

**EVALUATION OF ACTINOBACTERIA FROM LESS STUDIED SOURCES
FOR ANTI-MYCOBACTERIAL ACTIVITY**

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Engineering / technology Degree in Biotechnology

By

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This is to certify that this project report is the bonafide work of SUDHIR SAKTHI RAJA A (39230049) who carried out the project entitled "Evaluation of actinobacteria from less studied sources for anti-mycobacterial activity" under my / our supervision from

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ABSTRACT

Tuberculosis (TB), one of the oldest known human diseases, is still one of the major causes of mortality, since two million people die each year from this malady. Emergence of drug resistance in TB is becoming a major problem in management of the disease. Therefore, New antituberculosis drugs are needed to be developed to shorten treatment of tuberculosis, to improve treatment of drug-resistant tuberculosis, and to provide more efficient and effective treatment of latent tuberculosis infection. In the present study, we assessed the antimycobacterial capabilities of Soil actinobacteria isolated from Kashmir soil samples.

A total of 40 actinobacterial cultures were isolated from soil sample. All the actinobacterial cultures were evaluated for antimycobacterial activity by agar plug assay against *M. smegmatis*. Among which four cultures showed activity against *M. smegmatis*. These potential cultures were selected for further study. Crude extracts of the potential cultures were prepared using agar surface method. Microplate Alamar Blue Assay (MABA) was carried out to check the activity of crude extracts against *M. smegmatis* and *M. tuberculosis*(H37RA), Extracts with an inhibition of more than 50% were regarded as potential. Two cultures showed 60.5% and 60.8% of inhibition respectively. Characterization and taxonomy of potential cultures were studied, which showed that the potential actinobacterial culture belong to streptomyces genus. Whole genome sequencing will be carried out to confirm the species.

LIST OF ABBREVIATION

S.No	ACRONYM	EXPANSION
1	SCA	Starch Casein Agar
2	AIA	Actinomycete Isolation Agar
3	ISP2	International Streptomyces Protocol 2
4	SP	Sodium Pyruvate
5	YEME	Yeast Malt Agar
6	MTB	<i>Mycobacterium tuberculosis</i>
7	M.smeg	<i>Mycobacterium smegmatis</i>
8	Tb	Tuberculosis
9	TLC	Thin Layer Chromatography
10	Sp	Species
11	μg	Microgram
12	HIV	Human Immuno Virus
13	WHO	World Health Organization
14	INH	Isonicotinic acid hydrazide
15	RIF	Rifampicin
16	AIDS	Acquired Immuno Deficiency Syndrome
17	MDR	Multi-Drug Resistant
18	PAS	Para-amino salicylic acid
19	PZA	Pyrazinamide

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CHAPTER – 1

1. INTRODUCTION

1.1 TUBERCULOSIS

Mycobacterium tuberculosis (MTB) is the bacterium that causes the chronic granulomatous illness tuberculosis, which is highly contagious and airborne. It is a disease that mostly targets the lungs. Because of the pathogen's slow growth and immune resistance, treating TB has always been difficult. The history of tuberculosis is very old and it has afflicted many human beings. Recent molecular genetics research has revealed that the progenitor of *M. tuberculosis* existed 3 million years ago. (Ahmad, 2011) (Barberis *et al.*, 2017a) When a person with TB coughs or sneezes, the germs that cause the disease are transmitted. The majority of persons who carry the germs that cause tuberculosis do not exhibit any symptoms. When symptoms do show up, they frequently include a fever, weight loss, night sweats, and a cough (which can occasionally have a bloody tint). For people with no symptoms, treatment is not always necessary. A lengthy course of treatment comprising numerous antibiotics will be necessary for those who have active symptoms. The global effort to reduce TB is seriously threatened by drug-resistant TB. The prevalence of multidrug-resistant tuberculosis (MDR-TB), which is defined as *M. tuberculosis*-related tuberculosis that is resistant to both of the first-line antitubercular medicines, rifampicin and isoniazid, has significantly grown in recent years. An MDR-TB infection that is also resistant to a fluoroquinolone and one of three injectable second-line drugs (amikacin, kanamycin, or capreomycin) is known as XDR-TB. MDR-TB has a worse prognosis than drug-susceptible TB, which has a higher fatality rate, lower cure rates, lengthier but less effective treatments with harmful side effects, high costs, and challenging logistics in many nations. MDR-TB treatment success rates are 48–64% and XDR-TB rates are 20–40%, respectively. (Heyckendorf, Lange and Martensen, 2014)

Numerous factors contribute to the spread of drug resistance, including inadequate public health infrastructure, ineffective treatment, ineffective infection control, and the HIV epidemic. These factors include unmonitored therapy, nonadherence, incorrect use of anti-TB agents, and delays in the diagnosis of drug resistance.

1.2 STATISTICS

In 2021, 10.6 million persons worldwide (95% uncertainty interval : 9.9-11 million), or 134 cases (95% UI: 125-143) per 100,000 people, developed TB. 6.7% of all TB cases were among HIV-positive individuals. Geographically, the WHO areas of South-East Asia (45%), Africa (23%) and the Western Pacific (18%) had the highest percentages of TB cases in 2021, while the Eastern Mediterranean (8.1%), the Americas (2.9%), and Europe (2.2%) had the lowest percentages (WHO Report, 2022). 7,882 cases of TB were reported in the United States in 2021, with an incidence rate of 2.4 cases per 100,000 people (TB Incidence and Mortality: 1953–2021). The number of TB cases reported in the United States has increased, according to TB data from 2021. India's TB incidence for the year 2021 is 210 per 100,000 population – compared to the baseline year of 2015 (incidence was 256 per lakh of population in India); there has been an 18% decline which is 7 percentage points better than the global average of 11%. Between 2005 and 2019, there were fewer TB-related deaths worldwide each year, but in 2020 and 2021, the trend was the opposite. HIV-negative deaths were estimated to total 1.6 million in 2021, up from the best estimates of 1.5 million in 2020 and 1.4 million in 2019, and back to the level of 2017. HIV-positive deaths were estimated to total 187 000 in 2021, up from the best estimates of 158 000-218 000. The reduction from 2015 to 2021 was just 5.9%, reversing the progress previously achieved towards the first milestone of the End TB Strategy, which projected for a 35% drop between 2015 and 2020. (WHO Report, 2022)

1.3 CHARACTERISTICS OF MTB

MTB, which causes human tuberculosis (TB), are the first cause of death linked to a single pathogen worldwide. In the last decades, evolutionary studies have much improved our knowledge on MTB history and have highlighted its long co-evolution with humans (Ahmad and Mokaddas, 2009). Its ability to remain latent in humans, the extraordinary proportion of asymptomatic carriers (one-third of the entire human population), the deadly epidemics and the observed increasing level of resistance to antibiotics are proof of its evolutionary success. (BañUls *et al.*, 2015) *Mycobacterium tuberculosis* are aerobic, non-spore-forming, non-motile bacillus with a high cell wall content of high-molecular-weight lipids. These are slow growers; it takes about 15 – 20 hours. They have an unusual, waxy coating on its cell surface because of the presence of mycolic acid, due to which it

renders the cells resistive to Gram staining. This makes them weakly Gram – positive. Numerous MTB genetic signatures demonstrate that these bacteria have influenced the evolution of humans as well as being a paradigm of human adaptation (Magombedze, Dowdy and Mulder, 2013). Some authors propose that MTB infection may have a protective effect against both active TB disease and other pathologies because of the imbalance between the number of asymptomatic carriers and the number of patients with active TB. Given the level of suffering and mortality brought on by TB, it would be improper to classify these infectious organisms as commensals or symbionts. (Bañuls et al., 2015). The phenotypic variety of the pathogen is influenced by anatomical, physiological, and immunological variation in the microenvironments, which creates a very complicated interaction between the host and the pathogen. Phenotypic heterogeneity, which is defined as nongenetic diversity seen in specific individuals within a clonal population, can be advantageous, particularly in varying harsh environmental situations. This is especially true for MTB infections, because the bacteria may survive and can create a chronic infection in the host that lasts a lifetime. The varied and diverging paths of specific lesions within a single host have been shown in recent research in tuberculosis patients and in animal models. (Dhar, McKinney and Manina, 2016). With the increasing advancement in resistant and multi resistant cases, there would be a greater demand for new bioactive compounds in future.

