

**EXPLORING ACTINOBACTERIA ISOLATED FROM HIMALAYAN
REGION FOR NEW ANTIMYCOBACTERIAL COMPOUNDS
PROJECT WORK**

Submitted in partial fulfillment of the requirements for the award of
Bachelor of science degree in microbiology

By

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

SATHYABAMA

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

CATEGORY-1 UNIVERSITY BY UGC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **KEERTHANA.R (41300018)** who carried out the project "**EXPLORING ACTINOBACTERIA ISOLATED FROM HIMALAYAN REGION FOR NEW ANTIMYCOBACTERIAL COMPOUNDS**" under our supervision from October 2023 to April 2024.



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I, **KEERTHANA.R (41300018)** hereby declare that the Project Report entitled "**EXPLORING ACTINOBACTERIA ISOLATED FROM HIMALAYAN REGION FOR NEW ANTIMYCOBACTERIAL COMPOUNDS**" done by me under the guidance of **Dr.P.PRAKASH** (internal guide) and **Dr. M. RADHAKRISHNAN** (external guide) at centre for drug discovery and development, sathyabama research park, Sathyabama Institute of Science and Technology, Chennai is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in **Microbiology**.

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ABSTRACT

Mycobacterium tuberculosis (M.tb), also known as Koch's bacillus, is a species of pathogenic bacteria in the family Mycobacteriaceae and the causative agent of tuberculosis. First discovered in 1882 by Robert Koch. Humans are the only known reservoirs of *M. tuberculosis*. The route of transmission is through aerosols released by infected individual. Globally 1.8 billion people are infected and 80% of people lead to death due to TB. The burdensome challenge faced is the emergence of resistance in TB infection, this makes the management of the disease very difficult. Though certain drugs are available for the management but development of resistance makes the disease management a difficult process. Therefore it is very important to find a molecule which can help in the management of the same. Actinobacteria are one of the largest bacterial phyla in the domain bacteria. The results of culturable and unculturable studies revealed that the members of this phylum are widely distributed in wide range of living and nonliving sources in diverse ecosystems including the extreme one. In recent years, there are several novel actinobacterial genera, and species were reported from living and non-living sources of entire environment. We have been investigating bioactive potentiality of actinomycetes isolated from certain less explored Indian ecosystems against mycobacterial pathogens. Actinomycetes were isolated from the soil samples of himalayan region and screened against *M.smegmatis* by agar plug method and, the isolates which showed anti-TB activity were further selected for extraction. The anti-TB activity of the extracts will be screened against [H37RA] Strain through a liquid based assay - Microplate Alamar Blue Assay(MABA).

Keywords: *Mycobacterium tuberculosis*, Microplate Alamar Blue Assay, *M.smegmatis*, bioactive potentiality, H37RA Strain

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LIST OF ABBREVIATION

ACRONYM	EXPANSION
ISP2	International Streptomyces Project 2 media
AIA	Actinomycetes Isolation Agar
SCA	Starch Casein Agar
NA	Nutrient agar
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
TB	Tuberculosis
<i>M.TB</i>	<i>Mycobacterium tuberculosis</i>
EA	Ethyl acetate
CF	Chloroform
DCM	Di-chloro methane
<i>S. lavendule</i>	<i>streptomyces lavendule</i>
LJ medium	lowenstein jensen medium
RPM	Rotation per minute
RNA	Ribonucleic Acid
RF	Retention factor
DNA	Deoxyribonucleic acid

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CHAPTER 1

INTRODUCTION

1.1 TUBERCULOSIS

Tuberculosis (TB), resulting from infection with *Mycobacterium tuberculosis* (*M.tb*), stands as one of the primary causes of mortality among infectious diseases in humans, predominantly impacting the lungs. From a pathological standpoint, TB is identified by necrotizing granulomatous inflammation in affected organs. The transmission of *M.tb* infection within the population primarily happens through the inhalation of contaminated aerosols originating from individuals with active pulmonary disease. In humans, exposure to *MTb* can result in either primary active disease or asymptomatic latent infection (LTBI). TB can be broadly classified into three periods: the early period, current period, post-independence period.

1.1.1 Early period

During the early period of TB control, significant developments occurred including the establishment of sanatoriums, with India pioneering the concept with its first open-air facility in Tiluana, near Ajmer, Rajasthan, in 1906. Additionally, the first TB dispensary was established in Bombay in 1917, marking a crucial step in patient care. By 1925, chest radiology became instrumental in diagnosing TB, enabling the detection of deep-seated areas of consolidation. The BCG vaccine, developed by Albert Calmette and Camille Guérin in 1906, further advanced efforts against TB. India initiated a nationwide mass BCG campaign in 1951, marking a significant milestone in the country's fight against the disease.

1.1.2 Post-independence initial nationwide TB control programs

District TB centers (DTCs) have been established with the goal of integrating TB control initiatives into existing government health services, aiming to address the TB issue within communities in a cost-effective manner. Shortly after the establishment of the Anantapur DTC, it became clear that while case detection was feasible, the primary challenge in combating TB lay in ensuring patients remained on

continuous treatment until they were cured. Building upon the district TB center model, the Indian government launched the National TB Control Program (NTCP) in 1962. The availability of effective TB drugs such as Streptomycin (1944), PAS (1946), Thiacetazone (1950), Isoniazid (1952), and Rifampicin (1966) followed the period of India's independence.

1.1.3 Current period

The World Health Organization (WHO) declared tuberculosis (TB) a global emergency and introduced the DOTS strategy, urging all nations to embrace it. This strategy rested on five core principles: securing political commitment and sustained funding for TB control programs, diagnosing TB through sputum smear examinations, ensuring a consistent supply of high-quality anti-TB medications, supervising drug intake directly, and maintaining precise reporting and documentation of all registered cases.

1.2 MULTIDRUG-RESISTANT TUBERCULOSIS

The emergence of multi-drug-resistant tuberculosis (MDR-TB) signifies resistance to two key anti-TB medications: Isoniazid and Rifampicin, along with resistance to certain fluoroquinolones and second-line injectable TB drugs (amikacin, kanamycin, or capreomycin). These strains do not respond to the standard six-month treatment with first-line drugs and may require over two years of treatment with less effective, more toxic, and costly medications. Both MDR-TB and extensively drug-resistant TB (XDR-TB) pose significant challenges to the effectiveness of TB control programs.

