)	:High
Syringe Insertion Speed	:High
Injection Mode	:Normal
Pumping Times	:5
Inj. Port Dwell Time	:0.3 sec
Terminal Air Gap	:No
Plunger Washing Speed	:High
Washing Volume	:8uL
Syringe Suction Position	:0.0 mm
Syringe Injection Position	:0.0 mm
Use 3 Solvent Vial	:1 vial

[GC-2010]

Column Oven Temp.	:100.0 °C
Injection Temp.	:250.00 °C
Injection Mode	:Split
Flow Control Mode	:Linear Velocity
Pressure	:73.0 kPa
Total Flow	:24.0 mL/min

Column Flow	:1.00 mL/min
Linear Velocity	:37.2 cm/sec
Purge Flow	:3.0 mL/min
Split Ratio	:20.0
High Pressure Injection	:OFF
Carrier Gas Saver	:OFF
Splitter Hold	:OFF

Oven Temp. Program

Rate	Temperature(°C)	Hold Time(min)
-	100.0	4.00
2.00	140.0	0.00
6.00	270.0	4.00

< Ready Check Heat Unit >

Column Oven	: Yes
SPL1	: Yes
MS	: Yes

< Ready Check Detector(FTD) >

< Ready Check Baseline Drift >

< Ready Check Injection Flow >

SPL1 Carrier	: Yes
SPL1 Purge	: Yes

< Ready Check APC Flow >

< Ready Check Detector APC Flow >

External Wait	:No
Equilibrium Time	:1.0 min

[GC Program]

[GCMS-QP2010 Ultra]

IonSourceTemp	:220.00 °C
Interface Temp.	:240.00 °C
Solvent Cut Time	:3.50 min
Detector Gain Mode	:Relative to the Tuning Result
Detector Gain	:1.00 kV +0.00 kV
Threshold	:0

[MS Table]

--Group 1 - Event 1—

Start Time	:3.50min
End Time	:49.00min
ACQ Mode	:Scan
Event Time	:0.30sec
Scan Speed	:2500
Start m/z	:45.00
End m/z	:700.00
Sample Inlet Unit	:GC

[MS Program]

Use MS Program	:OFF
----------------	------

ii. 16S rRNA Gene sequencing:

METHODOLOGY:

Genomic DNA isolation: DNA isolation from Microbial samples were done using the Biobee Spin EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd.

Protocol:

1. Lysis/homogenization: Cells grown in monolayer should be lysed by suspending 1-3 colonies aseptically and mixed with 500 µl of lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting.
2. Add 4 µl of RNase and 500 µl of neutralization buffer into it. 3. Vortex the content and incubate the tubes for 30 minutes at 65°C in water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.
4. Centrifuge the tubes for 10 minutes at 10,000 rpm.
5. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml micro centrifuge tube without disturbing the pellet.
6. Add 600 µl of Chloroform Isoamyl Alcohol and do hand mixing vigorously.
7. Centrifuge the tubes for 10 minutes at 10,000 rpm. Carefully, transfer 600 µl of aqueous phase into a fresh 2ml micro centrifuge tube.
8. Binding : Add 600 µl of binding buffer to the content and mix thoroughly by pipetting and incubate the content at room temperature for 5 minutes.
9. Transfer 600 µl of the contents to a spin column placed in 2 ml collection tube.
10. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through. 11. Reassemble the spin column and the collection tube then transfer the remaining 600µl of the lysate.
12. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through.
13. Washing : Add 500 µL washing buffer I to the spin column. Centrifuge at 10,000 rpm for 2 mins and discard flow- through
14. Reassemble the spin column and add 500µl washing buffer II and Centrifuge at 10,000 rpm for 2mins and discard flow-through. Dry spin the tube for 5 minutes at 10,000 rpm.
15. Transfer the spin column to a sterile 1.5-ml micro centrifuge tube 16. Elution: Add 100 µl of Elution buffer at the middle of spin column. Care should be taken to avoid touch with the filtrate.
17. Incubate the tubes for 2 minutes at room temperature and Centrifuge at 10,000 rpm for 2 minutes. The buffer in the micro centrifuge tube contains the DNA.
18. DNA concentrations were measured by Qubit flurometer 3.0 or 1% Agarose Gel Electrophoresis.

PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a singlestranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix

- Taq DNA polymerase is supplied in 2X Taq buffer
- 0.4mM dNTPs,
- 3.2mM MgCl₂ and
- 0.02% bromophenol blue.

Table 4.3 PRIMER DETAILS

Primer Name	Sequence Details	Number of Base
27F	5' AGAGTTTGATCTGGCTCAG 3'	20
1492R	5' TACGGTACCTTGTTACGACTT 3'	20

Add 5 µL of isolated DNA in 25 µL of PCR reaction solution (1.5 µL of Forward Primer and Reverse Primer, 5 µL of deionized water, and 12 µL of Taq Master Mix).

Perform PCR using the following thermal cycling conditions.

1. Denaturation The DNA template is heated to 95°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. Annealing The mixture is cooled to anywhere from 55°C . This allows the primers to bind (anneal) to their complementary sequence in the template DNA.