1.4 ACTINOBACTERIA

The phylum Actinobacteria contains the order Actinomycetales, which is composed of Gram-positive bacteria known as actinomycetes. They contain individuals of major economic and medical significance, such as filamentous actinomycetes like *Streptomyces* species, which are known to create a variety of bioactive secondary metabolites and form symbiotic relationships with higher creatures like plants and insects. It is well known that there are many different types of actinobacteria in both common and uncommon habitats. They are the ruthless creators of tens of thousands of naturally occurring chemicals with biological activity. (Berdy, 2012; Velho-Pereira and Kamat, 2013; Balagurunathan et al., 2014; Dhakal et al., 2017). About 13,700 of the 33,500 microbial bioactive compounds discovered between 1940 and 2010 are produced by actinobacteria. The discovery of new broad spectrum of antibiotics has dramatically decreased over the past 20 years, according to numerous major pharmaceutical corporations. This has prevented new antibiotic classes with novel mechanisms of action from entering the market.

(Genilloud, 2017). This made it impossible to examine common terrestrial sources, especially for actinobacteria, and motivated researchers to look for potentially novel bioactive compounds in unusual and severe settings, such the marine environment. (Berdy, 2005; 2012; Lam, 2006; Raveh et al., 2013). The most prevalent bacterial population in soils is made up primarily of the varied group of bacteria known as actinobacteria. While certain family members are being studied primarily for their potential as sources of bioactive compounds, many elements of their roles in their natural habitat, including interactions with other creatures, are yet unexplored. (Jagannathan *et al.*, 2021)

1.4.1 Actinobacteria in tuberculosis research

Microbes' secondary metabolites are a rich source of complex compounds with a wide range of biological activities and difficult chemical skeletons. The order Actinomycetales (actinomycetes), a group of bacteria, has an unrivalled capacity for biosynthesizing biologically relevant chemicals, making these common soil dwellers assets with biotechnological promise. More than half of the antibiotics obtained from bacteria are produced by actinobacteria alone, and the majority of these compounds come from just two species (*Streptomyces* and *Micromonospora*). (Bérdy, 2005, 2012). Many unique Actinobacteria with strong biological activity profiles have already been discovered by uncommon habitat surveys (Bull, Goodfellow and Slater, 1992; Peláez, 2006). These understudied ecosystems include marine, desert, forest, and severe settings (such hyperarid, high-temperature, and high-saline environments), as well as unique bacteria that coexist with higher creatures in symbiotic or mutualistic partnerships. This strategy has already led to the discovery of novel substances (Maldonado *et al.*, 2005; Hozzein and Goodfellow, 2007; Freundlich *et al.*, 2010; Wang *et al.*, 2013), some of which contain anti-TB metabolites, such as abyssomicins and diazaquinomycins from Actinobacteria (Mullowney *et al.*, 2014, 2016).

1.4.2 Streptomyces

Streptomyces's ability to develop distinct structural and biological variations enables a variety of uses for the organism in biotechnology, agriculture, and medicine. (A. Van der Meij et

al.,2017).C Hardisson and M B Manzanal were the first to explore sporogenesis in *Streptomyces* on a sizable number of species (seven), a general picture of this process utilising their research as well as results are already published.(Mendez *et al.*, 1985) The synthesis of the sporulation septum and the development of the arthrospore are the two fundamental phases in the sporogenesis of *Streptomyces*. the ultrastructural analysis of the sporulation septum formation proves that there are three main forms within this genus. The septum of Type I is distinct because it is created from the start by two distinct cross walls, because there is a deposit of material before the synthesis of the double annulus, which completes the septum, Type II is identified. There are two subcategories that fall under this class. The deposits in the first are wedge-shaped. In the second, the sporulation septum, which the double annulus is hardly visible in, is almost totally made up of the deposits, which are of a distinct shape and are extremely well developed. The septum of Type III, represented by *Streptomyces cinnamonensis*, is distinct because only one cross wall makes up the septum. *Streptomyces* species are a source of fresh pharmaceutical chemicals that continue to draw attention. *Streptomyces* species' systems biology and biotechnology for the creation of secondary metabolites. (Hwang *et al.*, 2014)

CHAPTER – 2

2. LITERATURE REVIEW

2.1 HISTORY OF TUBERCULOSIS

Tuberculosis is a chronic granulomatous disease caused by the bacterium *Mycobacterium tuberculosis*; occasionally human tuberculosis can be caused by *Mycobacterium bovis* and *Mycobacterium africanum* also (Smith, 2003). The disease, tuberculosis is historically known as consumption or wasting disease or white plague and has affected humans for centuries. The word “tuberculosis” is derived from the word “tubercle” which means a small lump or nodule. Until mid-1800s, people thought that tuberculosis was hereditary. They did not realize that it could be spread from person to person through air. Also, until 1940s, there was no treatment for tuberculosis. For many people, a diagnosis of tuberculosis was often a slow death sentence. Robert Koch announced the discovery of a staining method to demonstrate the organism in 1882 (Barberis *et al.*, 2017b). Ehrlich soon improved upon the method by staining the organism with an aniline dye basic fuchsin and decolorizing it in a mineral acid, hence the “acid fast” tag. Later Ziehl and Neelsen improved upon the method. In the same year, Ziehl changed the mordant to carbolic acid and incorporated it with basic fuchsin dye to form carbol fuchsin. Thus, the standard stain for demonstrating acid fastness was formulated – the Ziehl Neelsen stain (Bodal *et al.*, 2015). Yet, half a century passed before drugs were discovered that could treat tuberculosis. Until then, many people were sent to sanatoriums; special rest homes where they followed a prescribed routine every day. Those people who could not afford to go to sanatoriums, died at home. A break-through came in 1944, when an American scientist Salmon Waksman and one of his assistants, Albert Schatz, discovered Streptomycin, a drug used to treat tuberculosis. (Esposito, Zinzula and Tramontano, 2007) In the same year, Jorgen Lehman, synthesized para-amino salicylic acid (PAS) and later in 1952, Gerhard Domagk discovered isonicotinic acid hydrazide (INH). Combination therapy with these agents resulted in predictable cure for 90-95% patients in 24 months. The replacement of PAS by ethambutol in 1960 allowed reduction in duration of treatment to 18 months. The next major advance in therapy was the discovery of rifampicin and the combination therapy of INH, ethambutol and rifampicin resulted in predictable cure in >95% cases in just 8-9 months. Later ethambutol was replaced by pyrazinamide (PZA) and this when given along with INH and rifampicin, cure was attained in 6 months (Jindani, Doré and Mitchison, 2003). With the discovery of

drugs, the second half of 20th century saw a continuous drop in morbidity and mortality from tuberculosis in industrialized nations. The incidence increased dramatically with the spread of AIDS, by approximately 12% between 1985 and 1995 (Olsen *et al.*, 1998) .A new threat is the increase of multidrug resistant (MDR) tuberculosis strains, which are resistant to principal anti-tubercular drugs, isoniazid and rifampicin, and may be resistant to other anti-tubercular drugs as well. Thus, resistance has become a major issue and the rapid diagnosis of cases and early initiation of treatment are needed to bring the disease under control. (Rossolini *et al.*, 2014)