1.3 PATHOGENESIS

The pathophysiology of pulmonary TB involves *Mycobacterium tuberculosis* (*M.tb*) entering the respiratory tract and lungs of the host, triggering an innate immune response and being engulfed by key immune cells like macrophages and dendritic cells. *M.tb* then replicates within these cells while more immune cells are recruited. Although the host can potentially eliminate *M.tb* at this stage, it often leads to the

formation of solid granulomas. These granulomas consist of foam cells from macrophages and necrotic immune cells, eventually forming caseous granulomas that rupture and release bacteria, leading to active TB development. The released Mtb bacteria become infectious aerosol droplets, initiating a new cycle of infection. Depending on the infection and the immune response, TB can be classified as primary tuberculosis, the initial infection, or secondary tuberculosis, resulting from the reactivation of latent infection. (Sandhu, G. K. 2011c)

Table 1.1: Current global clinical pipeline of new tuberculosis drugs based on information provided by the Working Group for New TB Drugs

PRE-CLINICAL	PHASE 1	PHASE 2	PHASE 3
OTB-658	GSK-286	WX-081	Bedaquiline
FNDR 20364	TBAJ587	OPC-167832	Delamanid
Sanfitrenum	TBI-223	Suetzolid	Refapentine
TB47	BVL-GSK098	BTZ-043	Pretomanid
GSK839	PBTZ-169	Rifampin	Clofazimine
MBX-4888A	TBAJ-876	Delpazolid	Rifampicin
	TBI-166	GSK 3036656	

1.4 ACTINOMYCETES

Approximately 60% of Earth's living matter comprises micro-organisms, with an estimated two to three billion species coexisting on the planet, profoundly impacting all living organisms. The microbial realm, a valuable resource, holds the potential to contribute significantly to sustainable development if managed wisely. These organisms play a crucial role in maintaining ecological equilibrium worldwide (Kuhad, 2012). Although microorganisms serve as vectors for contagious diseases, they also play numerous beneficial roles, spanning from involvement in biogeochemical cycles to the production of various items such as food, beverages, and pharmaceuticals. Microorganisms are acknowledged as abundant sources of biologically active compounds, which are extensively utilized in fields such as medicine, agriculture, and

industry (Monciardini *et al.*, 2014). Due to their distinctive and versatile characteristics, microorganisms are viewed as promising contenders for addressing intricate challenges in the realms of science and technology. Over the past eight decades, they have been harnessed to propel advancements in various fields including medical and food technology, genetic engineering, environmental preservation, agricultural biotechnology, as well as in the treatment of agricultural and municipal waste (Higa and Parr, 1994). The primary categories of microorganisms comprise bacteria, fungi, and a particular bacterial group known as actinomycetes. Within the microbial realm, actinomycetes stand out as the most economically and biologically important bacteria due to their ability to generate a wide array of bioactive compounds, including antibiotics, antitumor agents, immunosuppressants, inhibitors, enzymes, and substances that promote plant growth. Undoubtedly, actinomycetes represent a significant opportunity for the development of further medically, agriculturally, and industrially valuable compounds (Chaudhary *et al.*, 2013). Actinomycetes, widely dispersed microorganisms, secrete a diverse array of natural compounds in their environment, aiding in pathogen defense and contributing to humus formation and decomposition. They are particularly intriguing due to the bioactive substances they produce, which have proven significant in both biotechnological and pharmaceutical realms, expanding our understanding of microbial diversity. These aerobic Gram-positive bacteria typically form branching, non-fragmenting hyphae and asexual spores. Representing one of the dominant bacterial phyla, actinomycetes are classified into five classes, nineteen orders, and fifty families according to the Bergey's Manual of Systematic Bacteriology (Goodfellow *et al.*, 2012).

1.5 OCCURRENCE AND DISTRIBUTION OF ACTINOMYCETES IN NATURE

Actinomycetes are found everywhere, in abundance, ranging from polar regions to the equator, inhabiting nearly every habitat. Most actinomycetes function as saprophytes, while others act as parasites or endophytes. Due to their unique physiology and nutritional requirements, they can thrive in diverse environmental conditions. This versatility allows them to break down various organic substances,

making them widespread throughout nature. Actinomycetes are present in a multitude of natural and human-made environments, including both aquatic and terrestrial ecosystems (Kurtboke, 2017). Actinomycetes have been discovered in a wide array of habitats, including marine and freshwater sediments, springs, glacial regions, caves, mangrove forests, mining sites, compost pits, and soil. In recent years, researchers have successfully isolated numerous actinomycetes from various ecological settings. Studies conducted in familiar environments often yield known actinomycete species upon isolation. Conversely, exploring previously uncharted or less-studied ecosystems such as marine environments, deserts, forests, caves, hills, and plantations has proven fruitful in discovering new and unique actinomycetes. Consequently, actinomycetes have been extensively investigated across diverse ecological landscapes and in previously untapped regions worldwide (Manikkam *et al.*, 2014).

1.5.1 Aquatic ecosystems

Aquatic ecosystems dominate the planet, with marine habitats covering approximately two-thirds of the Earth's surface. Studies conducted in these environments have revealed the existence of varied populations of actinomycetes. These microorganisms are frequently encountered in various marine ecosystems worldwide, including seawater, sandy shores, deep-sea regions, mangroves, coastal sediments, and oceanic formations (Kamjam *et al.*, 2017). Marine ecosystems are home to distinct genera like *Salinospora*, *Salinibacterium*, *Marinispora*, *Solwaraspora*, and *Serinicoccus*. Similarly, numerous studies highlight the variety of actinobacteria present in freshwater environments globally (Zothanpuia *et al.*, 2018). Actinomycetes are commonly found in various aquatic habitats like rivers, streams, and lakes. Studies indicate that only a small portion of actinomycetes, particularly Streptomyces, are present in the water itself, whereas greater populations thrive within sediments, with *Micromonospora* being particularly abundant (Shah *et al.*, 2017).

