# EXTRACTION AND IDENTIFICATION OF SECONDARY METABOLITES PRODUCED BY SOIL ACTINOMYCETES AND THEIR BIOCHEMICAL CHARACTERIZATION

***Project work submitted to Central University of Punjab***
***For the degree of***
***M.Sc. Life Sciences with specialization in Microbial Sciences***

***Submitted by***


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## DECLARATION

I declare that the project report entitled ***"Extraction and identification of secondary metabolites produced by soil Actinomycetes and their biochemical characterization"*** has been prepared by me, under the guidance of Dr. Pramod Kumar Kushawaha, Assistant Professor, Department of Biochemistry and Microbial Sciences, Central University of Punjab. No part of this project has formed the basis for the award of any degree or fellowship previously.

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## CERTIFICATE

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## ABSTRACT

***Extraction and identification of secondary metabolites produced by soil Actinomycetes and their biochemical characterization.***

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The worldwide use of antibiotics has rapidly increased since the discovery of penicillin in 1928 by Alexander Fleming, for treating terrible bacterial diseases. But in due course of time, over-consumption of antibiotics has led to an enormous selective pressure on bacteria, which has forced them to adapt to the antibiotics and hence gain development of resistance. In the past years, the excessive use of antibiotics has led to significant evolution in microorganisms where naturally occurring genes resistant to the antibiotic have been spreading fast, thus, the need for new antibiotics has increased. In the stated work, samples were collected from the mountainous sites of Himachal Pradesh and were analyzed for the presence of secondary metabolites. The bacterial species were characterized by biochemical tests and metabolites produced by them were identified.


(Jahnavi Kumari Singh) (Dr. Pramod Kumar Kushawaha)

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Since, every end marks a new beginning; there is yet another milestone to reach to. Time may perish our association but our contributions to one another will remain intact in our memories till the end of our times.

(Jahnavi Kumari Singh)

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## LIST OF ABBREVIATIONS

| Sr.No. | Full form | Abbreviation |
|---|---|---|
| 1. | Guanine + Cytosine | G+C |
| 2. | Acquired Immunodeficiency Syndrome | AIDS |
| 3. | Centigrade | $^{o}C$ |
| 4. | Millimeter | Mm |
| 5. | Ribonucleic Acid | RNA |
| 6. | Deoxy Ribonucleic Acid | DNA |
| 7. | Gas-Chromatography Mass-Spectrophotometry | GC-MS |
| 8. | Example | e.g. |
| 9. | Starch Casein Agar | SCA |
| 10. | Nutrient Broth | NB |
| 11. | Negative | -ve |
| 12. | Positive | +ve |
| 13. | Indole; Methyl-Red; Voges-Proskauer; Citrate | IMViC |
| 14. | Hydrogen Peroxide | $H_2O_2$ |
| 15. | Oxygen | $O_2$ |
| 16. | Rotations Per Minute | Rpm |
| 17. | Round Bottom Flask | RBF |
| 18. | Micro liter | µl |
| 19. | Milliliter | Ml |
| 20. | Minute | Min |

# CHAPTER I

# INTRODUCTION

## 1. INTRODUCTION

Actinomycetes are well known for the production of life saving drugs *i.e.* antibiotics; which are the secondary metabolites produced during the later growth phase. They are less dominant than bacteria and more prominent than fungi, thus, originally they were considered to be the intermediates between fungi and bacteria. Actinomycetes approximately make up to 10-50% of the total microbial populace as analyzed by the plating methods in virgin and the cultivated lands as well.

Actinomycetes are filamentous bacteria which show positive result for gram staining, have high G+C content (69-78%) in DNA with highly specific growth cycle (Cummins and Harris, 1958).Residing in a broad range of environments, unlike other bacteria, Actinomycetes are exclusive in their morphology with specific branching substrate and also possess aerial mycelium having conidia formation, which are similar to those of fungi (Robinson *et al.*, 2016). For this reason, they are also known as ray fungi. They produce mycelium which might be aerial or may unfold on the substrate on which the microorganism is growing. The mycelia in some species may break to form rod or coccoid shapes, or others may form spore, along with the sporangia or spore cases that may be found on aerial hyphae.The substrate spores and mycelium can be pigmented, which comes out to be one of the amazing and attractive aspects of these microbes.

On agar plates, they form lichenoid, leathery or powdery colonies. They possess cell wall characteristic of bacteria and filamentous nature of fungi. Others which are known as the Non-Actinomycetes are referred to as the Rare Actinomycetes. Majority of these organisms are free living (Ceylan, Okmen and Ugur, 2008). Although terrestrial samples have been screened extensively, only a small fraction of the Actinomycetes taxons have been located (Baltz *et al.*, 2005). Taxonomic characterization of Actinomycetes producing novel metabolites is an essential step in any screening program.

Many a times, however, taxonomic study of the microorganism is initiated, only when the metabolite it produces is of much interest, *i.e.*, when a description of the producing microbe is needed for the patent application.

Identification of isolates to the species level will often give the researcher a clue as to whether or not a metabolite is novel (Labeda, 1987).Among the potential sources of natural products, bacteria have been proven to be an ultimate source with a surprisingly small group of taxa accounting for the large variety of compounds discovered.Actinomycetes are one of the major constituents of soil microorganisms, together with some bacteria as well as some fungi. For decades, microbial products have been one of the major precursors for discovery of novel drugs.

70% of the 22,000 known secondary metabolites till now have been reported to be contributed by Actinomycetes only, and majority of them have been found to be produced by the genus *Streptomyces* alone ( Subramani and Aalbersberg, 2012). They have a crucial role in organic matter turnover and carbon cycle as they play a major role in decomposition of organic materials like cellulose and chitin (Scannell *et al.*, 1971). Also, they have been recognized as prolific producers of secondary metabolites with diverse biological activities. Some of them are industrially exploited as antibacterial or antifungal agents (antibiotics), antitumor compounds, insecticides, or herbicides.However, with the increasing microbial resistance in opposition to routinely used antibiotics and the presence of still uncured diseases like cancer or AIDS, there is a very spontaneous need for discovery of newer bioactive compounds.

So, there is a need to screen Actinomycetes in the hope of getting novel antibiotics, which have not been discovered yet and are active against drug-resistant pathogens.

Important reservoirs of bioactive compounds are the so-called cryptic or silenced secondary metabolite biosynthetic clusters which have been discovered during the analysis of bacterial chromosome sequences.

Cryptic secondary metabolite clusters refer to the groups of genes that might be responsible for the biosynthesis of a secondary metabolite but are probably not expressed in the conditions routinely used in laboratory; therefore the products synthesized remain unknown. The sequencing of bacterial genomes revealed the presence of many such clusters and it was already shown that in some cases the identification of their products led to the discovery of new compounds (Gross, 2007).

Actinomycetes have been acknowledged to produce diverse metabolites from their complex biochemical processes. Over 23,000 microbial secondary metabolites are known including the beta lactam antibiotics like penicillin, cephalosporin and some form shikimic acid like chloramphenicol (Barrios-Gonzalez *et al*., 2003).On looking over the data, approximately 42% of these metabolites are known to be contributed by Actinomycetes, 16% by fungi and other bacteria.

Most commercially important biomolecules are obtained from diverse genera like *Streptomyces, Saccharopolyspora, Amycolatopsis, Micromonospora* and *Actinoplanes*. These metabolites which are released into surroundings have extensive biological properties such as antiviral, immunosuppressive, antitumor, insecticidal, anti-inflammatory, antioxidant, enzyme inhibitory and diabetogenic. Those that produce antibacterial and antifungal metabolites may also pose several effects on the immediate surrounding; of inhibiting other microorganisms from growing being the major one. Such groups of Actinomycetes with antagonistic properties are referred to as the biological antagonistic types. They are of special interest since they are the ones whose metabolites are exploited for the manufacture of antibiotics like, penicillin, erythromycin, streptomycin, tetracycline, vancomycin and amphotericin etc.

Moreover, bacterial products are significant not only because they pose useful pharmacokinetic properties required for clinical improvement, but also due to assured therapeutic activities (Kekuda *et al*., 2010).

### 1.1. Knowledge gap:

Discovery of penicillin in 1928 by Alexander Fleming rapidly increased the use of antibiotics for treating terrible bacterial diseases. But, in due course of time, over-consumption of antibiotics has led to an enormous selective pressure on bacteria, which have been forced to adapt to the antibiotics and hence gained resistance. This acquired resistance elicited many researchers to explore novel antibiotics that would be effective against microbes.

Although most of the antibiotics have been extracted from Actinomycetes, still many Actinomycetes remain unexplored. Many antibiotics are being discovered regularly but resistance against them is also developing simultaneously, so need for novel and effective one is continuously being felt.

### 1.2. Hypothesis:

Isolation of antibiotic producing Actinomycetes and identification of secondary metabolites produced by them during fermentation period. Knowledge about metabolites may be exploited for further developmental studies.

### 1.3. Objective:

- Isolation of antibiotic producing Actinomycetes from mountainous soil of Dharamshala (H.P.).
- Biochemical characterization of the isolated Actinomycetes.
- Extraction and identification of secondary metabolites produced by the isolated Actinomycetes.