2.2 GLOBAL BURDEN OF TUBERCULOSIS

In 2015, an estimated 9.6 million people developed tuberculosis and 1.5 million died from the disease. WHO declared tuberculosis as a global public health emergency nearly 20 years ago. (Chaisson, Frick and Nahid, 2022) The Millennium Development Goal target of 2015 for halting and reversing the tuberculosis epidemic by 2015 has been met globally. Tuberculosis incidence has fallen by an average of 1.5% per year since 2000 and is now 18% lower than the level of 2000. The tuberculosis death rate dropped 47% between 1990 and 2015. An estimated 43 million lives were saved through tuberculosis diagnosis and treatment between 2000 and 2014. Ending the tuberculosis epidemic by 2030 is among the health targets of the newly adopted Sustainable Development Goals. Tuberculosis-HIV co-infection and drug resistant tuberculosis aggravate tuberculosis situations globally. One in three HIV deaths is due to tuberculosis. Global level targets for HIV testing among tuberculosis patients and provision for antiretroviral therapy to those who are HIV positive have not reached. (Shankar *et al.*, 2014)

2.3 BRIEF MICROBIOLOGY

The organism belongs to the Family: Mycobacteriaceae, Order: Actinomycetales and Class: Actinomycetes. Genera that are closely related to the members of the genus *Mycobacterium* include *Nocardia*, *Rhodococcus* and *Corynebacterium* (Jurado *et al.*, 2009). The genus *Mycobacterium* comprises of slow growing organisms, which are acid fast. They are non-motile, straight or slightly curved rods and may occasionally demonstrate branching. The organisms are aerobic and have Gram positive cell wall although they do not Gram stain well. Mycobacteria contain lipid rich cell surface which includes true waxes and glycolipids. A 60-90 carbon chain, mycolic acid, is a component unique to Mycobacterial cell wall. Mycolic acid is responsible for the following characteristics of *Mycobacterium*: $\frac{3}{4}$ Acid fastness $\frac{3}{4}$ Failure to stain by Gram stain $\frac{3}{4}$ Resistance to the action of antibodies and complement (Sander *et al.*, 2004) Another important component of the cell wall is lipoarabinomannan (LAM), a carbohydrate structural antigen on the outside of the bacterium. LAM is immunogenic and it also facilitates survival of Mycobacteria within macrophages (Sander *et al.*, 2004).

2.4 PATHOGENESIS

Tuberculosis is spread from person to person through respiratory droplets generated during coughing or sneezing or talking of a patient having pulmonary or laryngeal tuberculosis. These droplet nuclei may also be generated by aerosol treatment, sputum induction, aerosolization during bronchoscopy and through manipulation of lesions or processing of tissue or secretions in the laboratory. These minuscule droplets can remain air-borne for minutes to hours after expectoration. The number of bacilli in the droplets, virulence of the bacilli, exposure of the bacilli to UV light, degree of ventilation and occasions for aerosolization, all influence transmission (Knechel, 2009). Once inhaled, the majority of the bacilli are trapped in the upper part of the airways, where the mucus-secretory goblet cells exist. The mucus produced catches the foreign body. The cilia present on the surface of the cells constantly beat the mucus and its entrapped particles upwards, for removal. This provides an initial physical defense that prevents infection in most persons exposed to tuberculosis. Bacteria that bypass the mucociliary system reach the alveoli within the lungs. These

bacteria are quickly surrounded and engulfed by alveolar macrophages. These macrophages form the next line of defence of the body to destroy the invading Mycobacteria and prevent infection. Patients with tuberculosis also generate larger particles containing numerous bacilli. But these particles do not serve as effective vehicles for transmission of infection because they do not remain airborne, and, if inhaled, do not reach the alveoli. These large particles are trapped in the mucus blanket on the walls of the upper airways, and are carried to oropharynx. These are then either swallowed or expectorated. The organism phagocytosed by alveolar macrophages, multiplies slowly within the macrophages. The infected macrophage produces cytokines and chemokines, which attracts other phagocytic cells, including monocytes, other alveolar macrophages, and neutrophils. (Duque and Descoteaux, 2014) These cells eventually form a nodular granulomatous structure called the 'tubercle'. If the bacterial replication is not controlled, the tubercle enlarges and the bacilli enter local draining lymph nodes, usually the hilar or mediastinal lymph nodes. This leads to lymphadenopathy, a characteristic clinical manifestation of pulmonary tuberculosis. The lesion produced by expansion of the tubercle into the lung parenchyma together with lymph node involvement is called the Ghon complex. Unchecked bacterial growth may lead to hematogenous spread of the bacilli to produce disseminated tuberculosis. Certain organs like bone marrow, liver and spleen are notably resistant to subsequent multiplication of these bacilli. Organisms deposited in the upper lung zones, kidneys, bones and brain find environments that favor the growth of the bacilli and the bacteria multiplies several times before specific cellular immunity develops and limits multiplication. (Smith, 2003)

2.5 ACTINOBACTERIA ISOLATED FROM THE RARE HABITATS

A study conducted by A Hussain in year Antituberculous activity of 'Actinobacteria isolated from the rare habitats' helps us to know about the discovery and in vitro potential

of uncommon actinobacteria against Mycobacterium TB for its metabolites in the Northwestern Himalayas. (Hussain *et al.*, 2017) The study found that one of the creative methods for finding new natural products is to explore untapped natural sources. This work also provided compelling evidence that rare and powerful actinobacterial strains can create bioactive chemicals with antagonistic action, and that the inhibitory potential of these metabolites can be investigated. The pH, temperature, humidity, oxygen level, and other environmental factors may change from those seen in typical environmental samples in unusual extreme environments, causing some bacteria to adapt or mutate. Because the abundant, intermediate, and uncommon sub-communities may have different rates of development and activity and consequently respond to environmental factors in different ways, it is possible that these sub-communities were largely influenced by different environmental conditions. (Dong *et al.*, 2020)

2.5.1 Actinomycetes isolated from unexplored regions of sundarbans mangrove ecosystem

Sohan Sengupta and Arnab Pramanik isolated actinomycetes from the mangrove ecosystem of the Sundarbans. It is exceptional for its ecological dynamics and frequently flooded with low, moderate, or high salinity water. This ecosystem, which is well located at the transition between the terrestrial and marine environments, is home to an abundant and diverse range of microorganisms (Ghosh *et al.*, 2010). Antimicrobial activity against fifteen test species, including three phytopathogens, was examined in 54 isolated actinomycetes. Nine isolates with different morphologies and biological activity underwent polyphasic identification research. Eight isolates showed the most resemblance to the genus *Streptomyces*, according to 16 s rDNA sequencing, whereas one sample only showed a 93.57% similarity to *Streptomyces albogriseolus* NRRL B-1305(T). Three particular strains of eight isolates, including phyto-pathogenic fungus, were discovered to have a noticeably higher level of antibacterial potential. The strain SMS_SU21 was ultimately determined to be the most promising one since it demonstrated antibacterial

activity with a MIC value of 0.05 mg ml⁻¹) and antioxidant activity with an IC₅₀ value of 0.242 0.33 mg ml⁻¹). (Sengupta *et al.*, 2015)