3. Extension The reaction is then heated to 72° C , the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

Table 4.4 PCR CONDITION

STAGES	TEMPERATURE	TIME	25 cycles
Initial Denaturation	95°C	2 min	
Denaturation	95°C	30 sec	
Annealing	50°C	30 sec	
Extension	72°C	2 min	
Final extension	72°C	10 min	
Hold	4°C	∞	

Purification of PCR Production

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ 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).

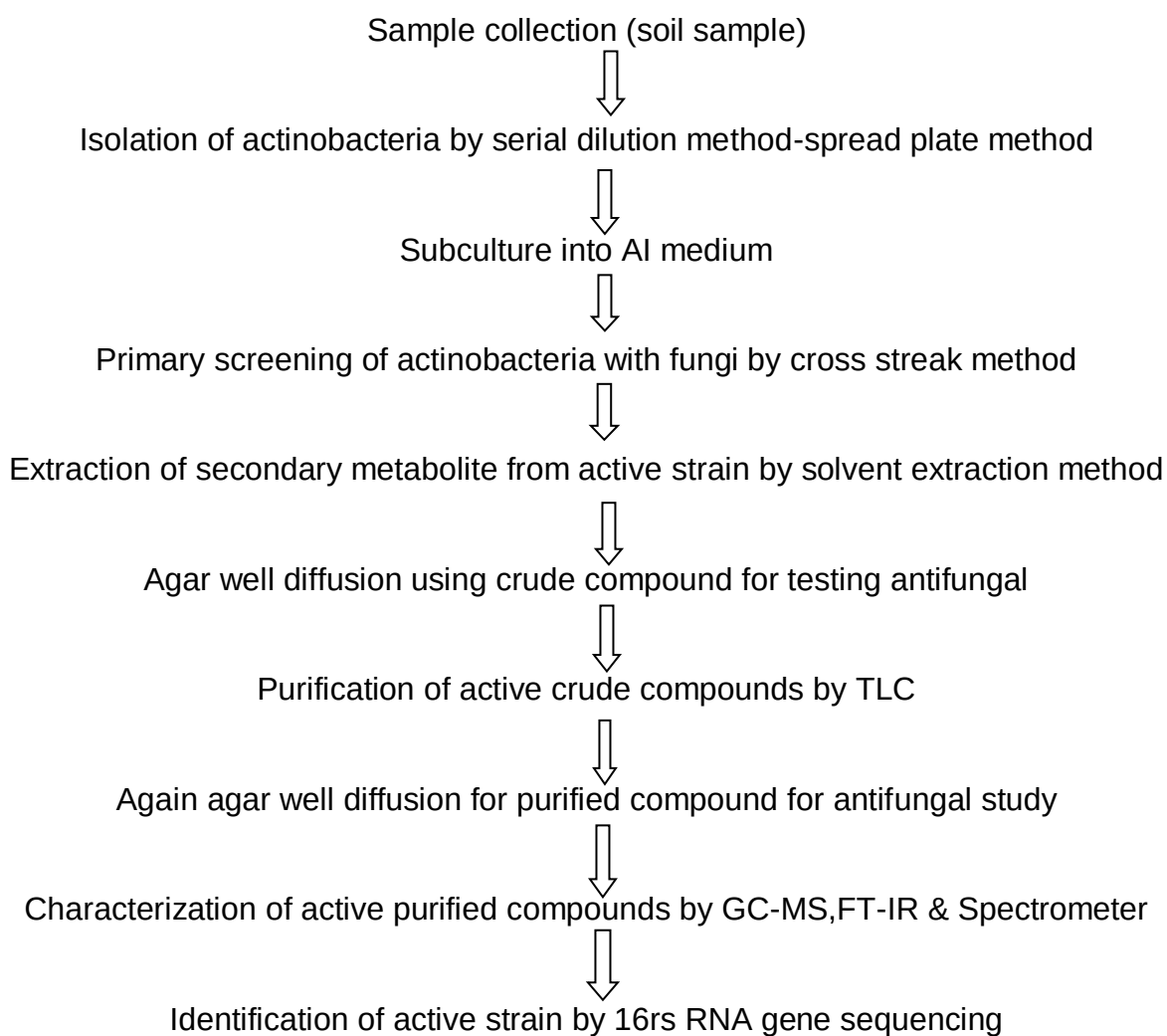
Bioinformatics protocol:

1. The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2. The program MUSCLE was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.
3. 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 TreeDyn method was used for tree rendering.

CHAPTER 5

5.1 WORKFLOW

ANTIFUNGAL COMPOUNDS FROM ACTINOBACTERIA



CHAPTER 6

6.1 RESULTS AND DISCUSSION:

6.1.1 Isolation of actinomycetes from soil sample:

Several distinct colonies were formed in a petridish, out of which 10^{-1} and 10^{-2} shows the clear colonies of actinomycetes species.