# CHAPTER II

# REVIEW OF LITERATURE

## 2. REVIEW OF LITERATURE

Actinomycetes are differentiated by a very complex life cycle belonging to the phylum Actinobacteria, which has been recognized as one of the largest taxonomic segments among the 18 broad lineages discovered under the Domain Bacteria (Ventura *et al.*, 2007).

Like many other soil microbes, they behave as mesophilic bacteria in laboratory with an optimum growth rate at 25°C to30°C (Bizani and Brandelli, 2002). They have been extensively found in both terrestrial and aquatic environments, and have also been known to play a key role in recycling refractory biomaterials by decomposing complex mixtures of different polymers in dead plants, animals and fungal materials. They also serve a very significant role in biodegradation of soil and humus formation as they are responsible for recycling of the nutrients associated with recalcitrant polymers like keratin, chitin and lignocelluloses (Good fellow and Williams 1983; McCarthy and Williams 1992; Stach and Bull, 2005). They also produce numerous volatile substances like geosmin known for the characteristic “wet earth odor” (Wilkins *et al.*, 1996) and has a range of physiological and metabolic properties.Variety of the order actinomycetes spreads upto about 40 families, 170 genera and 2000 species which have been validly explained and published till now.

The bioactive secondary metabolites produced by all the microbes is approximately 23,000 in number, of which 10,000 are contributed by Actinomycetes, thus representing 45% of all bioactive microbial metabolites discovered(Berdy, 2005).

Among Actinomycetes, approximately 7,600 compounds are contributed by *Streptomyces* species only (Berdy, 2005). Thus, *Streptomyces* has emerged out as a major antibiotic-producing organism exploited by the pharmaceutical industry (Berdy, 2005). This group has came out as a good contributor of medically important antitumor drugs like peptides (bleomycin and actinomycin D), aureolic acids (mithramycin), anthracyclines (aclarubicin, daunomycin and doxorubicin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins, etc (Newman and Cragg, 2007; Olano *et al.*, 2011).

Marine microorganisms embody a complex and wide assemblage of microscopic existence forms, of which approximately only 1% has been introduced into research studies (Charan *et al.*, 2004). They have also been found in symbiotic relation with significant marine invertebrates, especially sponges (Piel, 2004; Kim and Fuerst, 2006).

**Structure of Actinomycetes:**

The Actinomycetes are a large group of aerobic bacteria, since they closely resemble fungi in aspect of their morphology; most probably this resemblance develops from adaptation to the same habitat. Fine structural studies of Actinomycetes spores during germination have been confined to the genera *Streptomyces* (Kalakoutswl and Agre, 1973). The latter genus produces endospores which behave in a comparable manner to those of *Bacillus*, a new wall layer being synthesized inside the cortex of the spore and developing to form the germ-tube wall.

In the *Streptomyces* species studied, the spores had a two-layered wall and the inner one extended to form the germ-tube wall. It is yet not clear whether the layer is formed by reorganization of wall materials existing in the dormant spore or develops during germination. Ultra structural modifications during the germination of fungal spores have been studied more extensively.

Some conflicting results have been obtained and closely related species have been reported to fall into different groups. This may be partially because of the use of different fixatives like potassium permanganate giving inferior results to those obtained with osmium tetroxide or aldehydes. Marked changes in spore wall layers can also be brought by the hydration during specimen preparation. On growing over an agar-surface, the branches developing into a network of hyphae grow both on the surface and under the surface of the agar.

The under-surface hyphae are called substrate hyphae, whereas the on-surface hyphae are called aerial hyphae. The aerial hyphae extend above the substratum and reproduces asexually. Septum divides the hyphae into elongated cells (approximately 20 mm and longer) containing bacterial chromosomes *i.e.* nucleoids.

Moreover, Actinomycetes are non-motile, but when motality is present, it is limited to flagellated spores.

**Cell Wall Composition:**

The composition of cell wall varies significantly among different groups and is of considerable taxonomic importance.

Different cell wall types have been distinguished in these bacteria on the basis of the two significant features of peptidoglycan composition and its structure.

These features are:

- Presence of Diaminopimelic acid and sugar molecules;
- Presence of glycine in interpeptide bridges (Characteristic sugar patterns are found only in cell wall types II-IV of Actinomycetes having meso-diaminopimelic acid).

**Molecular Approach:**

One of the most influential methods to taxonomy is through the study of nucleic acids. As a result of this measure, either the cistron products directly or the genes themselves on comparison yield good data.

Molecular science, which has every identification and classification, has its origin within the early supermolecule crossbreeding studies. Although a new stand in the introduction of supermolecule sequencing techniques has been achieved.

Importance of phyletic studies along with 16S rDNA sequences is taking over the science of bacterium.

Sequences of 16S rDNA have contributed by providing actinomycetologists with a phyletic tree which helps in providing the premise for identification and conjointly offers investigation of evolution of Actinomycetes. 16S rDNA analysis starts by analyzing DNA (Hopwood *et al.*, 1985) and amplifying the gene coding for 16S rRNA exploitation by the enzyme chain reaction. The isolated DNA fragments are sequenced directly.

The sequencing reactions are done in DNA Sequencer so as to work out the order during which the bases are organized at intervals along the length of sample. A computer is simultaneously used for finding out the sequence for identification and phyletic analysis. Though, analysis of 16S rDNA generally allows identification of organism upto the genus level only.

**Role of *Streptomyces*:**

*Streptomyces* is the largest genus of the Actinobacteria; family Streptomycetaceae. There are numerous antibiotics derived from *Streptomyces* which are used as antibacterial agents. *Streptomyces* derived antifungals are mostly macrolide polyenes (large ring structure having lots of conjugated Carbon-Carbon double bonds) and include illustrious members as:

- Nystatin (first Actinobacteria-sourced antifungal for humans, by *S. noursei*);
- Amphotericin B (by *S. nodosus*, originally isolated from a sample of Venezuelan soil) and;
- Natamycin (by *S. natalensis*)

Other antibacterial antibiotics of include:

- Erythromycin (a macrolide that often substitutes for penicillin when people be allergic to it, made by *S. erythraea*);
- Tetracycline ( a longstanding acne drug that makes you light-sensitive, by *S. rimosus*);
- Chloramphenicol (cheap, effective, but can cause aplastic anemia, by *S. venezuelae*);
- Vancomycin (a relatively ginormous glycopeptide, by *S. orientalis*) and;
- Thienamycin (by *S. cattleya*, modified to get imipenem; first carbapenem beta-lactam antibiotic).

Many metabolites produced by a way of Actinomycetes have proven to be toxic to be used as antibiotics for humans, apart from their toxicity towards cells (specifically dividing cells), they have also been reinvented as chemotherapeutic drugs; for e.g., plicamycin (made by *S.plicatus*), actinomycin-D (the original), mitomycin (aziridine made by *S. lavendulae*), bleomycin (glycopeptide made by *S. verticullus*).

Initially during 1950's; isolation of antibiotic actinomycin from *Streptomyces antibioticus* of soil, stimulated extensive screening of terrestrial Actinomycetes, where they live primarily as saprophytes, water and colonizing plants show marked chemical and morphological diversity, but from a distinct evolutionary line.

From about last 70 years, tremendous screening of Actinomycetes has led to the re-discovery of acknowledged bioactive compounds from the terrestrial, marine, and mangrove environments as well.Therefore, it has have become a need that Actinomycetes from unexplored environments ought to now be used as a source of desired novel metabolites.In passing years, Actinomycetes discovered from different types of environments (sediments, sponges, tunicates, marines, neuston, mountains and many others) have gained immense attention.

Any significant exploitation of the chemical diversity of these microbial resources relies on right understanding of their organic variety and related key factors that maximize the possibility of valid identification of novel molecules.

**Ecological Importance:**

Actinomycetes are responsible for digestion of most of the resistant carbohydrates like cellulose and chitin. Being significantly well adapted to survival in harsh environments, some are able to grow at elevated temperatures (>50°C) and are essential to the composting method.They are also responsible for the pleasant odor of freshly formed soil.

**Infections:**

Bacteria of the Actinomycetes genus are normal commensal members of human oral cavities. They may cause serious infections when they attack on tissue due to abberations in the oral mucosa. The disease has been found to be less universal, but is still present in areas of United States. Additionally, *Nocardia* species may also be involved.

**Human Health Importance:**

Antibiotics produced by Actinomycetes are best recognized and most valuable. Some of them are chloramphenicol, gentamycin, erythromycin, vancomycin, tetracycline, amphotericin, nystatin, novobiocin, neomycin, etc.

Among antibiotics some target bacterial ribosomes and are utilized in treating respiratory infections, for example in treating the Legionnaires disease, tetracycline and erythromycin are used. Vancomycin antibiotic attacks on deadly organisms such as Methicillin Resistant *Staphylococcus aureus* (MRSA) and bacterial cell walls. For leprosy and tuberculosis treatment; Rifamycin is used which targets bacterial RNA polymerase. Amphotericin is one of the minority antibiotics that attack fugal membranes. Antibiotics obtained from these microorganisms generally do not affect human cells and for that reason have lesser side effects. On the other hand, metabolites like Adriamycin prevent DNA replication, because of this it is used in cancer treatment, although rapamycin is used against immune system to facilitate organ transplants.