2.5.2 soil actinomycetes isolated from Kashmir

Although understudied yet with a rich biodiversity, the Kashmir area of the Northwestern Himalayas was chosen for the current study due to its actinomycete isolation and antibacterial potential. The soil samples were taken from various high-altitude districts of Kashmir, and a total of 121 morphologically distinct actinomycetes were isolated. In an initial screening, the fermented broth of a culture of 27 isolated actinomycetes that had been grown for 10 days demonstrated antibacterial activity against at least one of the tested bacteria. (Shah *et al.*, 2017) The outcomes showed that among the studied microorganisms, the crude extracts of 6 isolates displayed the best antibacterial activity against *S. aureus*. The powerful strains designated as IIIM06, IIIM37, IIIM42, IIIM55, IIIM57, and IIIM62 were identified using the 16S rRNA gene sequencing method. Strong activity was evident in the activity results for the IIIM06 and IIIM55 strains. It was discovered that the actinomycete isolate IIIM55 contained the active ingredient alborixin and had a MIC of 0.25 g/ml against *S. aureus*. (Shah *et al.*, 2016). The IIIM06 isolate, known as *S. pratensis*, had considerable activity against *S. aureus* ATCC 29213 with MIC 0.25 g/ml and was also very active against the H37Rv strain of *M. tuberculosis* with MIC 0.0625 g/ml. As a result, the isolate IIIM06 was chosen for further chemical analysis. This isolate was found to be closely related to the genus *Streptomyces* and to have the highest resemblance (100%) to the *S. pratensis* when the 16S rRNA gene sequence of strain IIIM06 (1334 nucleotides) was compared to sequence in Gene Bank. Based on the similarity of a 1334 nt consensus length of the 16S rRNA gene, a phylogenetic tree was created using the neighbor-joining technique. Column and flash chromatography were used to separate the *S. pratensis* IIIM06 large-scale fermented broth's crude ethyl acetate extract, which had the highest antibacterial activity. (Keller *et al.*, 2010), (Gubbens *et al.*, 2014) (Shah *et al.*, 2017)

2.5.3 Soil actinobacteria from forest ecosystems of Northeast India

Northeast India is widely renowned for its vast biodiversity because it is a part of the Indo-Burma mega-biodiversity hotspot. The area has a variety of climatic, edaphic, and altitudinal differences that lead to a large range of natural niches. Northeast India serves as a bridge between the Indo-Malayan, Indo-Chinese, and Indian bio-geographical areas. It harbours a variety of India's flora and fauna (Myers *et al.*, 2000). The microbiological diversity is likewise understudied in this region of the planet, unlike the floral and faunal variety. In species found in biodiversity hotspots, the local environment may have an impact on the emergence of novel secondary metabolic pathways ('Applications of Fungal Ecology in the Search for New Bioactive Natural Products', 2007). The biodiversity of terrestrial ecosystems on earth is thought to be greatest in forests. They are the largest source of unique microbes and their priceless natural products that is currently available (Strobel *et al.*, 2004; Kim, Lee and Lee, 2014). Priyanka Sharma and Debajit Thakur isolated 54 actinobacterial cultures from the Pobitora Wildlife Sanctuary and 53 cultures from Kaziranga National Park in Assam, India. They exhibited unique colony morphology, mycelia, colour, and pigment synthesis, and they were connected with sparse to profuse sporulating capabilities. 39 isolates from Pobitora Wildlife Sanctuary and 38 isolates from Kaziranga National Park make up the 77 actinobacteria (71.9%) that showed positive activity. A total of 51 isolates (66%) demonstrated antimicrobial activity against *Staphylococcus aureus* MTCC 96, 49 isolates (64%) demonstrated antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, 60 isolates (78%) demonstrated antimicrobial activity against *Candida albicans* MTCC 227, 59 isolates (77%) demonstrated antimicrobial activity against *Escherichia coli* MTCC 40. Additionally, against all four test bacteria, 29 isolates (37.5%) exhibited antibiotic activity. At the amino acid level, PKS-I sequences and their closest matches shared 56–68% similarity in a BLASTP search. The gene products responsible for the formation of concanamycin A, tautomycin, chlorothricin, nanchangmycin, oligomycin, and rifamycin are closely related to the amino acid sequences of PB-10, PB-32, PB-47, PB-52, PB-64, and Kz-24, which correspond with the KS domain. 20 actinobacterial PKS-II sequences had 68–95% amino acid identity with their closest matches. It was projected that actinorhodin, naphthocyclinone, jadomycin B,

and landomycin would be their closest match pathway products. Chemical composition of the isolates were characterized by GC-MS.. (Sharma and Thakur, 2020)

2.5.4 Soil actinobacteria isolated from dry land soil

Actinobacteria are extensively distributed in fresh waterways, sewage, air, surface soils, deep soils, fresh waters, and the surface of plants, but are more frequently found in soil than other sources. In soil, *Streptomyces* is more prevalent than other genera (Goodfellow and Williams, 1983; Sanglier *et al.*, 1993; Jeya, Kiruthika and Veerapagu, 2013). The primary possible source of antibacterial chemicals since the 1940s has been soil (Protasov *et al.*, 2017). The soil's pH, temperature, type, amount of organic matter, cultivation, aeration, and moisture content are a few of the variables that affect their population density (Davies and Williams, 1970; Jeya, Kiruthika and Veerapagu, 2013). Actinobacteria have a significant role in the production of bioactive secondary metabolites with great commercial value, such as immunosuppressive chemicals and antibacterial, antiviral, antifungal, anti-cancer, and anti-parasitic uses (Bérdy, 2005; Bibb, 2005; K *et al.*, 2011; Kumar N *et al* 2010). In a study Majidzadeh isolated 18 distinct Actinobacteria strains, seven of which (198, 220, 278, 281, 293, 294 and 297) showed antibacterial activity against ATCC strains and *Bacillus subtilis* from sixty soil samples. There were four isolates with a 57.14 percent resistance to *Escherechia faecalis* (ATCC29212), three with a 42.85% resistance to *Streptococcus aureus* (ATCC25922), four with a 57.14 percent resistance to *Klebsiella pneumoniae* (ATCC700603), three with a 42.85% resistance to *Acinetobacter baumannii* (ATCC19606), four with a 57.14 percent resistance to *Pseudomonas aeruginosa* (AT CC27853).(Majidzadeh *et al.*, 2021)

2.5.5 Actinobacteria isolated from marine environment

Recent evidence strongly suggests that actinobacteria are widely spread in marine ecosystems, including sponges, fish, molluscs, mangroves, seaweed, and sediments in addition to seawater. These organisms are becoming more significant due to their development of novel bioactive compounds, such as antibiotics, antioxidants, cytotoxic, antitumor agents, immunosuppressive agents, cardiovascular, etc. (Hassan *et al.*, 2017) with their distinctive carbon skeletons, which also serve as a solid foundation for

synthesis. Actinobacteria create many biochemical compounds with several carbon skeletons, and it has been discovered that these compounds are the primary factor obstructing human disease at various places. The chemical makeup of compounds formed from actinobacteria is diverse, including peptides, alkaloids, polyketides, and terpenoids, as well as their biotechnological activities, which include antimicrobial, antitumor, cytotoxic, anti-parasitic, and immunosuppressive effects (Liang et al., 2016; Manivasagan, et al., 2014). Most of the time, there is no clear connection between the chemistry of the secondary metabolites produced by marine actinobacteria and their mode of action on disease in vivo (inhibition of cell mitochondrial electron transport chain, interaction with bacterial RNA polymerase to combat inflammation,). The potential of the secondary metabolites produced by marine actinobacteria has been compared to a gold mine. Most studies on secondary metabolites generated from actinobacteria have looked at their pharmacological effects. Future treatments for important diseases including cancer, drug-resistant bacteria, a variety of viral ailments, malaria, many infections, and inflammations may be made possible by actinobacteria. (Jagannathan *et al.*, 2021)

CHAPTER - 3

AIM

To evaluate actinobacteria from less studied sources for Anti-Mycobacterial properties.