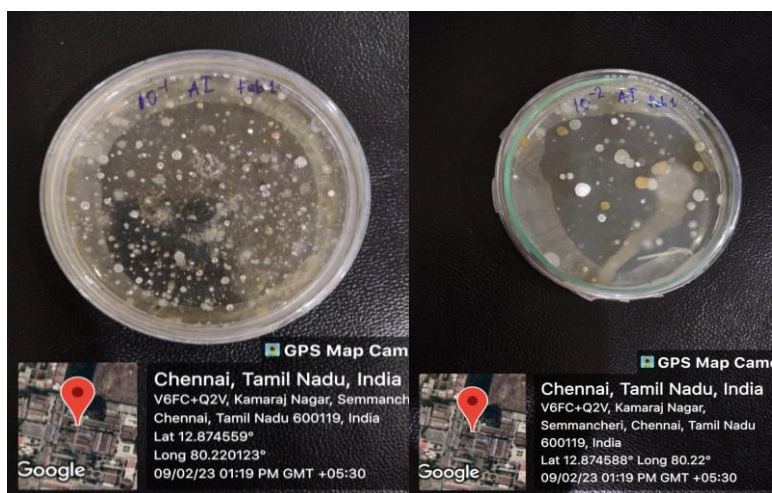


Figure 6.1.1.1 : isolation of actinomycetes

The colonies from these dilutions were subcultured onto actinomycetes isolation agar medium plate to form pure colonies by streak plate method.



Figure 6.1.1.2 : subculture

6.1.2 Antifungal activity:

Occurrence of inhibition zone against fungus *aspergillus*, *athelia*, *fusarium oxysporum*, *Trichoderma viride*.