**GC-MS (Gas chromatography–mass spectrometry):**

It is a method that analytically functions as a combination of both gas-chromatography as well as mass spectrometry. It helps in identification of different compounds within an unknown sample. Thus, it is used to detect tiny amounts of matter including that of samples obtained from planet Mars during probe missions in early 1970s.

**Applications:**

- Drug detection;
- Fire investigation;
- Explosive investigation;
- Environmental analysis;
- Identification of unknown samples;
- Identification of trace elements in materials;
- For airport security to detect substances in luggage or on human beings.

**Working:**

The GC-MS mainly has two principle building blocks: the gas chromatograph and the mass spectrometer.The gas chromatograph is composed of capillary which depends on dimensions of the column (diameter, length and film thickness) and the phase properties as well (e.g. 5% phenyl polysiloxane). As the sample passes through the column length, separation of the molecules depends on two factors; the distinction in the chemical properties between different molecules in a mixture and their relative affinity towards the stationary phase of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time). Further along the process, the MS (mass spectrometer) downstream captures, ionizes, accelerates, deflects, and detects the ionized molecules separately. This is done by segmenting every molecule into respective ionized fragments and then recognizing them on the basis of their mass/charge ratio.

**Modes of operation for GC-MS:**

1. Spectral mode – observe the mass spectrum every second or so during chromatogram – provides most information for research or method studies,
2. Total ion current – total sum of signal for all ions as single large signal – most sensitivity,
3. Selective ion monitoring – look at certain mass/charge ratios for compounds of interest – routine analysis.

# CHAPTER III

# MATERIAL AND METHODS

## 3. MATERIAL AND METHODS

### 3.1. SAMPLE COLLECTION:

An appropriate amount of sample was collected in polythene bags and were immediately stored at 4°C.

Site details:

1. Dharamshala (Glu Devi Temple and Triund), Himachal Pradesh;
2. Latitude: 32.2498 North;
3. Longitude: 76.3303 East;
4. Height: 2788.920 meters to 2869.387 meters above the sea level.

### 3.2. ISOLATION OF ACTINOMYCETES:

1. For isolation, the initial stage was heating the soil sample, for 30 to 60 minutes at 60°C.
2. After heating, the soil was diluted in distilled water and made upto $10^{-9}$ dilutions.
3. Then, spread plate technique was used for making plates of different dilutions.
4. Specific medium called Starch Casein Agar (SCA) was used for selective isolation of Actinomycetes.
5. 10µl from dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-5}$ was spread over SCA plates.
6. The plates were incubated for 5 days at 28°C to 30°C.
7. Colonies were observed later.

### 3.3. PREPARATION OF MASTER PLATES:

1. Random single colonies from each of the spread dilutions were streaked over SCA plates to get master plates of isolates from each dilution (4 colonies from $10^{-1}$, 3 colonies from $10^{-2}$, and 2 colonies each from $10^{-3}$ and $10^{-5}$ dilutions).
2. Plates were incubated at 28°C for 5 days.
3. Single colonies were observed later.

### 3.4. PRELIMINARY SCREENING:

1. Considering four isolates of only $10^{-1}$ dilution, loop full culture of each of the 4 isolates were inoculated in test tubes with 5ml NB along with a control tube.
2. Test tubes were incubated overnight at 37°C.
3. 5µl from each tube was spread over nutrient agar plates and incubated at 28°C to 30°C for 3 days.
4. After 3 day incubation, when good single colonies were observed, 100µl of 4 test organisms were spread over each plate.
5. Additional incubation of 24 to 30 hours was provided.
6. Zone of inhibition was observed and recorded.

### 3.5. GRAM STAINING:

The gram stain is a differential stain developed by Dr. Hans Christian Gram, a Danish physician in 1884. It is very useful stain for identifying and classifying bacteria into two broad groups; the gram-positive and gram-negative. The gram-positive bacteria have thick peptidoglycan cell wall (50-90% of envelope) and can retain the crystal violet-iodine complex formed during staining, and since gram-negative cells have thin layer of peptidoglycan (10% of envelope), so do not retain the purple stain and are counter-stained pink by safranin.

There are four main steps of gram staining:

- Primary staining (crystal violet) of the heat-fixed smear of bacterial culture (heat fixation kills some bacteria but is mostly used to affix the bacteria
- Addition of iodide, which binds to crystal violet and traps it in the cell;
- Rapid decolorization with ethanol or acetone;
- Counterstaining with safranin (Carbol fuchsin is sometimes substituted for safranin as it more intensely stains anaerobic bacteria).

Procedure-

1. A heat fixed smear of overnight culture was prepared over a clean dry slide.
2. The slide was flooded with crystal violet staining reagent and left it for 1 minute.
3. Slide was washed in a gentle and indirect stream of tap water for 2 to 3 seconds.
4. The mordant; Gram's iodine was added to the slide and left it for 1 minute.
5. Slide was washed in a gentle and indirect stream of tap water for 2 to 3 seconds.
6. The decolorizing agent (95% ethyl alcohol) was added and left it for 15 to 30 seconds.
7. Slide was flooded with counter-stain *i.e.* safranin and left it for 30 to 60 seconds.
8. Slide was washed indirectly in stream of tap water until color disappears.
9. Slide was blot dried gently with absorbent paper.
10. Observed the slide under a microscope and recorded the staining results.

### 3.6. IMViC TESTS:

#### 3.6.1. INDOLE TEST:

Some bacteria can produce indole from amino acid tryptophan using the enzyme tryptophanase. Production of indole is detected by using Kovac's reagent. Indole reacts with the aldehyde in the reagent to give a red color.

Procedure-

1. Bacterium was inoculated in the test tubes containing 5ml of tryptone broth.
2. The tubes were incubated at 37°C for 24 to 28 hours.
3. After incubation, few drops of Kovac's reagent were added to each tube.
4. Observed for the presence or absence of the reddish brown ring on the top.

### 3.6.2. MR (METHYL-RED) TEST:

The MR test is used to detect the production of sufficient acid during fermentation of glucose. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.All enteric bacteria initially produce the pyruvic acid from glucose metabolism. Some bacteria subsequently use the mixed acid pathway to metabolize pyruvic acid to other acids like lactic acid, acetic acid, and formic acid.

The bacteria are called methyl-red-positive, if they give a positive result for MR test. Other bacteria use the glycol pathway for pyruvic acid metabolism to natural end-products and are called methyl-red-negative.

Procedure-

1. Bacteria to be tested were inoculated in test tubes containing MR-VP broth.
2. Test tubes were incubated at 37°C for 24 hours.
3. After incubation, five drops of Methyl-Red reagent was added into test tubes.
4. Color change was observed.

### 3.6.3. VP (VOGES-PROSKAUER) TEST:

Voges-Proskauer test is used to demonstrate the ability of an organism to convert pyruvate to acetoin. Acetyl-methyl carbinol is an intermediate in the production of butylene glycol.

In this test, 2 reagents, Barrit's A ( 5% α-naphthol) and Barrit's B ( 40% Potassium Hydroxide; KOH) reagent are added to the incubated broth and exposed to atmospheric oxygen.If acetoin is present, it is oxidized by alpha-naphthol to produce the red color. Alpha-naphthol acts as a catalyst and also as a color intensifier.

Procedure-

1. Bacteria to be tested were inoculated in test tubes containing MR-VP broth.
2. Test tubes were incubated at 37°C for 48 hours.
3. Few drops of Barrit's A and Barrit's B reagent were added.
4. Color change was observed.

### 3.6.4. CITRATE TEST:

The citrate utilization test helps to test the ability of an organism to utilize citrate as a source of energy. The medium contains inorganic ammonium salts ($NH_4H_2PO_4$) as the only nitrogen source and citrate as the only carbon source**.**Bacteria that can grow on this medium produce an enzyme *i.e.* citrate-permease, which converts citrate to pyruvate**.**

Bacterial growth indicates citrate utilization, which serves as an intermediate metabolite for Krebs cycle**.**

Procedure-

1. Slants of Simon Citrate Agar were prepared.
2. Slants were inoculated gently with a well isolated colony in a zig-zag manner.
3. Slants were incubated aerobically at $37^0$C for up to 5 to 7 days.
4. Color change was observed.

### 3.7. OXIDASE TEST:

The oxidase test is used for the identification of the bacteria that can produce Cytochrome-C oxidase, an enzyme of the bacterial Electron Transport Chain (ETC). When this enzyme is present, it oxidizes the reagent to dark blue colored product. But, the reagent remains reduced and colorless, when the enzyme is absent.

Procedure-

1. The disc was taken and placed over the tissue paper.
2. Few drops of water were added on the disc.
3. A colony was picked up by a loop and made asa smear over the disc.
4. Color change was observed within 10 to 30 seconds.