OBJECTIVES

- Isolation of Actinobacteria from the selected rice field soil and orchard soil sample from Kashmir
- Primary Screening of isolated actinobacteria for Anti- *Mycobacterium smegmatis* activity by agar plug method
- Preparation of extract of potential actinobacterial culture
- Screening of potential actinobacterial extract for Anti-smegmatis and Anti-Tuberculosis (H37RA) by Microplate Alamar Blue Assay

CHAPTER - 4

4. MATERIALS AND METHODS

4.1 SAMPLE COLLECTION

Soil samples were collected from Rice cultivable soil and Orchard soil from Kashmir. The soil samples were packed in a air-tight container and preserved in cold temperature to mimic it's natural climatic conditions.

4.2 SELECTIVE ISOLATION PROCEDURE AND MEDIA

The soil samples were dried and were processed to four step surface sterilization method. 5mg of Rice cultivable soil sample and orchard soil samples were measured. Three types of media were used for selective isolation of actinobacteria, sodium pyruvate (SP) for enhancing the growth of dormant and rare actinobacteria. The measured soil samples were serially diluted from 10^{-1} to 10^{-5} and plated on starch casein, ISP2, AIA and SCA, with and without SP using L-rod by spread plate method. The plates were incubated at room temperature for 28 days for the growth of actinobacterial colonies (Radhakrishnan *et al.*, 2007).

4.3 RECOVERY, PURIFICATION, DE-REPLICATION AND PRESERVATION OF ACTINOBACTERIA

Colonies showing suspected actinobacterial morphology were recovered from the isolation plates using sterile L-shaped needle and inoculated on respective media. The plates were incubated at 28°C for 7 days. Morphologically different actinobacterial cultures were selected for study (Radhakrishnan *et al.*, 2014).

4.4 CHARACTERIZATION OF ACTINOBACTERIA

All of the actinobacterial cultures were inoculated into ISP2, AIA, SCA, ISP2.SP, AIA.SP, SCA.SP media to perform cultural characterisation. All of the plates were incubated for 7 to 14 days. At 28°C. Growth, colony uniformity, aerial mass colour, the presence of reverse side pigment, and the production of soluble pigment are among the cultural traits that have been observed (Shirling and Gottlieb, 1966).

4.5 ANTIMYCOBACTERIAL ACTIVITY OF SOIL ACTINOBACTERIA

AGAINST *M. smegmatis*

The agar plug method was used to assess the antagonistic activity of isolated actinobacteria against *M. smegmatis*. Before plating, 7H9 agar media was prepared, and the test pathogen was introduced. After the plates were prepared, an agar plug of 9 mm in diameter was cut from all the media plates, placed over a 7H9 plate, sealed, and incubated for 48 hours at room temperature to look for an inhibitory zone.

4.6 PRODUCTION OF BIOACTIVE METABOLITES

Bioactive compound from potential actinobacterial cultures was produced by agar surface fermentation (Radhakrishnan *et al.*, 2014). All the actinobacterial cultures were inoculated into their respective media and incubated at 28°C for 10 days for the production of secondary metabolites. During incubation, the extracellular metabolites were secreted into the agar medium. After scrapping the mycelial growth, the agar medium was cut into pieces and extracted using ethyl acetate at 1:2 ratio for 24 hours. The solvent portion was collected and concentrated by evaporating and quantified.

4.7 PHYSIOLOGICAL CHARACTERISICS

Basal agar medium recommended by Shirling and Gottlieb (1966) was used for carbon utilization studies. After sterilization of basal agar medium by autoclaving at 15 lb for 15 minutes, about 1% of various ether sterilized sugars were added and poured into petri plates. About one ml of well grown culture of actinobacteria was inoculated in different sugar containing basal agar plates. Growth was recorded after 7 days of incubation at 28⁰C. The same procedure was used to study the utilization of nitrogen sources including Peptone, Yeast extract, Malt extract, Potassium nitrate and Soybean by the potential actinobacteria. Sugars used in this study include glucose, fructose, sucrose, mannitol, inositol, xylose, rhamnose, raffinose, and cellulose (Hi media).

4.7.1 Assimilation of carbon source

To study the effect of carbon source the selected isolate was-quadrant streaked on Petri plates containing fermentation media with different 1% of carbon sources namely glucose, Lactose, Xylose, Starch, Mannitol, Ionositol, and Raffinase. The plates were further incubated, at 28 °C for 7 days and checked for growth of antimycobacterial activity.

4.7.2 Assimilation of nitrogen source

The effect of nitrogen source for the selected isolate was quadrant streaked on Petri plates containing fermentation media and replacing the nitrogen source alone. The nitrogen sources used are Soya bean, Malt, Peptone, Yeast and KNO₃. The plates were further incubated at room temperature for 7 days and checked for growth of antimycobacterial activity.

4.8 MICROPLATE ALAMAR BLUE ASSAY- MABA

MABA was performed to measure the cell survival and proliferation by keeping track of the environment's oxidation/reduction state. Alamar blue is a soluble redox dye that is nontoxic and stable in growth media, just like the tetrazolium salts. Because the reduced dye is pink and luminous when compared to the oxidised dye's blue colour, growth can be detected visually or with the aid of a fluorometer. Several laboratories have employed Alamar blue to evaluate the antibiotic susceptibility of *M. tuberculosis* and other mycobacterial species. (Ansar Ahmed, Gogal and Walsh, 1994; Collins and Franzblau, 1997; Franzblau *et al.*, 1998)

The assays were carried out as described by MRK *et al.*, with modifications. 100 microliters of mycobacterial suspension were added to 100µL extract containing culture medium. Growth Control wells were prepared using culture medium only and the mycobacterial suspension. For *M. tuberculosis*, the plates were sealed and incubated for seven days at 37 °C. and *M. smegmatis* were cultivated at 37 °C overnight. After this incubation period, 30 ul of 0.01% Alamar blue/resazurin solution was added to each well. The plates were incubated at 37 can additional 24 h. The MIC was determined as the lowest concentration of antimicrobial agent at which no growth was observed. Blue wells are due to the absence of growth whereas pink wells reflect the presence of growing mycobacteria. All experiments were performed in triplicate for each mycobacterial species and for each extract tested. Optical density was recorded in UV spectrophotometry(model/make) at 600nm and percentage of inhibition was calculated using the formula:

$$\text{Percentage of inhibition} = \frac{\text{control} - \text{test sample}}{\text{control}} \times 100$$

4.9 ANALYTICAL THIN LAYER CHROMATOGRAPHY

The number of components present in the crude pigmented extract was analyzed by analytical thin layer chromatography using ready-made silica gel coated plates (20mm X 20mm) (Merck). The crude extract was dissolved in small volume of ethyl acetate and spotted at the bottom of the TLC sheet using glass capillary tube. Chromatogram was run by using different solvent systems such as chloroform: methanol; dichloromethane: methanol; hexane: ethyl acetate and ethyl acetate; acetone in different ratio. The separated spots were observed under naked eye. R_f values of the separated compounds were calculated by adopting standard formula (Augustine *et al.*, 2005).