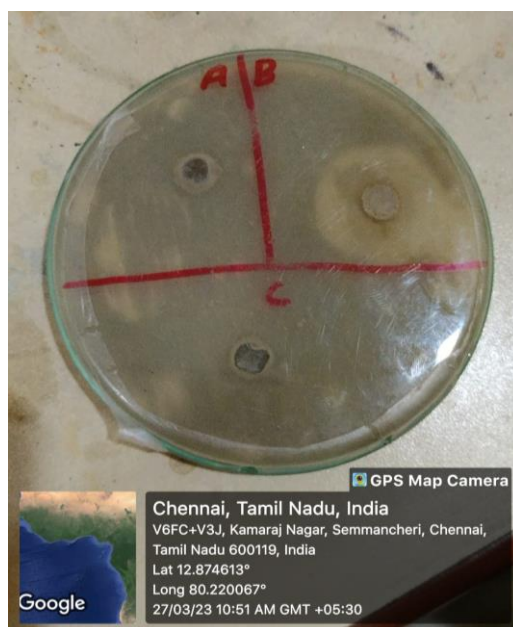


Figure 6.1.2.1 : antifungal activity 1



Figure 6.1.2.2 : antifungal activity 2

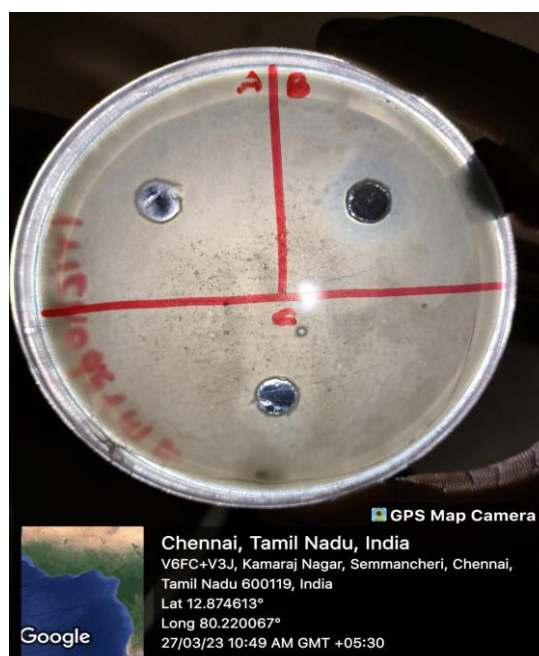


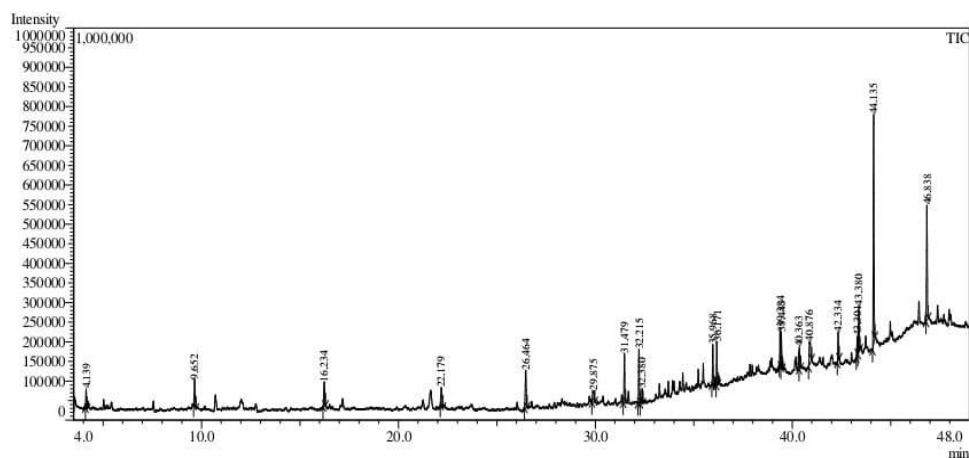
Figure 6.1.2.3 : antifungal activity 3

6.2.3 GC-MS ANALYSIS OF ACTIVE PURIFIED COMPOUNDS

Sathyabama Institute of Science and Technology, Centre for Ocean Research

3/28/2023 17:49:00

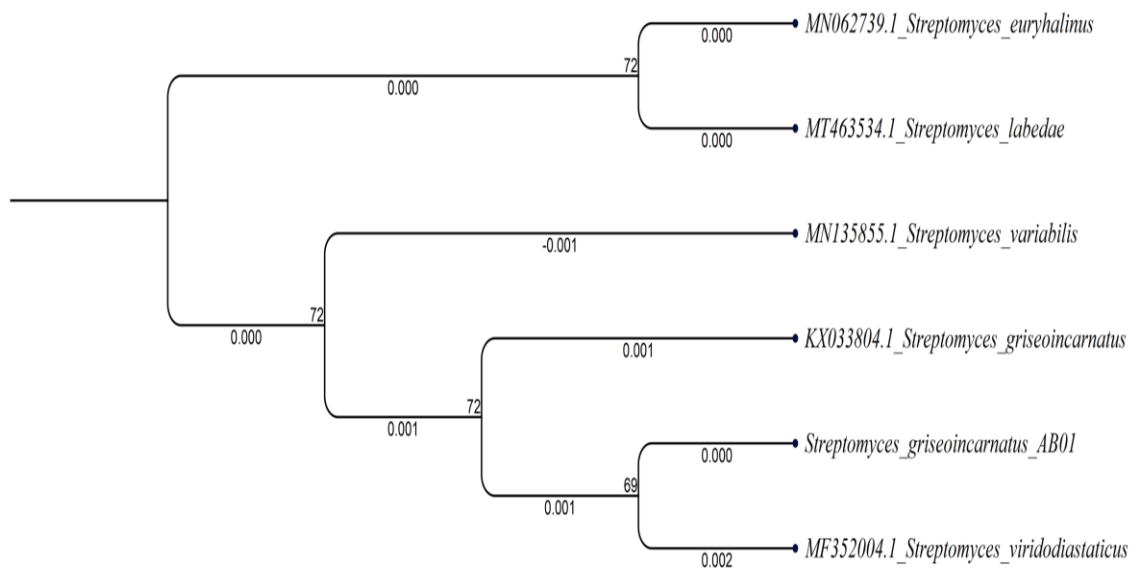
D:\DATA\March 2023\joevathy.qgd



Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	4.139	108693	1.70	OCTANE, 6-ETHYL-2-METHYL-
2	9.652	218786	3.43	Benzene, 1,3-bis(1,1-dimethylethyl)-
3	16.234	211729	3.32	1-Dodecanol
4	22.179	182734	2.86	2,4-Di-tert-butylphenol
5	26.464	344792	5.40	1-Hexadecanol
6	29.875	160738	2.52	Eicosane
7	31.479	327835	5.14	n-Nonadecanol-1
8	32.215	342680	5.37	1-EICOSANOL
9	32.380	108775	1.70	Heneicosane
10	35.968	242269	3.80	1-Nonadecene
11	36.171	263787	4.13	Behenic alcohol
12	39.384	215021	3.37	n-Tetracosanol-1
13	39.443	223900	3.51	1-Heptacosanol
14	40.363	136608	2.14	Glycidyl palmitate
15	40.876	291348	4.56	Myristic acid glycidyl ester
16	42.334	191394	3.00	Octacosanol
17	43.301	142351	2.23	1,8,11-Heptadecatriene, (Z,Z)-
18	43.380	356192	5.58	9-Octadecenoic acid (Z)-, oxiranylmethyl ester
19	44.135	1490133	23.34	Bis(2-ethylhexyl) phthalate
20	46.838	823503	12.90	8,12-Tetradecadienoic acid, 5-ethenyl-3,5,9,11-tetramethyl-
		6383268	100.00	

The NCBI Nucleotide BLAST analysis shown the isolated bacteria from soil shown 100% of query coverage and 99.52% of Sequence Identity to ***Streptomyces griseoincarnatus*** (KX033804.1). The 16S rRNA Gene Sequence of bacteria isolated from soil was curated and submitted to NCBI Genbank to retrieve the sequence accession number was acquired OQ826528.