1.5.2 Mangrove ecosystem

Actinomycetes are prevalent in marine environments, including mangrove ecosystems, showcasing diversity across various geographic locations characterized by differences in pH, temperature, salinity, moisture, and nutrient levels. Consequently, mangroves host a wide array of unique actinomycete genera. Thus far, studies have identified eight suborders, 11 families, and 24 genera, including the discovery of three new genera, within the order Actinomycetales from mangrove sediments (Xu *et al.*, 2014).

1.5.3 Hypogean ecosystem

Hypogean ecosystems like caves and mines offer inorganic energy sources due to their abundance of inorganic minerals, rather than organic materials (Tomczyk-Zak and Zielenkiewicz, 2015) examined the diversity of actinomycetes thriving in cave habitats, identifying species such as *Acidimicrobium*, *Pseudonocardia*, *Propionibacterium*, *Corynebacterium*, *Curtobacterium*, *Arthrobacter*, *Rhodococcus*, *Nocardia*, *Micrococcus*, *Blastococcus*, *Streptomyces*, *Kribbella*, *Gordonia*, and *Frankia*. Similarly, mining environments host a broad spectrum of actinomycetes, including *Catellatospora koreensis*, *Pseudonocardia kongjuensis*, *Kribbella aluminosa*, *Acidimicrobium ferrooxidans*, *Aciditerrimonas ferrireducens*, *Ferrimicrobium acidiphilum*, *Acidithrix ferrooxidans*, *Ferrithrix thermotolerans*, *Saccharothrix*, and *Nocardiopsis metallica* (Hurtado *et al.*, 2018).

1.5.4 Terrestrial ecosystem

Actinomycetes are Gram-positive bacteria that thrive aerobically, possessing DNA with a high guanine and cytosine content (>60%). They are widely spread in various environments, predominantly in soil. These bacteria inhabit diverse ecological niches, including acidic, alkaline, and extreme temperature conditions (such as thermophilic, mesophilic, and psychrophilic), as well as saline, low moisture, and polluted environments like soils contaminated with various substances such as heavy metals, minerals, oil, sewage, sludge, and pesticides. They can also be found in both

rhizosphere and non-rhizosphere soils, spanning forests, hills, mountains, and plantations. The evidence supporting their presence in these habitats is extensive (Mohammadipanah and Winck, 2016).

1.5.5 Desert ecosystem

Actinomycetes exhibit limited resilience to dehydration and high solute concentrations, yet they have been discovered in a wide array of extreme environments, including arid and hyper-arid deserts. Despite the sparse vegetation and minimal rainfall in these regions, actinomycetes exhibit considerable diversity. Various genera of actinomycetes can be found in desert habitats, encompassing *Streptomyces*, *Micromonospora*, *Saccharothrix*, *Streptosporangium*, *Cellulomonas*, *Amycolatopsis*, *Geodermatophilus*, *Lechevalieria*, *Nocardia*, and *Actinomadura* (Mohammadipanah and Wink, 2016).

1.6 HABIT OF ACTINOMYCETES

Actinomycetes are uncomplicated prokaryotic organisms, existing as unicellular, aerobic, Gram-positive bacteria characterized by a high proportion of guanine and cytosine nucleobases. They are differentiated from other bacteria by their filamentous hyphae. Most actinomycetes are immotile, though if motile, their movement is typically limited to flagellating spores. These organisms possess elongated multinucleate filaments that usually remain unfragmented, although in certain instances, they may break into smaller units known as arthrospores, measuring between 0.5 to 1.0 μm in diameter, occasionally reaching up to 2.0 μm . Actinomycetes exhibit diverse types of cell walls, with significant variations in cell wall composition among different groups, particularly regarding the sugars present in cell extracts (Wiley *et al.*, 2017). The hyphal network branches to create both substrate mycelium and aerial mycelium. Colonies can exhibit varying textures, appearing matte or glossy, with surfaces described as butyrous, friable, or mucoid. Colony morphology ranges from flat to low convex or domed with raised centers. Colony edges may be smooth (circular or broken), scalloped (crenate), or fringed (lobed or fimbriate). Aerial mycelium can display a spectrum of colors including white, yellow,

orange, brown, violet, red, blue, green, or gray, often producing pigments that diffuse into the surrounding medium. Within the aerial mycelium, chains of asexual, thin-walled spores called conidia or conidiophores are present. In some instances, spores are contained within a sporangium, referred to as sporangiospores. These chains of spores may exhibit straight, wavy (flexuous), or coiled configurations, with conidia-bearing filaments often showing spiral twists. Each conidium contains a nucleus and is enclosed by a tough outer wall that can be smooth, roughened, spiny, or hairy (Vobis, 1997).

1.7 CHARACTERIZATION OF ACTINOMYCETES

Actinomycetes are primarily distinguished based on their morphological features. Observing the colonies of actinomycetes growing on agar can offer valuable and quick hints for identifying their respective genera visually, following guidelines outlined in Bergey's Manual of Systematic Bacteriology (Goodfellow *et al.*, 2012) and International Streptomyces Project (Shirling and Gottlieb, 1976).

This involves examining colony traits like dimensions, form, surface texture, color uniformity across various growth media, and the presence or absence of aerial mycelia. Following this, pigment analysis entails investigating hues, dispersion properties of pigments, and the reverse side of colonies (Shirling and Gottlieb, 1966). The color and pigmentation of substrate mycelia are assessed using a standardized color reference key developed by (Tresner and Backus in 1963). The microscopic attributes of colonies utilizing Gram's staining method, as introduced by Gram in 1884, are examined through coverslip culture and slide culture techniques, as outlined by (Kawato and Shinobu in 1959). These characteristics are investigated for aerial mycelium (whether present or absent), substrate mycelium (fragmentation and sporulation), and sporangial attributes, as well as spore chain morphology (rectiflexibiles, retinaculiaperti, or spirales). Following established procedures, investigations into biochemical characteristics such as H₂S production, gelatin liquefaction, nitrate and hydrogen peroxide reduction (Gottlieb, 1960), along with tyrosine utilization (Lerch and Ettlinger, 1972), contribute to the identification process of actinomycetes. Additionally, physiological characteristics such as pH tolerance,