CHAPTER - 5

5. RESULTS AND DISCUSSION

5.1 ISOLATION OF SOIL ACTINOBACTERIA FROM THE KASHMIR SOIL SAMPLES

Totally 40 actinobacterial strains, 19 from Rice cultivable soil and 21 from Orchard soil from Kashmir were isolated. All the cultures showed good growth on ISP2, AIA and SCA media with and without SP.

5.2 PRELIMINARY IDENTIFICATION OF ACTINOBACTERIA

The isolated actinobacteria were grouped based on the morphological characteristics such as growth, consistency, aerial mass color, reverse side pigment, Soluble pigment, aerial mycelium, substrate mycelium. Most of the cultures had shown good growth except few. Majority of the cultures had shown powdery consistency, pasty and leathery consistency was shown by few of the cultures. Grey, white, light yellow, orange, dark brown pigments were also noticed. Aerial mycelium and Substrate mycelium were showed by all the cultures which were viewed under bright microscopy (Table:5.1, 5.2,5.3 and 5.4) (Figure:5.1)

S.No	MEDIA	DILUTION	NO OF COLONIES	WEEK1
1	SCA	10^{-3}	106	+
2	AIA	10^{-3}	TNTC	+
3	ISP2	10^{-3}	TNTC	+
4	SCA+SP	10^{-3}	100	+
5	AIA+SP	10^{-3}	118	+
6	ISP2+SP	10^{-3}	47	+
7	SCA	10^{-4}	15	+
8	AIA	10^{-4}	26	+
9	ISP2	10^{-4}	21	+
10	SCA+SP	10^{-4}	29	+
11	AIA+SP	10^{-4}	TNTC	+
12	ISP2+SP	10^{-4}	7	+
13	SCA	10^{-5}	7	+
14	AIA	10^{-5}	4	+
15	ISP2	10^{-5}	3	+
16	SCA+SP	10^{-5}	0	+
17	AIA+SP	10^{-5}	1	+
18	ISP2+SP	10^{-5}	2	+

Table:5.1 Rice soil sample

S.No	MEDIA	DILUTION	NO OF COLONIES	WEEK1
1	SCA	10 ⁻³	TNTC	+
2	AIA	10 ⁻³	TNTC	+
3	ISP2	10 ⁻³	22	+
4	SCA+SP	10 ⁻³	73	+
5	AIA+SP	10 ⁻³	101	+
6	ISP2+SP	10 ⁻³	TNTC	+
7	SCA	10 ⁻⁴	9	+
8	AIA	10 ⁻⁴	66	+
9	ISP2	10 ⁻⁴	TNTC	+
10	SCA+SP	10 ⁻⁴	35	+
11	AIA+SP	10 ⁻⁴	85	+
12	ISP2+SP	10 ⁻⁴	3	+
13	SCA	10 ⁻⁵	6	+
14	AIA	10 ⁻⁵	4	+
15	ISP2	10 ⁻⁵	2	+
16	SCA+SP	10 ⁻⁵	247	+
17	AIA+SP	10 ⁻⁵	4	+
18	ISP2+SP	10 ⁻⁵	Fungi	+

Table : 5.2 Orchard farm soil samples

S.No	Media	Dilution	Color	Texture
1	SCA	10^{-3}	White	Powdery
2	AIA	10^{-3}	White	Slimy
3	ISP2	10^{-3}	Yellow	Slimy
4	SCA SP	10^{-3}	Grey	Powdery
5	AIA SP	10^{-3}	White	Powdery
6	ISP2 SP	10^{-3}	Grey	Powdery
7	SCA	10^{-4}	White	Slimy
8	AIA	10^{-4}	Yellow	Slimy
9	ISP2	10^{-4}	Brown	Powdery
10	SCA SP	10^{-4}	Grey	Slimy
11	AIA SP	10^{-4}	Brown	Powdery
12	ISP2 SP	10^{-4}	Orange	Powdery
13	SCA	10^{-5}	White	Slimy
14	AIA	10^{-5}	Yellow	Slimy
15	ISP2	10^{-5}	Grey	Powdery
16	SCA SP	10^{-5}	Brown	Powdery
17	AIA SP	10^{-5}	White	Powdery
18	ISP2 SP	10^{-5}	Grey	Slimy

Table:5.3 Morphological Identification (Rice Samples)

S.No	Media	Dilution	Color	Texture
1	SCA	10 ⁻³	Yellow	Powdery
2	AIA	10 ⁻³	Brown	Powdery
3	ISP2	10 ⁻³	Grey	Powdery
4	SCA SP	10 ⁻³	Brown	Slimy
5	AIA SP	10 ⁻³	White	Powdery
6	ISP2 SP	10 ⁻³	Grey	Slimy
7	SCA	10 ⁻⁴	White	Powdery
8	AIA	10 ⁻⁴	Grey	Powdery
9	ISP2	10 ⁻⁴	Brown	Powdery
10	SCA SP	10 ⁻⁴	White	Slimy
11	AIA SP	10 ⁻⁴	Grey	Slimy
12	ISP2 SP	10 ⁻⁴	Brown	Powdery
13	SCA	10 ⁻⁵	Grey	Slimy

Table:5.4 Morphological Identification (Orchard Farm Samples)

5.3 SCREENING OF ACTINOBACTERIA FOR ANTIMYCOBACTERIAL ACTIVITY

Antimycobacterial activity of actinobacterial cultures were given in (Table:5.2 and Table 5.3). Of the 40 actinobacterial strains tested, O7 showed maximum zone of inhibition (19mm) against *M. smegmatis* tested in agar plug method (Figure:5.2). Agar plug methods are in use for many decades for the preliminary detection of antagonistic activity exhibited by the extracellular metabolites of actinobacteria (Balouiri *et al.*, 2016). The YEME agar media used for the recovery, growth and maintenance of actinobacteria support the bioactive metabolite production in agar plug method (Mohana *et al.*, 2014). Other cultures like O5, O8 and RI 12 also exhibited activity against *Mycobacterium smegmatis* with the zone of inhibition of O5 (12 mm), O8 (16mm), RI12 (14 mm)

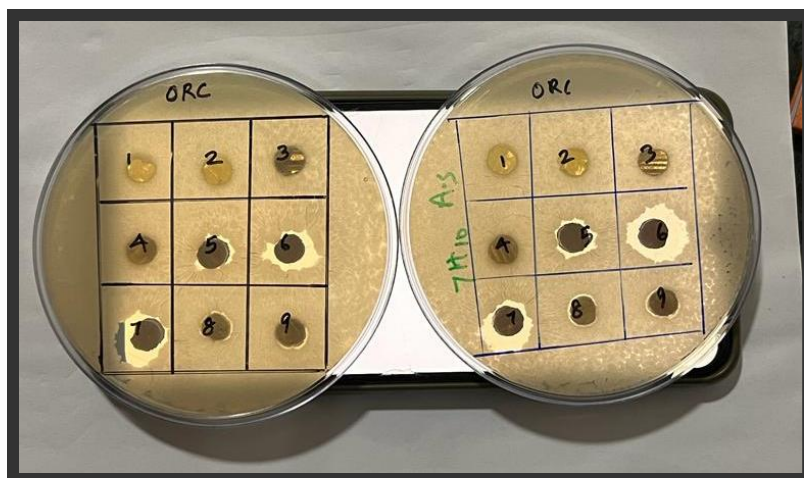


Figure:5.1 Agar Plug method for Antimycobacterial activity

5.4 PRODUCTION OF BIOACTIVE METABOLITES

Crude Extract of the potential cultures O5, O7, O8 and R12 were prepared by agar surface fermentation method to extract the secondary metabolite. (Figure 5.3)