38

> Contig Sequence_AB01

GATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACG
GGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGAC
GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGA
AAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTG
TCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT
CAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGC
GAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTG
GGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGC
CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCCG
ACAAGCGGCGGAGCATGTGGCTTAATTGCACGCAACGCGAAGAACCTTACCAAGGCTT
GACATACACCGGAAAGCTCCAGAGATGGTCCCCCCTTGTGGTTCGGTGTACAGGTGGT
GCATGGCTGTCGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCA
ACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCG
CCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCT
TGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATAACGCGAGGTGGA
GCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAA
GTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAAC
CCCTTGTGGGA

Streptomyces griseoincarnatus

CHAPTER 7

7.1 SUMMARY AND CONCLUSION

The goal of this work is to identify actinomycetes from soil samples and concentrate on their synthesis and characterisation of antifungal chemicals since streptomyces is thought to be the most significant bacterial source for natural antibiotics. By using the serial dilution method-spread plate method, actinomycetes are isolated. Actinomycetes isolation (AI) medium is used for subculture, and the cross-streak method is used for first actinobacterial screening. Solvent extraction is used to remove secondary metabolites from active strains. Agar well diffusion is used to investigate the antifungal effectiveness of crude compounds. The fungus species *Aspergillus niger*, *athelia*, *fusarium oxysporum*, and *Trichoderma viride* were all inhibited by pure extraction. TLC purification of active crude compounds, followed by another agar well diffusion to provide pure compounds for antifungal research. GC-MS characterization of the active, purified component. By sequencing the 16s RNA gene, the active strain was identified. *Streptomyces griseoincarnatus* was identified as the isolate based on morphological traits and 16S rRNA analysis.

In conclusion, according to 16S rRNA analysis the isolated bacteria from soil is identified as *streptomyces griseoincarnatus*. The isolate were shown antifungal activity against *aspergillus niger*, *fusarium oxysporum*, *Trichoderma viride* and produces antimicrobial compounds. Therefore ,this study shows that the isolated bacteria from soil has antifungal activity producing compounds.

REFERENCES

- [1] Wu, Y., Yuan, J., E, Y., Raza, W., Shen, Q., & Huang, Q. (2015). Effects of volatile organic compounds from *Streptomyces albulus* NJZJSA2 on growth of two fungal pathogens. *Journal of Basic Microbiology*, 55(9), 1104–1117.
- [2] Awla, H. K., Kadir, J., Othman, R., Rashid, T. S., & Wong, M.-Y. (2016). Bioactive Compounds Produced by *Streptomyces* sp. Isolate UPMRS4 and Antifungal Activity against *Pyricularia oryzae*. *American Journal of Plant Sciences*, 07(07), 1077–1085.
- [3] Sarmiento-Vizcaíno, A., González, V., Braña, A. F., Palacios, J. J., Otero, L., Fernández, J., Molina, A., Kulik, A., Vázquez, F., Acuña, J. L., García, L. A., & Blanco, G. (2016). Pharmacological Potential of Phylogenetically Diverse Actinobacteria Isolated from Deep-Sea Coral Ecosystems of the Submarine Avilés Canyon in the Cantabrian Sea. *Microbial Ecology*, 73(2), 338–352.
- [4] Raghava Rao, K. V., Mani, P., Satyanarayana, B., & Raghava Rao, T. (2017). Purification and structural elucidation of three bioactive compounds isolated from *Streptomyces coelicoflavus* BC 01 and their biological activity. *3 Biotech*, 7(1).
- [5] Ahsan, T., Chen, J., Zhao, X., Irfan, M., & Wu, Y. (2017). Extraction and identification of bioactive compounds (eicosane and dibutyl phthalate) produced by *Streptomyces* strain KX852460 for the biological control of *Rhizoctonia solani* AG-3 strain KX852461 to control target spot disease in tobacco leaf. *AMB Express*, 7(1).
- [6] OroobaMeteab Faja et al /International Journal of ChemTech Research, 2017,10(3): 212-224
- [7] Boukaew, S., & Prasertsan, P. (2020). Efficacy of volatile compounds from *Streptomyces philanthi* RL-1-178 as a biofumigant for controlling growth and aflatoxin production of the two aflatoxin-producing fungi on stored soybean seeds. *Journal of Applied Microbiology*, 129(3), 652–664.
- [8] Mani, A., Ravi, L., & Krishnan, K. (2018). Antibacterial and antifungal potential of marine *Streptomyces* sp. VITAK1 derived novel compound Pyrrolidinyl-Hexadeca-Heptaenone by in Silico docking analysis. *Research Journal of Pharmacy and Technology*, 11(5), 1901.