temperature requirements, salt tolerance, and the ability to utilize specific sugars (Arabinose, Glucose, Fructose, Galactose, Lactose, Maltose, Mannitol, Sucrose, Xylose) and amino acids (Cysteine, Arginine, Histidine, Tyrosine, Proline, Methionine, Asparagine, Tryptophan, Valine) for growth contribute to the identification of actinomycetes. While initial identification of isolates can be accomplished based on their morphological, biochemical, and physiological traits, relying solely on these traditional methods may not lead to a dependable classification of actinomycetes. Hence, rapid molecular techniques such as restriction fragment length polymorphism (RFLP), pulse field gel electrophoresis (PFGE), amplified ribosomal DNA restriction analysis (ARDRA), random amplification of polymorphic DNA (RAPD), DNA-DNA hybridization, and genus-specific primers are invaluable for distinguishing between rare and common genera of actinomycetes. A highly efficient molecular approach for taxonomic and phylogenetic analysis has emerged, utilizing 16S rRNA gene sequencing to identify strains up to the genus level (Cook and Meyers, 2003). Currently, while 16S rRNA gene sequencing typically enables identification up to the genus level, it is less effective for species-level identification in most instances. The contemporary systematic approach for actinomycetes, particularly through phylogenetic analysis using complete 16S rRNA gene sequences, represents a highly significant and potent tool for identification purposes. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis has also been employed to explore actinomycete diversity by examining their DNA fingerprints. Actinomycetes are a physiologically active group known for their secondary metabolites, which have been extensively researched and applied across various fields. While it's true that some actinomycetes can be pathogenic to humans, animals, or plants—examples include *Streptomyces somaliensis* causing *actinomycetoma*, *Actinomyces bovis* leading to lumpy-jaw disease in cattle, and *Streptomyces scabies* causing common scab in potatoes and sugar beets. The secondary metabolites derived from actinomycetes have diverse applications, serving as potential sources for novel compounds possessing properties such as antiviral, antibacterial, antiparasitic, antifungal, antitumor, immunosuppressant, biocontrol, agroactive, growth regulatory, enzymatic, enzyme inhibitory, and others (Solecka *et al.*, 2012).

To date, soil actinomycetes continue to be the primary reservoir for isolating bioactive metabolites, including antibiotics, enzymes, and compounds that either promote or inhibit plant growth(Dewi *et al.*, 2015) revealed that approximately 45% of all bioactive secondary metabolites derived from microorganisms originate from actinomycetes, with *Streptomyces spp.* alone responsible for around 75% of these compounds.

1.8 SIGNIFICANCE OF ACTINOMYCETES

Actinomycetes are a highly active group known for their secondary metabolites, which have been extensively studied and applied across numerous research fields. While a few actinomycetes can pose pathogenic risks to humans, animals, or plants—for instance, *Streptomyces somaliensis* causing actinomycetoma, *Actinomyces bovis* leading to lumpy-jaw disease in cattle, and *Streptomyces scabies* causing common scab in potatoes and sugar beets—the secondary metabolites originating from actinomycetes have broad applications. These compounds serve as promising sources for novel substances with diverse properties, including antiviral, antibacterial, antiparasitic, antifungal, antitumor, immunosuppressive, biocontrol, agroactive, growth regulatory, enzymatic, enzyme inhibitory, and other functions (Solecka *et al.*, 2012). Currently, soil actinomycetes continue to be the prime reservoir for extracting bioactive metabolites possessing antibiotic, enzymatic, and properties that either promote or inhibit plant growth, approximately 45% of all bioactive secondary metabolites sourced from microorganisms are derived from actinomycetes, with *Streptomyces spp.* accounting for roughly 75% of these compounds (Dewi *et al.*, 2015). Actinomycetes are recognized for their production of unique secondary metabolites that exhibit various bioactive properties. They have been extensively utilized for their antimicrobial attributes, including antibiotics, antibacterial agents, antifungals, antivirals, and antiparasitics, as well as for their anti-tumor, anticancer, anti-infective, immunosuppressive, and other bioactive compounds. According to the ABL Antibiotic Literature database, out of 34,000 natural compounds known for their antimicrobial, anticancer, and immunological activities, approximately 57% are sourced from actinomycetes (Velho-Pereira and Kamat, 2011; Janardhan *et al.*,

2014). Recently, the output of bioactive compounds from actinomycetes has declined due to the emergence of resistant pathogens, increased instances of multiple drug resistance, and the rediscovery of known compounds from the same species in familiar environments. As per the World Health Organization, the pace of emergence of drug-resistant strains surpasses the rate of new drug and antibiotic discovery. Actinomycetes have regained attention by isolating them from comparatively underexplored areas (Narasaiah *et al.*, 2014). The actinomycetes have converted their bioactive compounds into pharmaceutical drugs.

1.9 THE HIMALAYAN ECOSYSTEMS

The Himalayan ecosystems are one of distinct less explored habitat involving a mixture of trees, shrubs and crops. Among which, soil actinomycetes are preferred for their novel bioactive potentials. There are limited investigations concerning actinomycete diversity from rare ecosystem like Himalayas. Yet, most of the studies on actinomycetes in India have been explored for the isolation antimicrobial activities against different pathogens still we are unable to cope with the emerging problem of multidrug resistance in tuberculosis disease. In view of the above significance of actinomycetes secondary metabolites and as well as limited reports of actinomycete isolations from the less explored Himalayan region, the present investigation was undertaken to isolate and characterize actinomycetes from Himalayan soil sample and screen the strains for antimycobacterial activities.