### 3.8. CATALASE TEST:

It is a process of characterization of bacteria through which they are classified as aerobic and aerobic.Catalase is an enzyme which cleave $H_2O_2$ on bacterial culture, if there is a production of $O_2$ during the test then bacteria is aerobic otherwise the bacteria is anaerobic.

Procedure-

1. A bacterial colony was transferred over a clean dry glass slide using a loop.
2. Few drops of 3% $H_2O_2$ was added onto the slide and mixed gently.
3. Formation of bubbles was observed.

### 3.9. SECONDARY METABOLITE EXTRACTION:

1. One of the colonies (M2) producing zone of inhibition was sub-cultured in 5ml of NB at $35^oC$ to $37^oC$ for about 24 hours.
2. After incubation, 1ml of culture from the tube was inoculated into two flasks with 500ml NB in each flask (to prepare 1000ml culture in total).

**Solvent extraction method (Liquid-liquid extraction):**

It is a method to separate metal compounds, based on their relative solubilities in two different immiscible liquids, usually an organic solvent (non-polar) and water (polar). There is switching of different species from one liquid into another liquid phase, generally from aqueous to organic.The solvent which is enriched in solute(s) is referred to as extract whereas the feed solution that is depleted in solute(s) is called as the raffinate.

In sample preparation for chromatography, this technique is used for separation purposes by carrying out selective extraction of the analytes from the sample and also by extracting a small volume of solvent specific compounds that are expected to be present in the organic phase.

Procedure-

1. Both the flasks with culture inoculation were made to undergo shake flask fermentation, at 28°C for 5 to 7 days at 200rpm, till a good growth was observed.
2. After fermentation, the 1000ml culture was filtered by sterile whatman filter paper (grade1) aseptically.
3. The filtrate was centrifuged at 4500 rpm for 10 minutes and the supernatant was collected in a flask.
4. The solvent *i.e.* ethyl acetate, 1:1 (volume/volume), was added to the supernatant and shaken vigorously for about 1 hour until organic and aqueous phase got separated clearly.
5. Using separating funnel, the organic phase was separated and this process was repeated twice.
6. Finally, the organic phase was collected in Round bottom flask (RBF) of appropriate volume and was dried using rotary vacuum evaporator under reduced pressure at 40 to 45˚C, until a solid mass was obtained.

**3.10. SAMPLING FOR GC-MS:**

1. The solid substance obtained over the inner surface of the RBF was dissolved in an appropriate solvent *i.e.* methanol (approximately 3ml).
2. The obtained dissolved crude extract were filtered through a 0.22 micron syringe filter and collected in GC-MS vial.
3. GC-MS vial was given for analysis to Central Instrumentation Laboratory (CIL), at Central University of Punjab, Bathinda.
4. 10µl-20µl sample was suspended in GC-MS which was fortified with DB-5MS column. The parameters of GC-MS instrument were:
   - The initial temperature of the column was kept at 100˚C for 1 min.
   - Then sharply increased to 270˚C and finally held at 270˚C for 10min.
   - The flow rate of carrier gas *i.e.* Helium, was 0.1ml $min^{-1}$.

# CHAPTER IV
# RESULTS

# 4. RESULTS

**4.1.Screening of Actinomycetes:** After spreading the $10^{-1}$, $10^{-2}$, $10^{-3}$and $10^{-5}$ dilutions over the plates having selective media *i.e.* SCA; according to concentration of dilutions, growth was observed in scattered and dense patterns.

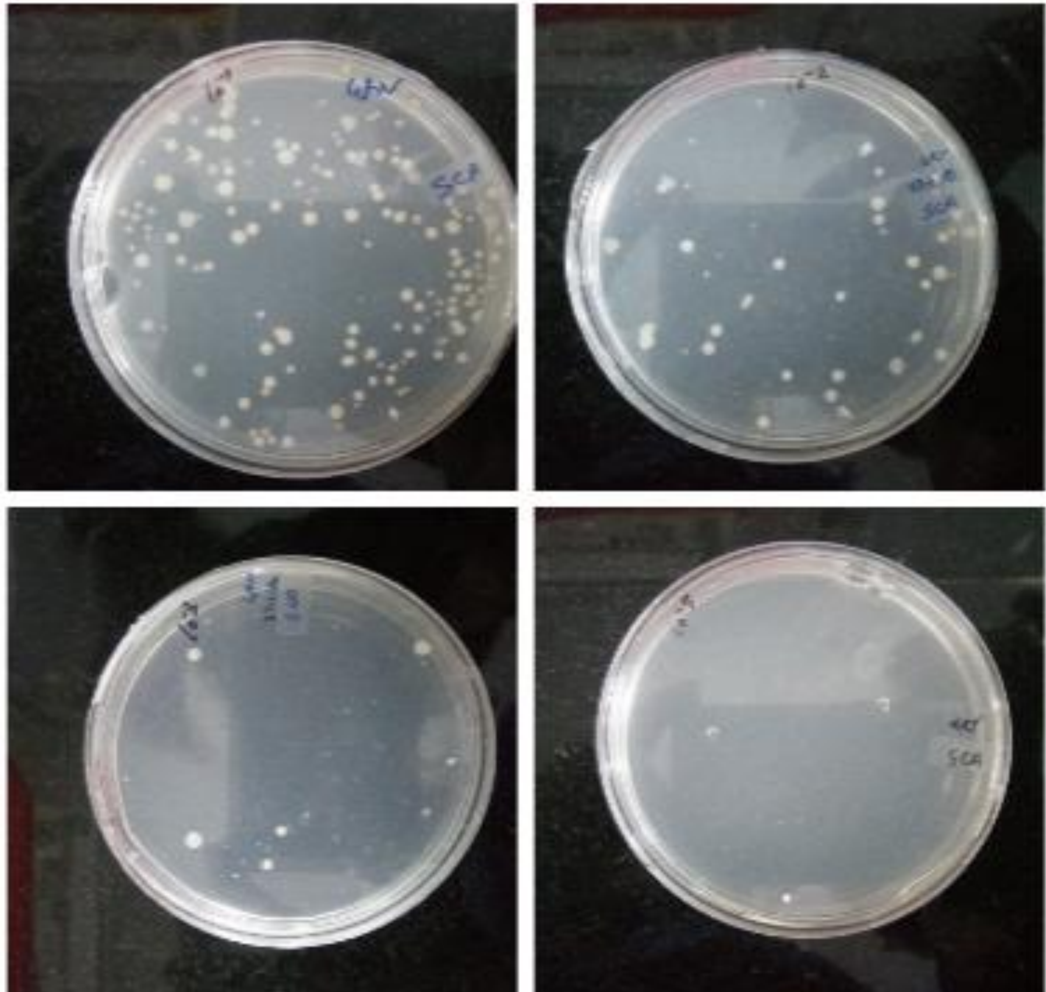


**Fig 4.1:**Isolated colonies obtained after spreading dilutions.

**4.2.Isolation of pure isolates:** Isolates were obtained after the streak plate techniqueof the isolated Actinomycetes of $10^{-1}$ dilution over SCA plates for selective growth. These isolates of $10^{-1}$ dilution were considered for further characterization of the unknown strains. Master plates were pepared and maintained for future reference in case of any mistake.