Figure:5.2 Potential Extracts

5.5 PHYSIOLOGICAL CHARACTERISTICS:

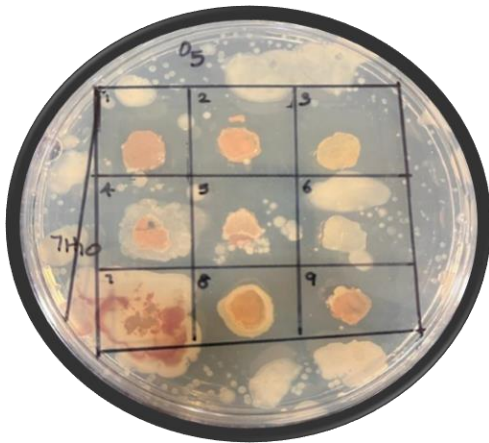


Figure:5.3

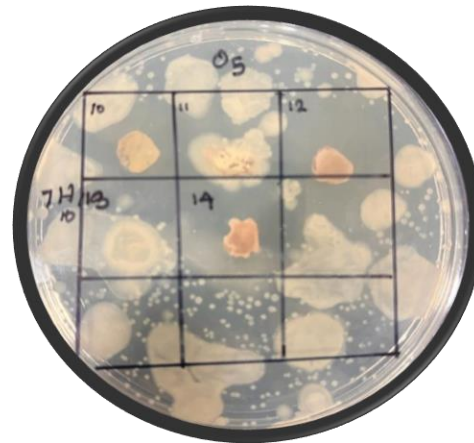


Figure:5.4

The anti-mycobacterial activity for O5 was observed in the culture that was grown with mannitol as its carbon source. In the figures 5.3 and 5.4 we can see, O 5 (2 and 9) showed anti-mycobacterial activity with the zone of inhibition of 16mm.

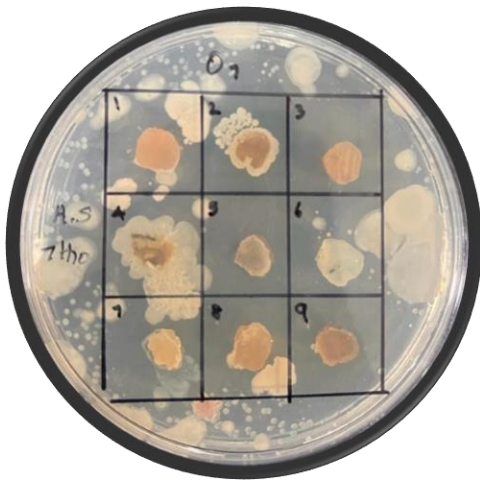


Figure:5.5

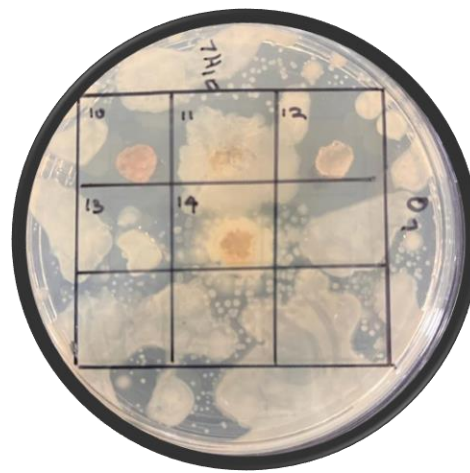


Figure:5.6

The anti-mycobacterial activity for O7 was observed in the culture that was grown with D-Glucose as its carbon source. In the figures 5.5 and 5.6, O 7(3 and 10) showed anti-mycobacterial activity with the zone of inhibition of 16mm and 14mm.

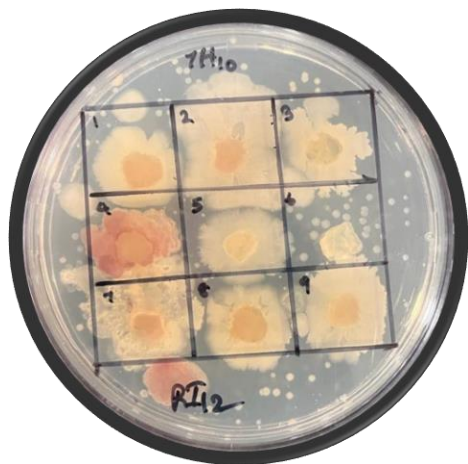


Figure:5.7

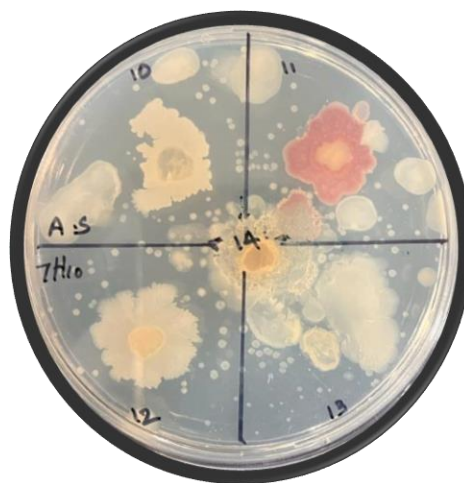


Figure:5.8

The anti-mycobacterial activity for RI12 was observed in the culture that was grown with KNO_3 as its nitrogen source. In the figures 5.7 and 5.8, RI 12 (6 and 13) anti-mycobacterial activity with the zone of inhibition of 15mm and 13mm.

5.6 TIME POINT ASSAY

All the potential cultures were able to grow well and utilize wide range of carbon sources, nitrogen sources.

The potential culture RI12 showed anti-mycobacterial activity against *Mycobacterium smegmatis*. The zone of inhibition was measured to be 19mm with a well size of 10mm.



Figure:5.9

5.7 MICROPLATE ALAMAR BLUE ASSAY-MABA

In vitro results for the *M. smegmatis* and *M. tuberculosis* H37Ra strains showed that O 7 and O 8 had 61.4% and 61.8% inhibition at 500 and 250 µg/mL towards *M. smegmatis* and O 7 had 50.6% of inhibition at 500 µg/mL towards H37Ra respectively.

$$\% \text{ of inhibition} = \frac{C-T}{C} * 100$$

Where, C – Control Value

T – Test Value

5.7.1 Microplate alamar blue assay for *Mycobacterium smegmatis*

Sample name	<i>M. smegmatis</i>	<i>M. tuberculosis</i> H37Ra	% of inhibition		
			500 µg/mL	250 µg/mL	125 µg/mL
O 7	0.379	0.842	52.3(<i>M. smegmatis</i>) 50.6(H37RA)	61.4	58.7
O 8	0.365	-	55.5	59.6	61.8