CHAPTER 2

LITERATURE SURVEY

2.1 TUBERCULOSIS

TB, historically known as consumption, gained scientific clarity when Robert Koch isolated its causative agent, leading to his *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), has afflicted humanity for centuries, prompting extensive research dating back over a hundred years. Despite this, TB remains a pressing global health challenge, particularly impacting marginalized populations, with links to poverty, malnutrition, and the prevalence of diseases like HIV/AIDS . Currently, TB accounts for an estimated annual incidence of 10 million cases and 1.3 million deaths worldwide. Coevolving with humans, *M. tuberculosis* has developed strategies for immune evasion, underscoring the urgent need for improved formulation of Koch's postulates and earning him the Nobel Prize in Medicine in 1905. It is now recognized that *M. tuberculosis* is part of the broader *M. tuberculosis* complex, including pathogenic species like *Mycobacterium bovis*, *Mycobacterium africanum*, and others, some of which can also infect humans(Singh, R., Kumar, P., and Tahlan, K. (2020).Tuberculosis (TB) has been a persistent affliction throughout human history, dating back over 17,000 years. Despite advancements in diagnosis and treatment, it remains a significant global health concern, ranking among the top infectious disease killers, second only to HIV. According to the World Health Organization (WHO), TB constitutes a worldwide pandemic and is a leading cause of death, especially among those with HIV. In India, the battle against TB can be divided into three distinct periods: an early era predating the discoveries of x-ray and chemotherapy, a post-independence phase marked by the introduction of nationwide TB control programs, and the current era, characterized by the WHO-supported TB control program. India's DOTS (directly observed treatment-short course) program is noteworthy for its rapid expansion and scale, being the largest in terms of patients initiated on treatment and second largest in population coverage globally. However,

significant challenges persist in controlling TB in India, including inadequate primary healthcare infrastructure in rural regions, unregulated private healthcare resulting in indiscriminate use of anti-TB drugs, the spread of HIV, lack of political commitment, and systemic corruption.

The emergence of multidrug-resistant TB (MDR-TB) poses an additional threat to TB eradication, often stemming from deficiencies in TB control programs. The WHO's "STOP TB" strategy aims to eliminate TB as a public health threat by 2050, providing a vision for global efforts to combat this ancient disease(Sandhu, G. K. 2011). In the contemporary era, Tuberculosis (TB) remains a significant public health challenge, particularly due to issues such as drug resistance, co-infection with diseases like AIDS and COVID-19, and the demanding and expensive treatment regimens. This review offers an overview of TB infection's pathogenesis, potential therapeutic targets, and associated regulators, covering existing frontline drugs, ongoing clinical trials, and compounds undergoing preclinical evaluation. A deeper understanding of *Mycobacterium tuberculosis* (*M.tb*) infection mechanisms and crucial biological targets holds promise for innovative treatment strategies. While many anti-TB medications focus on pathogen-related processes, there is growing interest in host-directed therapy (HDT), which addresses immune responses, survival mechanisms, and immunopathology. *M.tb*'s ability to adapt to the human host involves manipulation of host cellular mechanisms, and HDT seeks to disrupt this manipulation to improve treatment outcomes. This review offers valuable insights to guide future endeavors in the development of anti-TB drugs(Yang, J *et al.*, 2023). Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*, primarily affecting the lungs and transmitted through bacteria-containing air . The World Health Organization (WHO) reported 417,000–556,000 new Rifampicin Resistance (RR-TB) cases in 2018, with 78% progressing to Multi-Drug Resistance (MDR-TB). Extensively Drug-Resistant (XDR-TB) cases have been reported in 128 countries, necessitating new antimycobacterial drugs due to limited progress since the 1960s, despite existing treatment regimens(Gopalaswamy, R *et al.*, 2020).

Efforts to find new antimycobacterial drugs include exploring natural and synthetic compounds, with natural compounds contributing significantly to drug sales and experiencing a resurgence in discovery aided by bioinformatics and metagenomics. Actinobacteria, known for producing secondary metabolites, have yielded promising antimycobacterial compounds, such as cyclic peptides and those from marine sources. Indonesia's rich biodiversity, including diverse Actinobacteria, presents a promising avenue for antimycobacterial discovery, yet exploration remains limited. This study investigated Actinobacteria from *Curcuma zedoaria* endophytes and Pramuka Island's mangrove system in Indonesia, aiming to identify novel antibacterial compounds. Among the isolates, InaCC A758 showed potential against *M. tuberculosis* strain H37RV. Taxonomic analysis, along with gene cluster analysis for secondary metabolite production, was conducted on InaCC A758. Metabolite profiling via Gas Chromatography-Mass Spectroscopy (GC-MS) and High-Resolution Mass Spectroscopy (HR-MS) revealed the diversity of secondary metabolites produced and their novelty (14, 15). Bioassay-guided isolation and structural elucidation using UV-Vis, Fourier-transform infrared (FT-IR), and Nuclear Magnetic Resonance (NMR) spectroscopy were performed to identify active antimycobacterial compounds from InaCC A758. (Rakhmawatie, M. D., et al 2021).

2.2 ACTINOMYCETES

Actinomycetes, a diverse group of microorganisms, constitute their own phylum and are widely distributed across various ecosystems worldwide. They can be found in a multitude of environments, including but not limited to soil types such as desert sands, thermal regions, arid landscapes, snow-covered soils in glaciers, mining sites like coal or ore deposits, caves and rocky crevices, semi-aquatic areas like mangroves and swamps, mountainous regions, grasslands, wetlands such as bogs and moors, and forested or plantation areas like teak, rubber, mulberry, palm, banana, tea, cocoa, and coffee plantations. The composition and abundance of actinomycetes in a specific habitat are significantly influenced by various environmental factors such as temperature, pH levels, moisture content, aeration, organic matter content, nutrient availability (both macro and micro), as well as soil

cultivation practices, depth, slope, and other soil-related factors (Ghorbani-Nasarabadi, 2013 and Arya and Singh, 2016).

2.2.1 Occurrence and distribution of actinomycetes

Actinomycetes are extensively found throughout nature and are particularly prevalent in soil environments. Despite several decades of soil exploration, a relatively small fraction of the earth's surface has been thoroughly examined, resulting in the discovery of only a limited number of actinomycetes taxa (Velho-Pereira and Kamat, 2013). The exploration of the majority of the world's biodiversity remains necessary to uncover unidentified actinomycetes existing in nature. Actinomycetes have been utilized commercially in pharmaceuticals, agriculture, and nanotechnology sectors due to their beneficial properties. Despite their economic importance, our understanding of their distribution and ecological roles in natural settings remains limited. conducted research on the distribution and functions of actinomycetes in soil, providing insights into the characteristics of these soil-dwelling microorganisms (Moncheva *et al.*, 2003). Geographic location and soil characteristics are key factors influencing the distribution variability of actinomycetes. These factors include temperature, pH levels, organic matter content, moisture levels, soil depth and type, as well as aeration and cultivation practices (Arya and Singh, 2016). The study focused on examining how actinomycetes are distributed at different depths in relation to various soil physicochemical properties (Krishna *et al.*, 2012). The study observed a decline in the population of actinomycetes as the depth of the soil increased. Additionally, the research explored the biodiversity of actinobacteria within the paddy field ecosystem and conducted correlation coefficient analyses to examine the relationship between actinobacterial diversity and soil physicochemical properties. These analyses revealed a positive correlation with nitrogen, phosphorus, copper, and potassium (Priyadarshini and Dhanasekaran, 2015). In India, as there is a geographic variation in soil type and their contents, hence, the distribution of actinomycetes varies in these regions including desert, mountains, hills, forests, grasslands and plantation soils (Table 2.1).