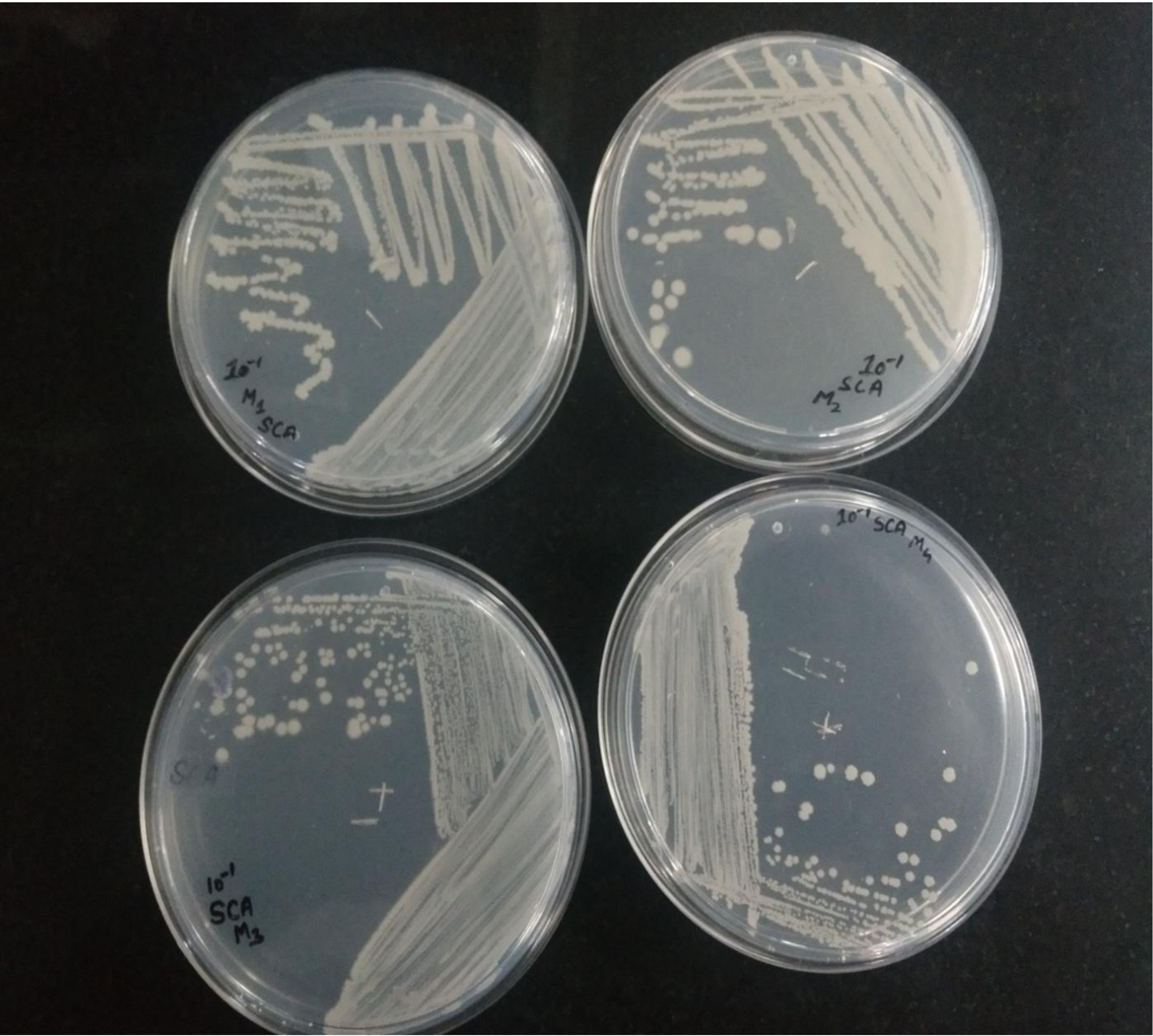


**Fig 4.2:** Four isolates of $10^{-1}$ dilution.

**4.3.Primary screening:** Zone of inhibition was obtained after spreading known pathogenic strains over well grown streaked plate of each isolate.Among the four considered isolates, 2 of them *i.e.* M1 and M3 showed activity against *Pseudomonas putida,* whereas rest two, M2 and M4 showed activity against *Salmonella enterica*.The diameter of clear zone indicated the amount of inhibition towards the known pathogenic strains.None of them showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

**1. TABLE OF SUSCEPTIBILITY TEST:**

| **Isolates** | **Test organism** | **Zone of inhibition** | | | | |
|---|---|---|---|---|---|---|
| | | Colony **1** | Colony **2** | Colony **3** | Colony**4** | Colony **5** |
| Isolate **M1** | *Pseudomonas Putida* | 3mm | 3mm | 3mm | 3mm | 3mm |
| Isolate **M2** | *Salmonella Enterica* | 2.5mm | 3mm | 2mm | 3mm | 3mm |
| Isolate **M3** | *Pseudomonas Putida* | 2mm | 2.5mm | 2mm | 2mm | 2mm |
| Isolate **M4** | *Salmonella Enterica* | 1mm | 1.5mm | 1.5mm | 1mm | 1.5mm |

**4.4. Morphological characterization:** Gram staining of the samples showed +ve result as blue color was seen after the staining *i.e.* the color of crystal violet was retained by the cells. Therefore, even after treatment with decolorizer, and subsequent washing, color remained intact and cellsdid not efficiently take up the counter stain *i.e.* safranin, which is pink in color.

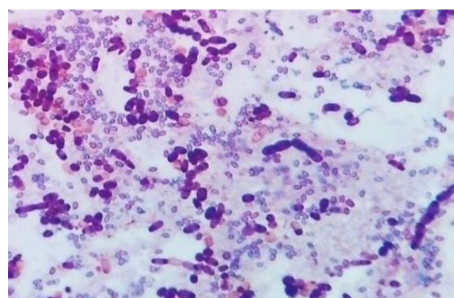

**Fig 4.4:** Gram staining (gram positive).

## 4.5.Biochemical characterization: (IMViC test, Oxidase test and Catalase test)

- Formation of reddish brown ring on top indicates +ve result for indole test. M1, M3 and M4 showed +ve result whereas M2 showed -ve result.

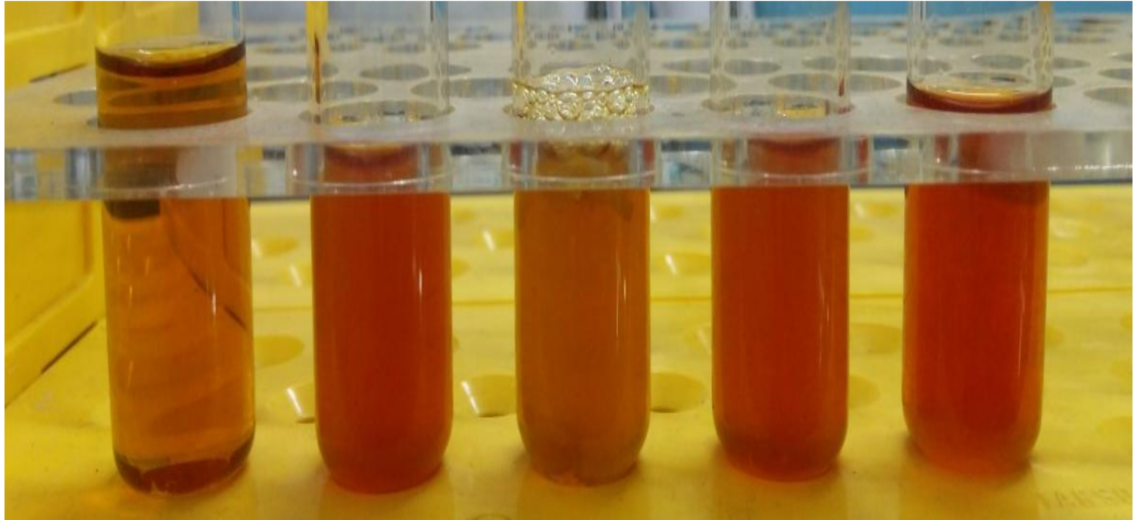

**Fig 4.5(a):** Indole test results.

- Formation of yellowish ring on topof the samples indicated -ve MR results.

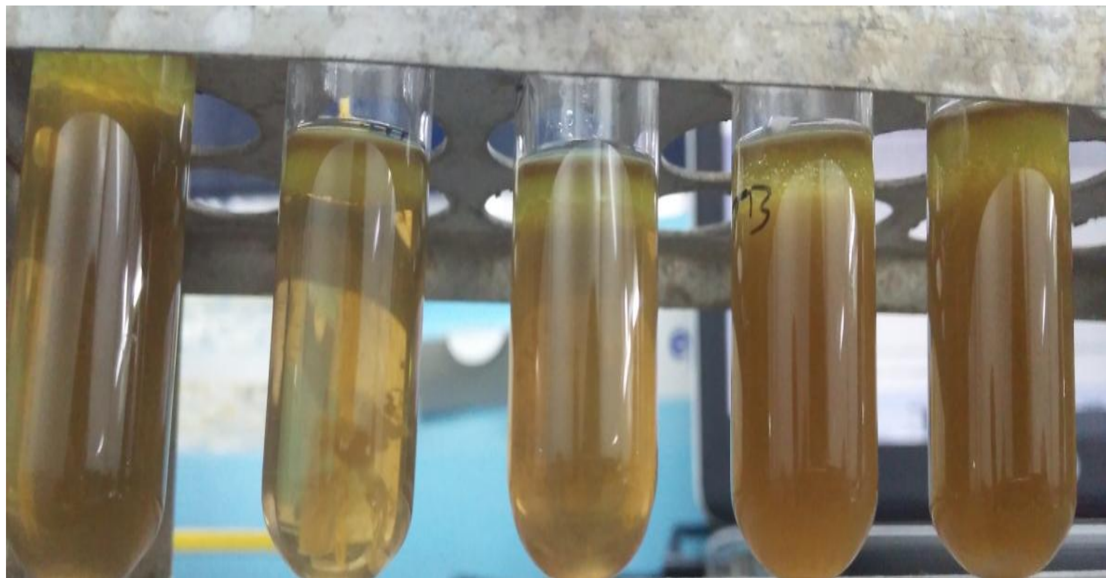

**Fig 4.5(b):** Methyl-Red test results.

- No formation of red color indicated -ve VP results for all the samples.