- [9] Al-rubaye, T. S., Risan, M. H., & Al-rubaye, D. (2020). Gas chromatography-mass- spectroscopy analysis of bioactive compounds from *Streptomyces* spp. isolated from Tigris river sediments in Baghdad city. *Journal of Biotechnology Research Center*, 14(1), 63–71.
- [10] Lyu, A., Yang, L., Wu, M., Zhang, J., & Li, G. (2020). High Efficacy of the Volatile Organic Compounds of *Streptomyces yanglinensis* 3-10 in Suppression of *Aspergillus* Contamination on Peanut Kernels. *Frontiers in Microbiology*, 11.
- [11] Yang, M., Lu, L., Pang, J., Hu, Y., Guo, Q., Li, Z., Wu, S., Liu, H., & Wang, C. (2019). Biocontrol activity of volatile organic compounds from *Streptomyces alboflavus* TD-1 against *Aspergillus flavus* growth and aflatoxin production. *Journal of Microbiology*, 57(5), 396–404.
- [12] Patel, J. K., Madaan, S., & Archana, G. (2018). Antibiotic producing endophytic *Streptomyces* spp. colonize above-ground plant parts and promote shoot growth in multiple healthy and pathogen-challenged cereal crops. *Microbiological Research*, 215, 36–45.
- [13] Dutta, J., & Thakur, D. (2020). Evaluation of Antagonistic and Plant Growth Promoting Potential of *Streptomyces* sp. TT3 Isolated from Tea (*Camellia sinensis*) Rhizosphere Soil. *Current Microbiology*, 77(8), 1829–1838.
- [14] Rammali, S., Hilali, L., Dari, K., Bencharki, B., Rahim, A., Timinouni, M., Gaboune, F., El Aalaoui, M., & khattabi, A. (2022). Antimicrobial and antioxidant activities of *Streptomyces* species from soils of three different cold sites in the Fez-Meknes region Morocco. *Scientific Reports*, 12(1).
- [15] Vijay, K., Sree, K. K., Devi, T. S., Soundarapandian, S., Ramasamy, V., & Thangavel, K. (2020). Computational Biology Approaches Revealing Novel Target in Vascular Wilt Pathogen *Fusarium oxysporum* f. sp. *lycopersici* for the Ligands of Marine Actinobacterial Origin. *Journal of Pure and Applied Microbiology*, 14(1), 363–373.
- [16] Haider Khan, I., & Javaid, A. (2019). ANTIFUNGAL, ANTIBACTERIAL AND ANTIOXIDANT COMPONENTS OF ETHYL ACETATE EXTRACT OF QUINOA STEM. *Plant Protection*, 3(3), 125–130.
- [17] Haider Khan, I., & Javaid, A. (2019). ANTIFUNGAL, ANTIBACTERIAL AND ANTIOXIDANT COMPONENTS OF ETHYL ACETATE EXTRACT OF QUINOA STEM. *Plant Protection*, 3(3), 125–130.

- [18] Boukaew, S., Cheirsilp, B., Prasertsan, P., & Yossan, S. (2021). Antifungal effect of volatile organic compounds produced by *Streptomyces salmonis* PSRDC-09 against anthracnose pathogen *Colletotrichum gloeosporioides* PSU-03 in postharvest chili fruit. *Journal of Applied Microbiology*, 131(3), 1452–1463.
- [19] Ortega, H. E., Ferreira, L. L. G., Melo, W. G. P., Oliveira, A. L. L., Ramos Alvarenga, R. F., Lopes, N. P., Bugni, T. S., Andricopulo, A. D., & Pupo, M. T. (2019). Antifungal compounds from *Streptomyces* associated with attine ants also inhibit *Leishmania donovani*. *PLOS Neglected Tropical Diseases*, 13(8), e0007643.
- [20] Bobek, J., Filipová, E., Bergman, N., Čihák, M., Petříček, M., Lara, A. C., Kristufek, V., Megyes, M., Wurzer, T., Chroňáková, A., & Petříčková, K. (2022). Polyenic Antibiotics and Other Antifungal Compounds Produced by Hemolytic *Streptomyces* Species. *International Journal of Molecular Sciences*, 23(23), 15045.
- [21] Abeer, F.; Imad, H. H. and Mohanad, J. K. (2017) A Review: Uses of Gas Chromatography-Mass Spectrometry (GC- MS) Technique for Analysis of Bioactive Natural Compounds of Some Plants. *Int J Toxicol and Pharma Res.* 9(1): 81-85.
- [22] Abussaud, M. J. and Saadoun, I. M. (1991). *Streptomyces* flora of some Jordan valley soils, characteristics and seasonal distribution. *Dirasat.* 18:66-75.
- [23] Aghighi, S., Bonjar, G. H. S., Rawashdeh, R., Batayneh, S., and Saadoun, I.(2004). First report of antifungal spectra of activity of Iranian Actinomycetes strains against *Alternaria solani*, *Alternaria alternate*, *Fusarium solani*, *Phytophthora megasperma*, *Verticillium dahliae* and *Saccharomyces cerevisiae*. *Asian J. Plant Scien.* 3 (4): 463-471.
- [24] AL-Kateb, H. and Costello, B. L. (2013). Analysis of the volatiles in the headspace above the plasmodium and sporangia of the slime mould (*Physarum polycephalum*) by SPME-GCMS. arXiv preprint arXiv:1307.8017.
- [25] Anderson, A. S. and Wellington, E. (2001). The taxonomy of *Streptomyces* and related genera. *Int J Syst Evol Microbiol.* 51 (3): 797–814.
- [26] Andrew, M. (2007). Role of advances in chromatographic techniques in phytochemistry. *Phytochemistry.* 68: 2785-2797.