Table 2.1: isolation of actinomycetes from various soil samples in india

STATES	REGIONS	ACTINOMYCETES	REFERENCES
Andhra pradesh	*Soil samples *Mangrove forest of coringa * Rhizospheric soil * Northern black soil of katheru ,Rajahmundry * Laterite soil samples from kandukuru parkasam * Rhizospheric soil,Acharya Nagarjuna university , guntur * Yarada hills, Vishakpatnam	<i>Streptomyces sp.</i> <i>S.viridis,s.albus</i>	Vijayalakshmi and bindhu ,2011 Deepti <i>et al.</i>,2012 Kumary and raju ,2012 Lanka <i>et al.</i>, 2013 Rao <i>et al.</i> , 2016 Kumari <i>et al.</i>, 2017
Assam	Kaziranga National Park	<i>Micromonospora spp.</i>	Talukdar <i>et al.</i> , 2012
Bihar	*Rhizoplane of herbaceous plants,muzaffarpur * soil samples ,patna	<i>Streptomyces sp.</i>	Salahuddin <i>et al.</i>, 2011 Kumari <i>et al.</i>, 2013
Chatisgarh	*Social forest area ,bhilai township * Forest area, bastar region	<i>Actinomycetes</i> <i>isolates</i>	Kulkarni and siddique, 2012 Singh and roymon,2018
Goa	*Soil	<i>Streptomyces</i> , <i>micromonospora</i> , <i>actinomadura</i>	Pereira and kamat,2011
Gujarat	*Terrestrial and coastal regions * Soil near medicinal plants in saurashtra region	<i>Actinomycetes</i> <i>isolates</i>	Ranjan <i>et al.</i> , 2014
Himachal pradesh	*Manali ice point *Soil samples from dalhousie rocks	<i>Streptomyces sp.</i>, <i>saccharothrix</i> <i>xinjiangensi</i>	Raja and prabakaran,2011 Jani <i>et al.</i>,2014

Jammu and kashmir	*Northwestern himalayas * High altitude ,kargil *Dal lake		Salaria <i>et al.</i> , 2016
Jharkhand	*Soil samples ,Ranchi dist	<i>Streptomyces spp.</i>	Shukla <i>et al.</i> , 2008
Kerala	*Mountain plantation sites *Kuruva island ,pookot lake , wayanad *Manjium plantation ,banks of checkkk dam and playground- mahatma gandhi university,kottayam *Tropical montane foresy ,eravikulam national park *Chambal territory	<i>Streptomyces spp.</i> , <i>nocardia</i> , <i>micromonospora</i> , <i>pseudonocardia</i> <i>,streptosporangium</i> , <i>nocardiosis</i> , <i>saccharomonospora</i> , <i>rhodococcus</i> , <i>actinosynema</i> , <i>nocardiodes,kitasatos</i> <i>poria, gordonia</i> <i>,intrasporangium,</i> <i>streptoalloteichus</i>	George <i>et al.</i> ,2012 Hamedani <i>et al</i> .,2012 Krishna <i>et al.</i> , 2012 Varghese <i>et al.</i> ,2012
Madhya pradesh	*Sheopur district *Garden and cultivated field soil *Playground ,rhizosphere of medicinal plants , agricultural field ,gwalior	<i>S.phaeochromogenes</i>	Chaudhary <i>et al.</i> , 2013 Saxena <i>et al.</i> ,2013 Jaden <i>et al.</i> , 2012
Maharashtra	*Soil samples from villages *Soil sediments ,mira road , mumbai *garden soil ,nagpur *Forest ,garden , pomegrante orchards	Actinomycete isolates	Kamble and kulkarni ,2012 Dhanasekaran,2013 Dharmik and gomashe ,2013 Chavan <i>et al.</i> ,2016
Manipur	*Soil samples from biotopes	Actinomycete isolates	Ningthoujam and thokcham ,2016
Odhisra	*Upland paddy of jeypore	<i>Streptomyces sp.</i>	Elamvazhunthi,2013

Punjab	*Rhizospheric and non rhizospheric soil *Rhizospheric soil *Agricultural soil, tangori		Kaur <i>et al</i> .,2013 Kamara and gangwar, 2015 Kaur <i>et al</i> ., 2016
Rajasthan	*Green forest cover,jaipur *Thar dessert soil,rajasthan	<i>S.althioticus</i>	Mathur <i>et al</i> .,2012 Radhakrishnan <i>et al</i> .,2013 Tiwari <i>et al</i> .,2015
Tamilnadu	*Rhizosphereic soil,Sennbathop forest,Srivilliputur range *Rhizospheric soil,Bharath university campus,chennai *Amrithi forest,vellore *Amrithi forest,vellore *Rathnagiri hills Siruvani forest *Soil samples(paddy field),perambalur *Siruvani forest *Agricultural soils,vengodu	<i>Streptomyces SPP</i> ; <i>S.lavendule</i> <i>S.olivochromogenes</i> <i>Micromonospora</i> , <i>Rhodococcus SP.</i>	Kumar <i>et al</i> .,2011 Thnagapandian <i>et al</i> .,2011 Gajendran <i>et al</i> .,2012 Nithya and ponmurugan.2012 Silambarasan <i>et al</i> .,2012 Velayudam and murugan.2012 Jeya <i>et al</i> .,2013 Raghunathan and padhmadas,2013