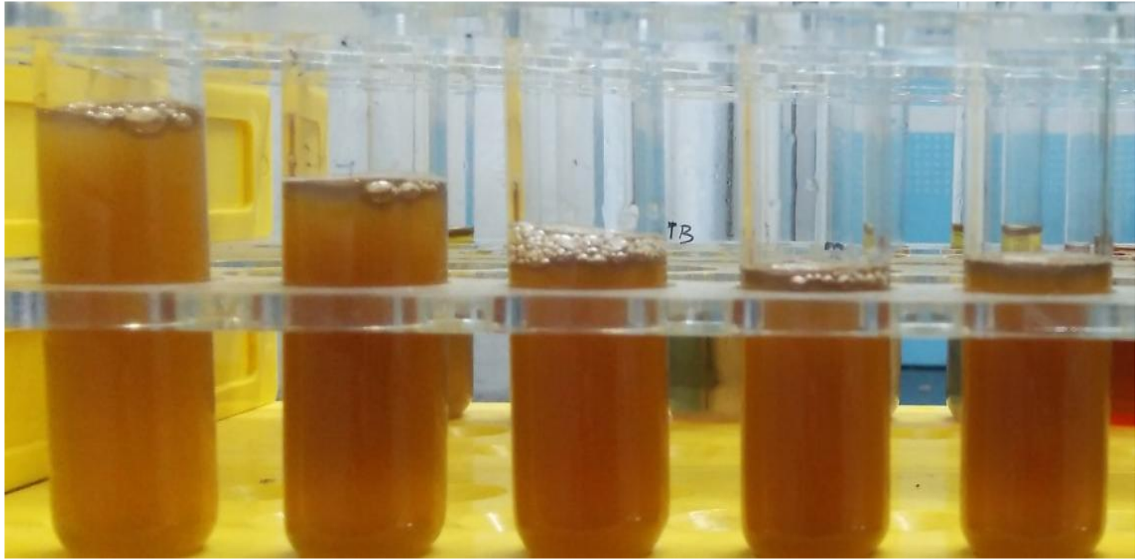


**Fig 4.5(c):** Voges-Proskauer test results.

- Color change of green colored simmons citrate agar into blue color indicates +ve result. But all of the 4 samples here indicated -ve result.

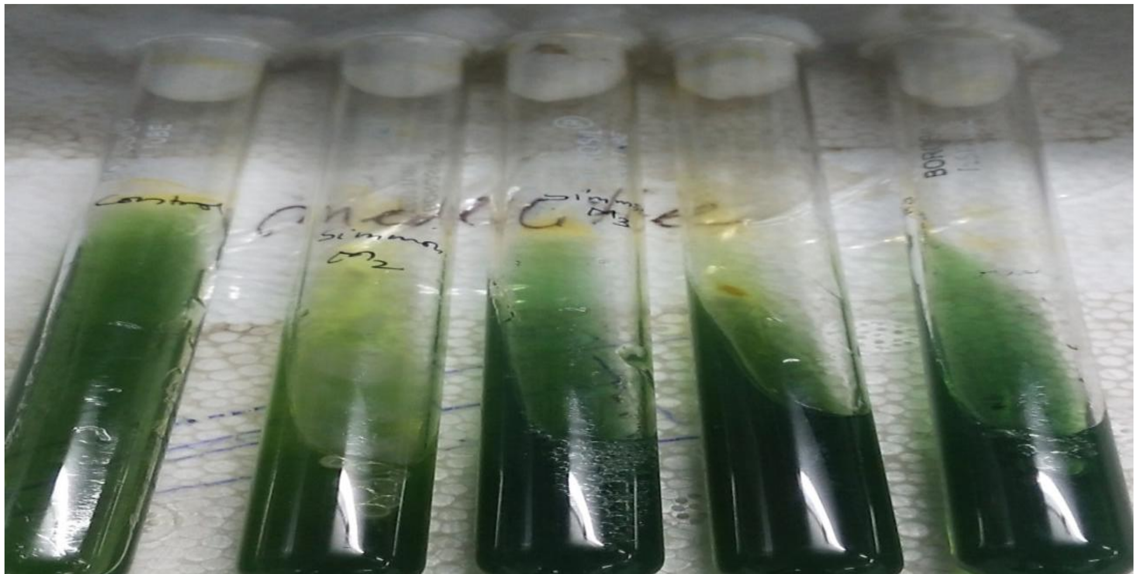

**Fig 4.5(d):** Citrate utilization test results.

- Color change of oxidase disc into dark blue indicated +ve result for oxidase test. M1, M2 and M3 gave +ve results whereas M4 gave –ve result.
- Formation of bubbles indicated +ve result for catalase test. M1, M3 and M4 gave +ve results whereas M2 gave –ve result with no or very less formation of bubbles.

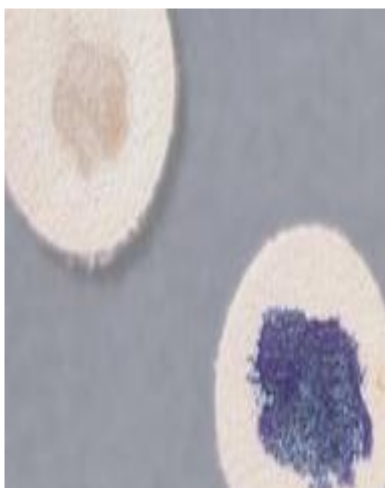

**Fig 4.5(e):**Oxidase test result.

**Fig 4.5(f):**Catalase test result.

**2.TABLE OF BIOCHEMICAL TEST RESULTS:**

| **Name of the test** | **M1** | **M2** | **M3** | **M4** |
|---|---|---|---|---|
| **Indole test** | +ve | -ve | +ve | +ve |
| **MR test** | -ve | -ve | -ve | -ve |
| **VP test** | -ve | -ve | -ve | -ve |
| **Citrate test** | -ve | -ve | -ve | -ve |
| **Oxidase test** | +ve | +ve | +ve | -ve |
| **Catalase test** | +ve | -ve | +ve | +ve |

**4.6.Processing of fermented cell culture:** Centrifugation of bacterial culture was done to remove cellular debris. Cellular components were preferably removed completely, to avoid any type of constriction in GC column, blockage in inlet or hindrance in detector jet.

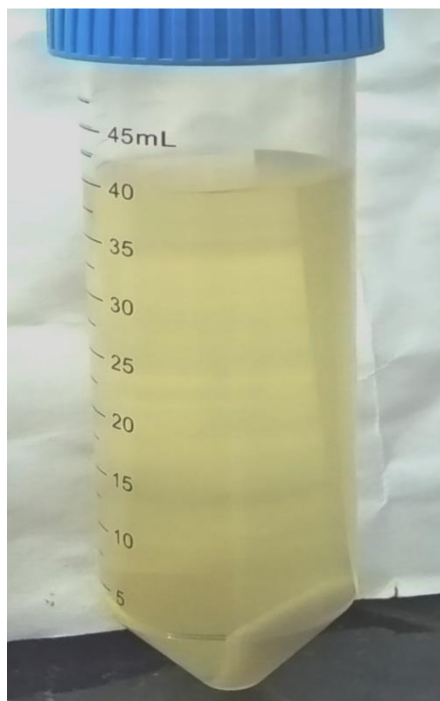


**Fig 4.6**: Culture with pellet after centrifugation.

**4.7.Solvent extraction method:** This method of extraction helped us to get the organic phase of ethyl acetate solvent which was expected to contain organic compounds and metabolites etc.

- The upper phase was obtained as ethyl acetate phase and the lower one as the aqueous phase.
- After shaking, the aqueous phase was removed from the separating funnel and the remaining clear phase was transferred into RBF of 500ml.
- The organic phase was dried in RBF using rotary vacuum evaporator and solid substance was obtained on the inner surface of the RBF.

**Fig 4.7(a):** Separation of aqueous and organic phase.

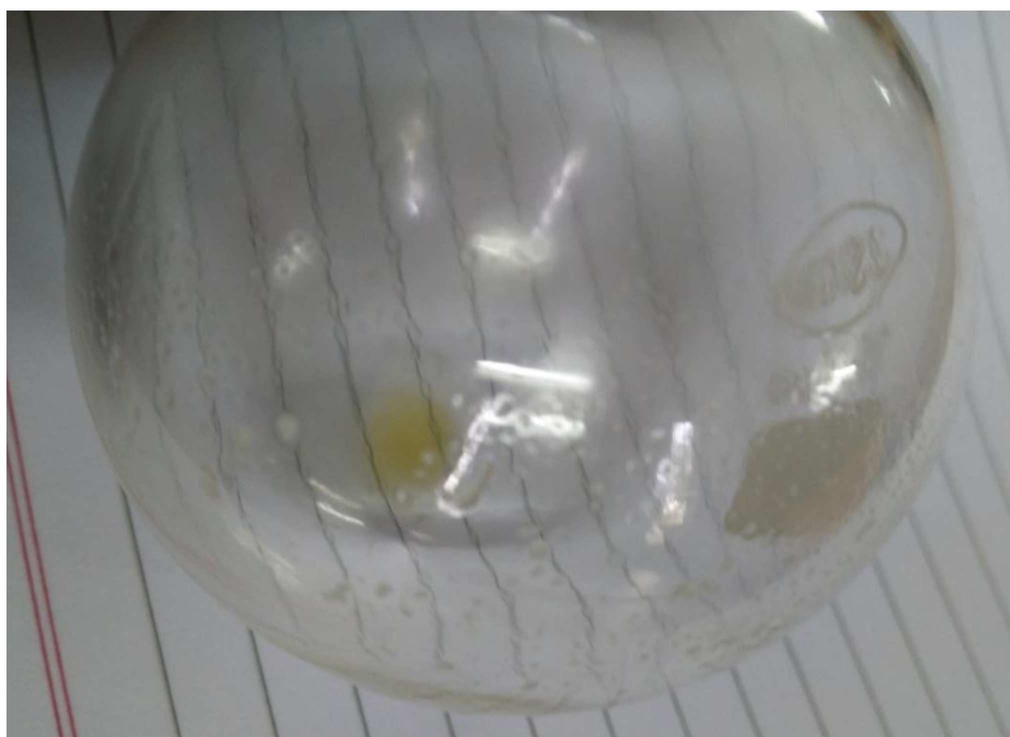

**Fig 4.7(b):** Dried compounds in RBF obtained from organic phase.

**4.8.GC-MS sampling and results:** The solid ubstance obtained over inner RBF surface was dissolved in 3ml solvent *i.e.* methanol andput into vial after filtering through 0.22 micron syringe filter.