Table:5.5 Optical density Reading under Uv spectrophotometry at 600 nm

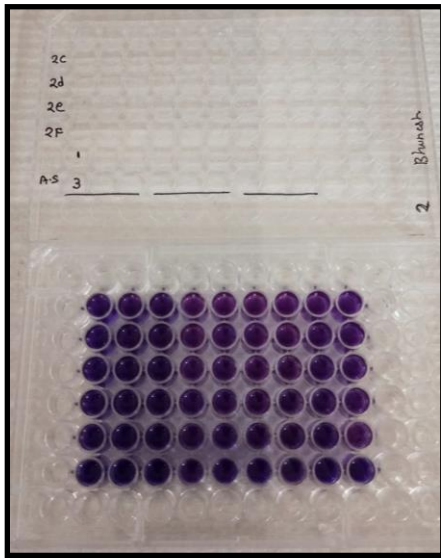


Fig:5.10 Dye addition for O 7, O 8

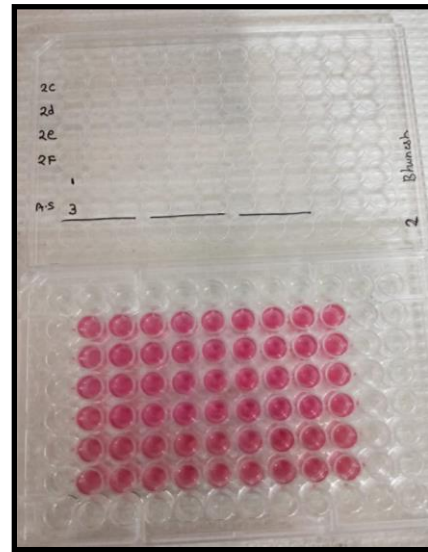


Fig:5.11 Dye incubation for O 7, O 8

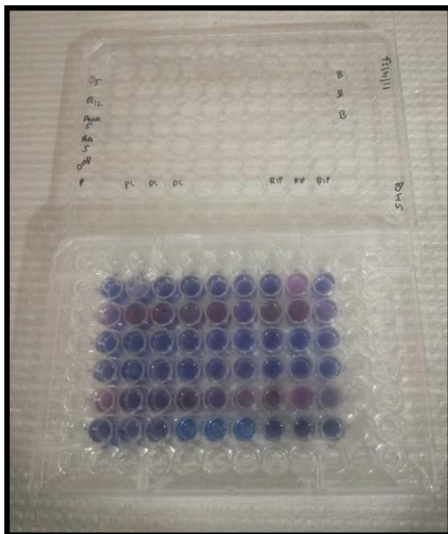


Fig:5.12 Dye addition for O 5, RI 12



Fig:5.13 Dye incubation for O 5, RI 12

5.7.2 Microplate Alamar Blue Assay for *Mycobacterium tuberculosis* H37Ra

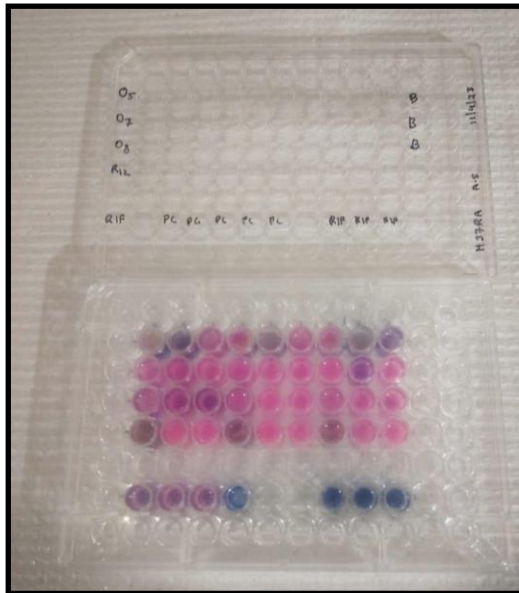


Fig:5.14 Dye addition *MTB H37RA*

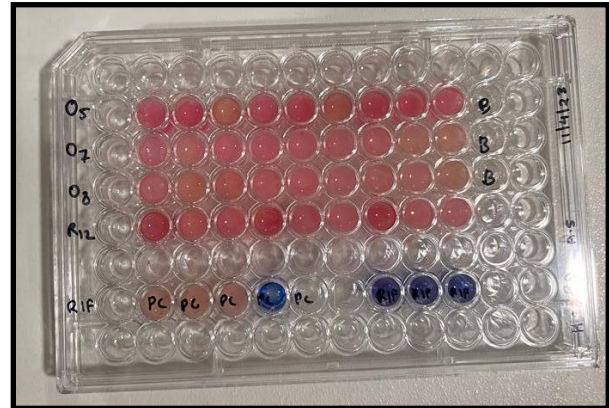


Fig:5.15 Dye incubation *MTB H37RA*

5.8 THIN LAYER CHROMATOGRAPHY

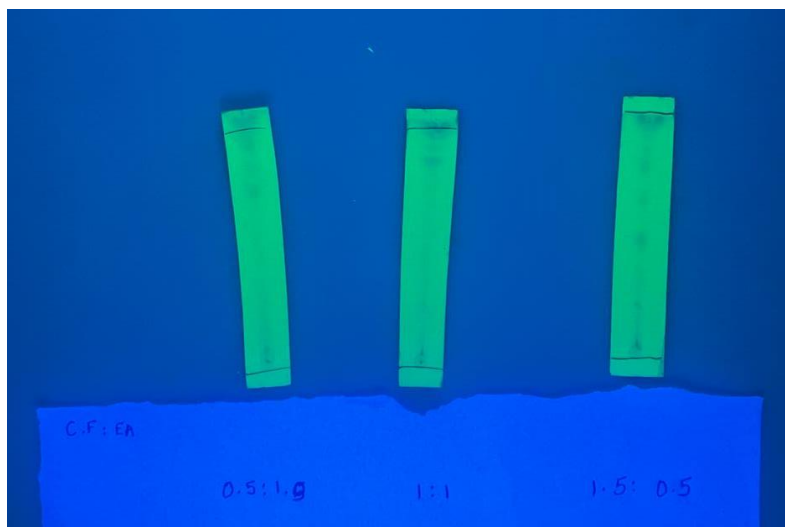


Fig:5.16 TLC

By performing TLC (Thin Layer Chromatography), we discovered that the potential culture O7 extract split into four bands in solvents containing chloroform and ethyl acetate in the ratios of 1:1 and 1.5:0.5.

CHAPTER – 6

6. CONCLUSION AND SUMMARY

Every year million people die from tuberculosis (TB), one of the oldest diseases known to exist in humans, it continues to be a leading cause of death. Drug resistance in TB is starting to pose a serious threat to the treatment of the illness. Therefore, there is a need for new antituberculosis medications in order to speed up the treatment of the disease, enhance the treatment of drug-resistant tuberculosis, and offer more effective and efficient care for latent tuberculosis infection. In the current work, we evaluated the soil actinobacteria that were isolated from soil samples from Kashmir for their antimycobacterial properties.

From the soil sample, 40 different actinobacterial cultures were recovered. Agar plug assays against *Mycobacterium smegmatis* were used to assess the antimycobacterial activity of all actinobacterial cultures. Four of the cultures among them shown activity against *M. smegmatis*. These potential cultures were chosen for additional research. Using the agar surface approach, crude extracts of the prospective cultures were created. To test the effectiveness of crude extracts against *M. smegmatis* and *M. tuberculosis* (H37RA), the Microplate Alamar Blue Assay (MABA) was used. Extracts with an inhibition of more than 50% were considered promising. Two cultures O 7 and O 8 demonstrated respective inhibition levels of 60.4% and 60.8%. One culture O 7 showed anti-tuberculosic activity against MTB H37RA with the percentage of inhibition of 50.6%. Studies on the characterization and taxonomy of possible cultures revealed that the probable actinobacterial culture belonged to the genus *Streptomyces*. To confirm the species, whole genome sequencing will be done.

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