2.2.2 Isolation of Actinomycetes

Actinomycetes, found across various ecological habitats, are a diverse microbial group that plays a vital role in promoting sustainable ecological development within different ecosystems. Their adeptness in modulating soil organic matter synthesis and decomposition, coupled with their capacity to enhance soil fertility, underscores their ecological significance. While isolating actinomycetes from mixed fungal cultures poses minimal challenge due to their distinctive physiological traits, separating them from true bacteria presents greater difficulty. To overcome this obstacle, diverse techniques have been devised to isolate actinomycetes and distinguish them from other Gram-positive bacteria. Despite streptomycetes often being predominant and thus easily isolated through conventional methods, there's a pressing need for selective isolation techniques to recover rare and novel actinomycetes while avoiding the reisolation of known and common genera. Consequently, researchers are exploring enhanced procedures for isolating actinomycetes from various habitats, including formulating nutrient substrates that preferentially support actinomycete growth and incorporating antibiotics into media to selectively inhibit non-actinomycete bacteria (Hayakawa, 2008). In contemporary research, soil pre-treatment techniques have emerged as pivotal in selectively isolating uncommon or new actinomycetes. For example, one method involves activating actinomycete spores through treating soil samples with yeast extract (Khanna *et al.*, 2011). There are various steps followed for the selective isolation of rare and novel actinomycetes, (Seong *et al.* 2001) conducted research on various pre-treatment techniques and specialized media to isolate uncommon actinomycetes from forest soil. They found that hair hydrolysate agar medium was the most effective medium, while wet-heating and phenol treatment of soil samples were determined to be effective methods for recovering *Actinoplanes*, *Micromonospora*, *Microbispora*, and *Streptosporangium* (Zhang 2011). They introduced two new isolation media, namely Zhang starch soil extract agar (ZSSE) and complex humic acid vitamin agar (CHV), designed to capture a broad range of uncommon actinomycetes using soil

extract as a key component. These media exhibited nutritional characteristics akin to standard agar media such as Gause No. 1, humic acid vitamin agar, and glycerol asparagine agar. ZSSE medium was particularly notable for its ability to simultaneously enhance diversity and specificity, surpassing typical agar mediums and facilitating the easy isolation of rare actinomycetes. On the other hand, CHV agar demonstrated effectiveness in isolating *Micromonospora* species.

2.3 IDENTIFICATION OF ACTINOMYCETES

Actinomycetes are identified by their filamentous hyphae, characterized by branching and forming both substrate and aerial mycelium. Reproduction occurs through spores, which are either produced at the tip of the vegetative mycelium or as distinct sporulating structures on the aerial mycelium.

2.3.1 Morphological characterization

Actinomycetes are primarily identified by examining the morphological characteristics of their colonies, which encompass size, shape, thickness, texture, and the presence or absence of aerial mycelia. Consistency in color across various media has been utilized as a criterion for characterizing actinomycetes. (Shirling and Gottlieb 1966) detailed pigment studies involving the examination of shades, the diffusive nature of pigments, and the reverse side of colonies. These characteristics were deemed significant for the characterization of actinomycetes. (Ambrawati *et al.*, 2012) characterized actinomycetes isolated from the rhizosphere of nut sedge using a method based on color grouping. This approach included evaluating the color of the aerial spore mass, the pigmentation of substrate mycelium, and the color of diffusible pigments.

2.3.2 Microscopical characterization

The utilization of coverslip culture and slide culture techniques has become significant for investigating the microscopic attributes of colonies employing diverse staining methods. Microscopic features such as the arrangement of aerial mycelium, curvature, branching, fragmentation pattern, and sporangial characteristics were

examined as part of this process (Kawato and Shinobu, 1959). For the first time, actinomycetes were examined using scanning electron microscopy (SEM), which proved instrumental in studying spore chain morphology, spore surface ornamentation, and routine organism identification. Subsequently, numerous strains were observed, revealing the diverse forms within this group. Advances were made in specimen preparation and instrument operation, leading to improved resolution at higher magnifications. Sample preparation involved the use of chemical fixatives and dehydration procedures (Skujiņš *et al.*, 2002). Kumar *et al.* (2011) introduced a novel, simple, and rapid technique for SEM examination of *Streptomyces* species. The cultures were cultivated on stubs and then coated with a gold film, bypassing the need for extensive fixing and dehydrating procedures. (Prakash and Nawani 2014) devised an enhanced preparation method for actinomycetes cultures for SEM, wherein cultures were grown on glass wool and subsequently dehydrated through lyophilization, eliminating the requirement for chemical fixatives and dehydrating agents.

2.3.3 Physiological and biochemical characterization

Recently, researchers have been engaged in the biochemical characterization of actinomycetes, focusing on their physiological and biochemical traits related to carbon and nitrogen utilization, as well as the hydrolysis of various sugars, proteins, amino acids, and organic compounds. Actinomycetes isolated from the tropical montane forest in Kerala exhibited the capability to utilize a wide range of carbon sources, with xylose, fructose, and mannose being the most preferred, while lactose was the least preferred. They also demonstrated the ability to decompose casein, tyrosine, and hypoxanthine, and showed potential for decomposing esculin, urea, and hippurate (Varghese *et al.*, 2012). (Quadri and Agsar, 2012) investigated how different carbon and nitrogen sources affect melanin synthesis, finding that the highest melanin production occurred when utilizing starch and tyrosine. The physiological and biochemical procedures encompassed details regarding growth under various pH levels, temperatures, and salt concentrations. Additionally, they examined the utilization of sugars (Arabinose, Glucose, Fructose, Galactose, Lactose, Maltose,

Mannitol, Sucrose, Xylose) and amino acids (Cysteine, Arginine, Histidine, Tyrosine, Proline, Methionine, Asparagine, Tryptophan, Valine), along with data on the metabolism of compounds (Li *et al.*, 2016).

2.3.4 Chemotaxonomic characterization

Chemotaxonomic characterization entails examining chemical variations and characteristics to classify and identify bacteria, including actinomycetes. In contemporary bacterial classification, chemotaxonomy has emerged as a crucial tool for distinguishing many actinomycetes, as morphologically similar isolates often exhibit differences in their diagnostic chemical compositions.