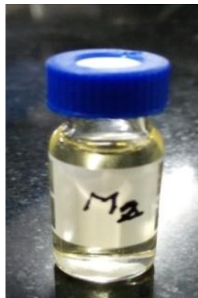


**Fig 4.8(a):** GC-MS sample.

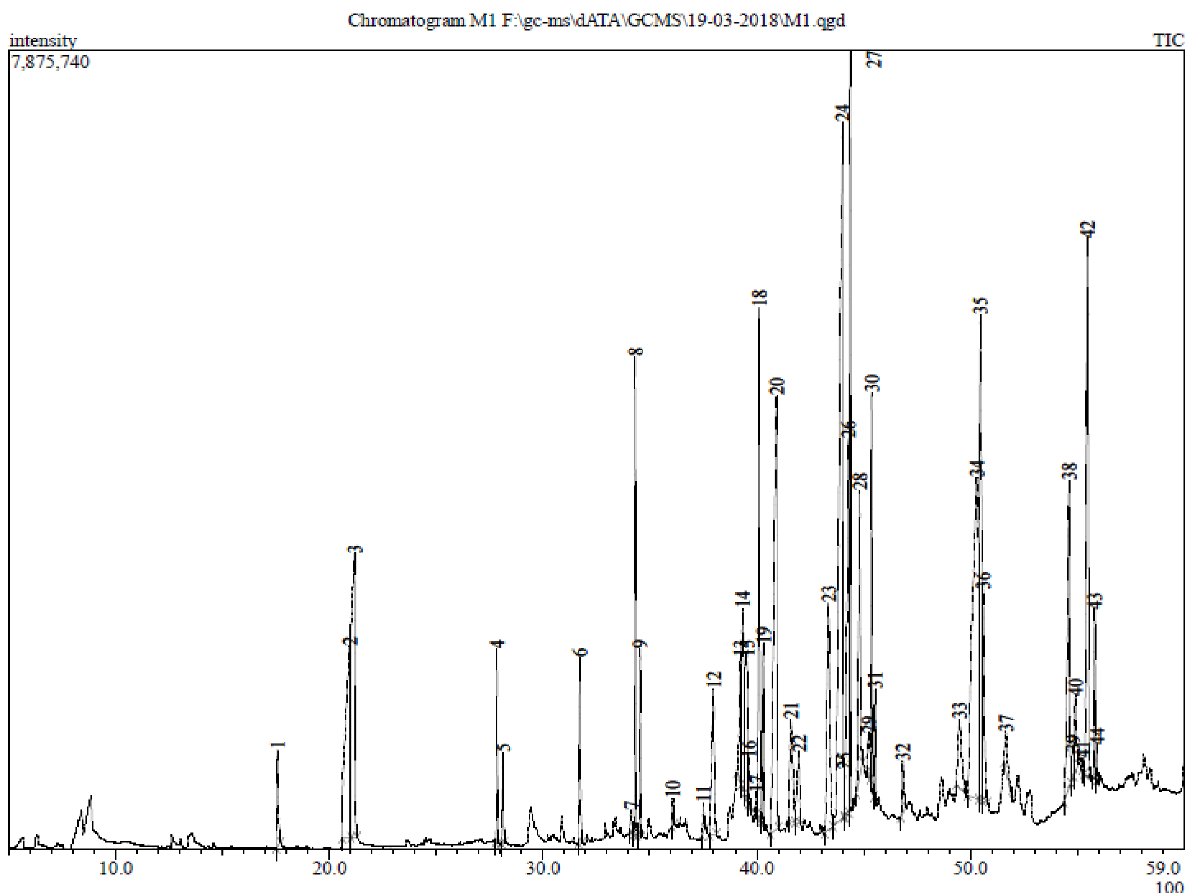


**Fig 4.8(b):** Peaks of different compounds.

- GC-MS result ofsample was obtained and presence of expected compounds was seen.The GC-MS analysis showed that the chemical composition of extract of isolate M2 contained 44 compounds.Many of the already known compounds were also identified, for e.g. 2-Piperidinone etc.
- Some of the other important compounds present in the sample were, hexadecane, dibutyl phthalate, tricosene, hexadecanoic acid etc.

Peak Report TIC

| 'eak# | R.Time | I.Time | F.Time | Area | Area% | Height | Height% | A/H | Mark | Name |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 17.562 | 17.500 | 17.717 | 4234950 | 0.69 | 951099 | 1.07 | 4.45 | | Phenylethyl Alcohol |
| 2 | 20.933 | 20.592 | 21.008 | 33142617 | 5.39 | 1891245 | 2.12 | 17.52 | | 2-Pentenoic acid, 4-hydroxy- |
| 3 | 21.173 | 21.008 | 21.308 | 28418623 | 4.62 | 2805196 | 3.15 | 10.13 | V | 2-Piperidinone |
| 4 | 27.835 | 27.767 | 27.908 | 5857813 | 0.95 | 1913603 | 2.15 | 3.06 | | 3-Hexadecene, (Z)- |
| 5 | 28.113 | 28.050 | 28.183 | 2751474 | 0.45 | 899416 | 1.01 | 3.06 | | Tetradecane |
| 6 | 31.720 | 31.642 | 31.842 | 7237285 | 1.18 | 1821664 | 2.05 | 3.97 | | Phenol, 2,4-bis(1,1-dimethylethyl)- |
| 7 | 34.109 | 34.050 | 34.208 | 1010598 | 0.16 | 221123 | 0.25 | 4.57 | | Diethyl Phthalate |
| 8 | 34.301 | 34.217 | 34.392 | 15237741 | 2.48 | 4718459 | 5.30 | 3.23 | | E-14-Hexadecenal |
| 9 | 34.531 | 34.392 | 34.608 | 6033320 | 0.98 | 1850784 | 2.08 | 3.26 | V | Hexadecane |
| 10 | 36.071 | 36.017 | 36.150 | 1013241 | 0.16 | 268298 | 0.30 | 3.78 | | Benzeneethanol, .alpha.-(phenylmethy |
| 11 | 37.494 | 37.433 | 37.575 | 1193811 | 0.19 | 290324 | 0.33 | 4.11 | | 2,2-Dimethyl-N-phenethylpropionami |
| 12 | 37.971 | 37.783 | 38.125 | 11581661 | 1.88 | 1429062 | 1.60 | 8.10 | | 3-Methyl-1,4-diazabicyclo[4.3.0]non: |
| 13 | 39.233 | 39.067 | 39.267 | 7289319 | 1.18 | 1227455 | 1.38 | 5.94 | | (3S,6S)-3-Butyl-6-methylpiperazine-2 |
| 14 | 39.348 | 39.267 | 39.417 | 13218246 | 2.15 | 1779688 | 2.00 | 7.43 | V | 2,5-Piperazinedione, 3-methyl-6-(1-m |
| 15 | 39.525 | 39.417 | 39.600 | 12391106 | 2.01 | 1405648 | 1.58 | 8.82 | V | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 16 | 39.625 | 39.600 | 39.725 | 1505970 | 0.24 | 465108 | 0.52 | 3.24 | V | 2-Chloro-2,5-dimethyl-5-propyl-2,5-d |
| 17 | 39.967 | 39.925 | 40.025 | 1044884 | 0.17 | 168777 | 0.19 | 6.19 | | 5-Octadecene, (E)- |
| Peak# | R.Time | I.Time | F.Time | Area | Area% | Height | Height% | A/H | Mark | Name |
| 18 | 40.111 | 40.025 | 40.200 | 18097975 | 2.94 | 5041024 | 5.66 | 3.59 | V | E-15-Heptadecenal |
| 19 | 40.301 | 40.225 | 40.383 | 5826713 | 0.95 | 1789183 | 2.01 | 3.26 | | Heptadecane, 2,6,10,15-tetramethyl- |
| 20 | 40.921 | 40.625 | 41.017 | 42471709 | 6.90 | 4273718 | 4.80 | 9.94 | | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 21 | 41.590 | 41.458 | 41.792 | 10299702 | 1.67 | 997096 | 1.12 | 10.33 | | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 22 | 41.965 | 41.792 | 42.050 | 5122798 | 0.83 | 708540 | 0.80 | 7.23 | V | 3,6-Diisopropylpiperazin-2,5-dione |
| 23 | 43.350 | 43.183 | 43.558 | 20380097 | 3.31 | 2237235 | 2.51 | 9.11 | | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 24 | 44.008 | 43.558 | 44.067 | 76158671 | 12.37 | 6865950 | 7.71 | 11.09 | V | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 25 | 44.083 | 44.067 | 44.117 | 1041715 | 0.17 | 462743 | 0.52 | 2.25 | V | 2,5-Piperazinedione, 3,6-bis(2-methyl |
| 26 | 44.291 | 44.117 | 44.317 | 25474766 | 4.14 | 3693300 | 4.15 | 6.90 | V | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 27 | 44.375 | 44.317 | 44.458 | 29062478 | 4.72 | 7506322 | 8.43 | 3.87 | V | Dibutyl phthalate |
| 28 | 44.811 | 44.658 | 44.950 | 16451144 | 2.67 | 2821650 | 3.17 | 5.83 | | n-Hexadecanoic acid |
| 29 | 45.241 | 45.158 | 45.300 | 2330458 | 0.38 | 399150 | 0.45 | 5.84 | | Carbonic acid, dodecyl ethyl ester |
| 30 | 45.380 | 45.300 | 45.475 | 13533139 | 2.20 | 3894388 | 4.37 | 3.48 | V | E-15-Heptadecenal |
| 31 | 45.538 | 45.475 | 45.608 | 3534838 | 0.57 | 1099161 | 1.23 | 3.22 | | Eicosane, 10-methyl- |
| 32 | 46.827 | 46.750 | 46.975 | 2815034 | 0.46 | 521624 | 0.59 | 5.40 | | 9-Hexadecenoic acid |
| 33 | 49.488 | 49.333 | 49.767 | 8378376 | 1.36 | 677774 | 0.76 | 12.36 | | 2,5-Piperazinedione, 3-methyl-6-(phe: |
| 34 | 50.292 | 49.858 | 50.375 | 59268464 | 9.63 | 3171795 | 3.56 | 18.69 | | 2,5-Piperazinedione, 3,6-bis(2-methyl |
| 35 | 50.458 | 50.375 | 50.567 | 39382063 | 6.40 | 4783502 | 5.37 | 8.23 | V | E-15-Heptadecenal |
| 36 | 50.592 | 50.567 | 50.792 | 10558141 | 1.72 | 2087951 | 2.34 | 5.06 | V | Benzeneacetic acid, 2-tetradecyl ester |
| 37 | 51.653 | 51.567 | 51.950 | 3449828 | 0.56 | 379196 | 0.43 | 9.10 | | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 38 | 54.611 | 54.367 | 54.708 | 23146740 | 3.76 | 3041660 | 3.41 | 7.61 | | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 39 | 54.767 | 54.708 | 54.833 | 1904017 | 0.31 | 273161 | 0.31 | 6.97 | V | 5-Nitroso-2,4,6-triaminopyrimidine |
| 40 | 54.913 | 54.833 | 55.042 | 4892724 | 0.79 | 748852 | 0.84 | 6.53 | V | Cyclo-(l-leucyl-l-phenylalanyl) |
| 41 | 55.262 | 55.225 | 55.317 | 336303 | 0.05 | 126234 | 0.14 | 2.66 | | 9-Octadecenamide, (Z)- |
| 42 | 55.484 | 55.325 | 55.608 | 31951806 | 5.19 | 5319433 | 5.97 | 6.01 | | Pyrrolo[1,2-a]pyrazine-1,4-dione, hex |
| 43 | 55.816 | 55.750 | 55.875 | 5267058 | 0.86 | 1674735 | 1.88 | 3.15 | | 1-Tricosene |
| 44 | 55.923 | 55.875 | 55.983 | 1138263 | 0.18 | 375361 | 0.42 | 3.03 | V | Heptadecane |
| | | | | 615437679 | 100.00 | 89077687 | 100.00 | | | |