- [27] Al-Saadi, A.; Noora, M. H. and Eman, M. J. (2013). Isolation and Identification of *Streptomyces* from Different Sample of Soils. *JBMS*. (1): 31-36.
- [28] Alva-Murillo, N.; Ochoa-Zarzosa, A. and López-Meza, J. E. (2012). Short chain fatty acids (propionic and hexanoic) decrease *Staphylococcus aureus* internalization into bovine mammary epithelial cells and modulate antimicrobial peptide expression. *Vet. Microbiol.* 155: 324–331.
- [29] Arumugam, T.; Senthil, K. P.; Kameshwar, R. et al. (2017). Screening of novel actinobacteria and characterization of the potential isolates from mangrove sediment of south coastal India. *Microb Pathog.* (107):225-33.
- [30] Atta, M. A. and Ahmad, M. S. (2009). Antimycin–A antibiotic biosynthesis produced by *Streptomyces* sp. AZ-AR-262: Taxonomy, fermentation, purification and biological activities. *J Basic Sci.* 3: 126-135.
- [31] Aue, W. P. (1976). Two-dimensional spectroscopy. Application to nuclear magnetic resonance. *The J Chem Phys.* 64: 2229.
- [32] Awais, M.; Shah, A.; Hamed, A. and Hasan, F. (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pak. J. Bot.*, 39: 1303-1312.
- [33] Babalola, O. O.; Kirby, B. M.; Le Roes-Hill, M.; Cook, A. E.; Cary, S. C.; Burton, S. G. and Cowan, D. A. (2009). Phylogenetic analysis of actinobacterial populations associated with Antarctic dry valley mineral soils. *Environ Microbiol.* 11: 566-576.
- [34] Bailey, S.; Baron, E. and Finegold, S. (1990). *Diagnosis Microbiology* (8th Ed.). The C.V. Mosby. USA.
- [35] Barka, E. A.; Vatsa, P.; Sanchez, L.; Gaveau-Vaillant, N.; Jacquard, C.; MeierKolthoff, J. P.; Klenk, H. P.; Clément, C.; Ouhdouch, Y. and van Wezel, G.P. (2016). Taxonomy, Physiology, and Natural Products of Actinobacteria. *Microbiol Mol Biol Rev.* 80 (1): 1–43.
- [36] Baskaran, R.; Mohan, P.M.; Sivakumar, K. and Kumar, A. (2016). Antimicrobial activity and phylogenetic analysis of *Streptomyces parvulus* dosmb-d105 isolated from the mangrove sediments of Andaman islands. *Acta Microbiologica et Immunologica Hungarica.* 63: 27– 46.
- [37] Bentley, S.D.; Chater, K. F.; Cerdeño-Tárraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M.A.; Kieser, H.; Harper, D.

- (2002). Complete genome sequence of the model Actinomycete *Streptomyces coelicolor* A3 (2). *Nature*. 417 (6885):141-7.
- [38] Berdy, J.; Horvathi, I. and Szentirmai, A. (1964). Antibiotics produced by *Streptomyces* II. The tautomeric transformation of Xanthomycin. *Z Allg Mikrobio*. 61: 232-5.
- [39] Blackburn, J. (2003). Nuclear Magnetic Resonance Spectroscopy. Chapter 13. Richland College, Dallas, TX. Dallas 7 County Community College District.
- [40] Blattner, F. R.; Plunkett, G.; Bloch, C. A.; Perna, N. T.; Burland, V.; Riley, M.; Collado-Vides, J.; Glasner, J. D.; Rode, C. K.; Mayhew, G. F.; Gregor, J.; et al. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science*. 277(5331):1453-62.
- [41] Buchanan and Gibbons., *Bergey's manual of determinative bacteriology*, (1974).
- [42] Bull, A. T. (2011). Actinobacteria of the extremobiosphere. In Horikoshi, K.; Antranikian, G.; Bull, A.T.; Robb; F. T.; Stetter, K.O. (eds). *Extremophiles Handbook*. Springer. Japan, Tokyo, 2: 1203–1240.
- [43] Bundale, S.; Deovrat, B.; Nashikkar, N.; Kadam, T. and Upadhyay, A. (2015). Optimization of Culture Conditions for Production of Bioactive Metabolites by *Streptomyces* spp. Isolated from Soil. *Adv. Microbiol.*, 5: 441-451.
- [44] Chater, K. F. (1984). Morphological and physiological differentiation in *Streptomyces*, in *Microbial development*. eds Losick, R.; Shapiro, L. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y), 89–115.
- [45] Chater, K. F. (1993). Genetics of differentiation in *Streptomyces*. *Annu Rev Microbiol.*; 47:685-713.
- [46] Chater, K. F. (1989). Multilevel regulation of *Streptomyces* differentiation. *Trends Genet*. 5 (11):372-7.
- [47] Chatwal, G. R.; Anand, S. K. (1979). *Instrumental methods of chemical analysis book*. Published by Himalaya Publishing House, Mumbai.
- [48] Chen, S.; Geng, P.; Xiao, Y.; Hu, M. (2012). Bioremediation of β -cypermethrin and 3-phenoxybenzaldehyde contaminated soils using *Streptomyces aureus* HPS-01. *Appl Microbial Biotechnology*. 94: 505–515.
- [49] Cockerill, Franklin R.; et al. (2012). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Ninth Edition. CLSI*. p. 12