**Fig 4.8(c):**Expected compounds corresponding to each peak.

# CHAPTER V

# DISCUSSION

## 5. DISCUSSION

Since, hilly region has been found to be a promising habitat for Actinomycetes species, and also there is a need to discover novel secondary metabolite, Actinomycetes are chosen for the study.

In the present study, Actinomycetes are isolated from the unknown mountainous sample and those with antimicrobial properties are identified. Out of the four known pathogenic test strains, isolated Actinomycetes show antimicrobial property against 2 test strains *i.e.Pseudomonas putida* and *Salmonella enterica*. After providing the shake flask fermentation, heavy cell growth is seen and cellular components are completely removed by either filtration or centrifugation. Further, on addition of the solvent, *i.e.* ethyl acetate, the organic compounds are expected to move to the upper organic phase of the mixture after shaking. On doing GC-MS analysis, some bioactive secondary metabolites have been seen to be produced by the Actinomycetes. At the end of the study, presence of many secondary metabolites present is determined in the unknown sample.

Actinomycetes give good growth on the selective media *i.e.* Starch Casein Agar, and according to the serial dilution, variations are observed in the growth pattern. On selecting random isolates from $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-5}$ dilutions, their master plates are maintained for further reference.Considering the 4 isolates of $10^{-1}$ dilution, antimicrobial screening is performed for them against 4 known pathogenic strains i.e. *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas putida* and *Salmonella enterica*. Isolates show inhibition against *Pseudomonas putida* and *Salmonella enterica* only. The amount of inhibition is clearly observed; also variation in diameter of different isolates against different test organisms is seen.

Gram staining gives the positive result which is already known for Actinomycetes. Although, on performing biochemical tests, some samples do show expected results but some variations have also been observed and recorded.Over the characterization steps, shake flask fermentation technique have been used for fermenting one of the strains out of four *i.e.* $M_2$, which also showed antimicrobial property.

Cellular components are removed by filtration using whatman filter paper (grade 1) and by centrifugation(it is necessary to completely remove cellular material to avoid any blockage in GC column).The organic phase, also known to be the ethyl acetate phase is already known to contain organic compounds or metabolites etc. Thus, after separating the organic phase it is made to dry in the RBF using a rotary vacuum evaporator under reduced pressure. Due to enough amount of the supernatant, dried compounds could be observed easily with naked eyes over the inner surface of RBF. The color of the obtained substance is little yellowish which is then made to dissolve in methanol and is syringe filtered. Lastly, it is collected in the sampling vial for GC-MS analysis.

GC-MS analysis shows the presence of expected compounds of secondary metabolites in the sample, predicted structures of identified compound fragments have been also provided in the complete report. As a part of qualitative study, many compounds have already been reported in previous research works (Khattab *et al.,* 2016).

Some of the commercially relevant compounds identified were as follows:

- 2-Piperidinone (or δ-valerolactam): A compound classified as a lactam. It is used as an intermediate in the preparation of therapeutic drugs and laboratory chemicals.
- Phenyl ethyl alcohol (or 2-phenyl ethanol): It occurs widely in nature and is found in a variety of essential oils. It is also a common ingredient in flavors and perfumery, particularly when the pleasant odor like that of rose is desired.It is used as a preservative in soaps and as an additive in cigarettes. It is of interest due to its antimicrobial properties.
- Hexadecane: Also known as cetane, it serves as a reference for fuel mixtures. It measures the detonation of the fuels like diesel.
- Dibutyl phthalate (DBP): It is a commonly used plasticizer due to its low toxicity and wide liquid range. Modified PVC (Poly Vinyl Chloride), used in plumbing for carrying sewerage and other corrosive materials, have DBP as a core constituent.

Some other already reported groups of antimicrobial compounds are aminoglycosides, glycopeptides, anthracyclines, macrolides, tetracyclines and polyenes ( Sharma *et al.,* 2014). Thus, the present study helps in identifying the secondary metabolites produced by the soil Actinomycetes of hilly region. For further studies, the data can be analyzed for studying the relevant metabolic compounds individually, their derivatives along with their therapeutic properties.

# CHAPTER VI

# SUMMARY

# 6. SUMMARY

The study was about the extraction and identification of secondary metabolites produced by randomly isolated strains from an unknown sample. In the above stated work, isolation of actinomycetes is performed using selective media. Further, the antibiotic producing strains, showing antimicrobial activity during screening are isolated and biochemical characterization is performed. After characterization, one of the strains is fermented further, after providing it a good growth by shake flask fermentation in standard conditions, cellular debris are removed completely to avoid any blockage or contamination in the GC column which may then give wrong or no result at all. Filtration as well as centrifugation techniques are used to assure complete removal of cellular components.

Over the process, ethyl acetate is added as an appropriate solvent for separation of organic compounds from the supernatant. The mixture of solvent and the supernatant is shaken for efficient separation of organic and inorganic compounds to obtain two clear liquid phases. The desired organic phase having secondary metabolites is separated, and dried in a rotary vacuum evaporator. Further, the obtained dried solid mass is dissolved in methanol, and prepared sample is sent for GC-MS analysis. The GC-MS result gives us the qualitative record of secondary metabolites present in the sample, their expected structures and possible derivatives.

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