- [50] Cordovez, V.; Carrion, V. J.; Etalo, D. W.; *et al.* (2015). Diversity and functions of volatile organic compounds produced by *Streptomyces* from a diseasesuppressive soil. *Front Microbiol.* 6:1081.
- [51] Czaplick, S. (2013). Chromatography in Bioactivity Analysis of Compounds. Column Chromatography. Chapter: Chromatography in Bioactivity Analysis of Compounds. InTech. Dean F. Martin, Barbara B. Martin
- [52] Dehnad, A.; Parsa, L.; Bakhshi, R. and Mokhtarzadeh, A. (2010). Investigation antibacterial activity of Streptomycetes isolates from soil samples, West of Iran. *Afri. J. Microbiol. Res.* 4 (14):1542–1549.
- [53] Deshmukh, M. B. and Sridhar, K. R. (2002). Distribution and antimicrobial activity of actinomycetes of a fresh water coastal stream. *Asian J Microbiol Biotech Environ Sci.* 4:335–340.
- [54] Diab, A. and Al Zaidan, A. (1986). Actinomycetes in the desert of Kuwait. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg.* 131 (3): 545 - 54.
- [55] El-Ahmady, N.; El-Bindary, A. A.; Abdel-Mogib, M. and Noura, S. N. (2017). *In vitro* activity, extraction, separation and structure elucidation of antibiotic produced by *Streptomyces anulatus* NEAE-94 active against multidrugresistant *Staphylococcus aureus*. *Biotechnol. Biotechnol. Equip.* 31 (2): 1-13.
- [56] Gerber, F.; Krummen, M.; Potgeter, H.; Roth, A.; Siffrin, C. and Spoendlin, C.(2004). Practical aspects of fast reversed-phase high-performance liquid chromatography using 3µm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice. *J Chromatogr A.* 1036 (2): 127–133.
- [57] Goshi, K.; Uchida, T.; Lezhava, L.; Yamasaki, M.; Hiratsu, K.; Shinkawa, H. and Kinashi, H. (2002). Cloning and analysis of the telomere and terminal inverted repeat of the linear chromosome of *Streptomyces griseus*. *J Bact.* 184:3411-3415.
- [58] Hain, T.; Ward-Rainey, N.; Kroppenstedt, R. M.; Stackebrandt, E. and Rainey, F. A. (1997). Discrimination of *Streptomyces albidoflavus* strains based on the size and number of 16S-23S ribosomal DNA intergenic spacers. *Int J Syst Bacteriol.* 47(1): 202-6

- [59] Hakvag, S., E.; Fjarvic, K. D.; Josefsen, E.; Lan, T. E.; Ellignsen, and Zotchev, S.B. (2008). Characterization of *Streptomyces* spp. Isolated from the sea surface microlayer in the Trondheim Fjord, Norway. *Mar drugs*. (6): 620-635.
- [60] Hardisson, C.; Manzanal, M. B.; Salas, J. A. and Suárez, J. E. (1978). Fine structure, physiology and biochemistry of arthrospore germination in *Streptomyces antibioticus*. *J Gen Microbiol*. 105 (2):203-14.
- [61] Hasani, A.; Ashraf, K. and Khosrow, I. (2014). Streptomycetes: Characteristics and Their Antimicrobial Activities. *Int. j. of Adv Biol and Biomed Res*. 2: (1), 63-75.
- [62] Holt, J. G.; Krieg, N. R.; Sneath, P. H. A.; Stanley, J. T. and Williams, S. T.(1994): *Bergey's Manual of Determinative Bacteriology*, 9th ed. Williams and Wilkins, Co., Baltimore.
- [63] Holly, C. G. (2007). NMR Exchange Spectroscopy. Department of Chemistry, Texas A and M University, College Station, TX 77843. Modern NMR Spectroscopy in Education, Chapter 13, pp 176–189. *ACS Symposium Series*, Vol. 969. Publication Date (Print): August 16,2007.
- [64] Hopwood, D. A. (1999). Forty years of genetics with *Streptomyces*: from in vivo through in vitro to in silico. *Microbiology*. 145 (9): 2183-202.
- [65] Hozzein, W. N. and Goodfellow, M. (2011). *Actinopoly sporaegyptensis* sp. nov, a new halophilic actinomycete. *Afr J Microbi. Res*.5:100-105.
- [66] Huang, Y.; Zhiyong, Z.; Hao, C.; Jianghua, F.; Shuhui, C.; Zhong, C. (2015).A high-resolution 2D J-resolved NMR detection technique for metabolite analyses of biological samples. *Sci Rep*. 5: 8390.
- [67] Jeener, J. (2007). Reminiscences about the Early Days of 2D NMR, in *Encyclopedia of Magnetic Resonance*, eds R. K. Harris and R. E. Wasylishen, John Wiley and **Sons**: Chichester.