2.3.5 Molecular Characterization

Traditional methods of classifying actinomycetes genera overlook many existing synonyms. Chemotaxonomy has been employed to establish correlations between the composition of actinomycete cell walls and their systematic positions. Over the past three decades, various approaches in chemotaxonomy have been explored to address this issue (Mabrouk and Saleh, 2014). Recently, advancements have incorporated molecular techniques such as comparative sequencing and DNA reassociation. Among these, the most effective method for determining the relatedness of higher taxa is the comparative examination of ribonuclease-resistant oligonucleotides derived from the 16S ribosomal RNA (rRNA).

2.4 ACTINOMYCETES AS SOURCES OF SECONDARY METABOLITES

Actinomycetes play crucial biogeochemical roles in terrestrial soils beyond their production of bioactive substances. They significantly contribute to the decomposition of organic matter in the carbon cycle, the conversion of mineral nutrients from inorganic to organic compounds, nitrogen fixation, enhancement of soil physical characteristics, and environmental conservation efforts. Moreover, they are key players in the breakdown and recycling of organic matter. Actinomycetes are highly esteemed for their remarkable capacity to produce a wide array of chemical compounds, including enzymes, antioxidants, antibiotics, antimicrobial substances

(such as antifungal, antiviral, and anti-parasitic agents), bio-control agents (such as antimicrobial pesticides, weedicides, larvicides, and insecticides), nutraceuticals, anti-infective and anti-tumor agents, plant growth regulators, and vitamins, among others (Ruanpanun and Chamswarng, 2016; Janaki *et al.*, 2016; Mukhtar *et al.*, 2017). Approximately 45% of bioactive secondary metabolites originate from actinomycetes, with *streptomycetes* alone accounting for approximately 75% of these compounds.

2.5 ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES

Actinomycetes are a valuable reservoir of numerous secondary metabolites, especially antimicrobial compounds with pharmaceutical and agricultural significance. Globally, the isolation and screening of potential actinomycete strains capable of producing promising antibiotics or antimicrobial agents have become a focal point for numerous research teams. Among the various genera of actinomycetes, *Streptomyces* and *Micromonospora* are recognized as the primary sources of antimicrobial compounds (Takahashi and Nakashima, 2018).

Furthermore, *Promicromonospora*, *Microbispora*, *Nocardia*, *Pseudonocardia*, *Streptosporangium*, *Nocardiopsis*, *Saccharomonospora*, *Streptomonospora*, *Arthrobacter*, *Kocuria*, *Microbacterium*, *Actinomadura*, *Saccharopolyspora*, *Actinopolyspora*, *Agromyces*, *Thermoactinomycetes*, *Nonomuraea*, *Spirillispora*, *Lechevalieria*, *Amycolatopsis*, *Isoptericola*, and others are known for their production of bioactive compounds with pharmaceutical importance (Jakubiek, Krzesnaik, 2018). (Rahman *et al.* 2011) and (Sharma *et al.* 2011) found that 53% and 38% of soil-dwelling actinomycetes displayed moderate to high antibacterial activity against pathogenic Gram-positive and Gram-negative bacteria, respectively. *Streptomycetes* from Agumbe soil were screened for their antagonistic effects against pathogenic bacteria and yeasts. The research concluded that the studied strains exhibited significant sensitivity to pathogens, with 35% of strains demonstrating broad-spectrum antibacterial activity, as evidenced by zones of inhibition ranging from 0 to 40mm (Gautham *et al.*, 2012; Kekuda *et al.*, 2012). A newly discovered species named *Nonomuraea monospora*, found in Thai cave soil, exhibited antimicrobial properties against a range of bacterial and fungal pathogens, as reported by (Nakaew *et al.*

2012). Meanwhile, research conducted by (Naine *et al.* 2012) and (Arafat *et al.* 2016) focused on isolating various strains of *Streptomyces* bacteria. These strains demonstrated potent antibacterial effects, particularly against certain pathogenic bacteria, with the most significant activity observed against *Pseudomonas aeruginosa*, showing a 20 mm inhibition zone at a concentration of 5 mg/ml.

2.5.1 Optimization of media and biochemical parameters for the production of antibiotic compounds

Enhancing the composition of culture media and optimizing physiological conditions are essential for the production of antibiotic compounds. Consequently, numerous studies have been dedicated to optimizing both the media and biochemical parameters to achieve maximum growth and secondary metabolite production. Among microbial genera, *Streptomyces* stands out as the primary source of secondary metabolites, including antimicrobial compounds. (Atta ,2011) assessed the bioactivity of *S. torulosus* against pathogenic bacteria and fungi. By refining the biochemical characteristics of the medium, it was found that antibiotic production peaked on the fifth day of incubation at a pH of 7.0 and a temperature of 30°C. Similarly, (Atta *et al.*,2012) investigated the biological activity of *S. lydicus* against bacterial and fungal pathogens. The optimization process involved response surface methodology utilizing Box-Behnken design and one-factor-at-a-time approaches, as outlined by(Souagui *et al.*,2015). Their investigation revealed that the optimal antifungal compound was achieved in a culture medium containing glucose (0.34%), yeast extract (0.38%), and sodium chloride (0.41%) at pH 10.0 on the ninth day, at a temperature of 30°C. Bundale *et al.* (2015) examined how culture conditions, including incubation time and temperature, pH levels, and various carbon and nitrogen sources, influenced both growth and the production of bioactive compounds.

2.5.2 Characterization of bioactive compounds isolated from actinomycetes

Research on extracting of secondary metabolites from *S. torulosus* using n-butanol, yielding a yellow-colored solid extract through the application of aqueous methanol and petroleum ether (Atta 2011) . Separation of the extract was examined

via thin layer chromatography (TLC) employing a solvent system of chloroform and methanol in a 24:1 (v/v) ratio. The isolated compounds were further purified through column chromatography with silica gel. The chemical structure of the purified compound was analyzed using ultraviolet (UV), infrared (IR), and mass spectral (MS) techniques, revealing its identity as the antibiotic tunicamycin. Similarly, (Atta *et al.*, 2012) investigated metabolite extraction from *S. lydicus* using chloroform. Separation and purification were carried out using TLC and column chromatography techniques. The compound's identification relied on UV, IR, MS, and Nuclear Magnetic Resonance (NMR) spectroscopic analyses, confirming its identity as natamycin, a polyene antibiotic. (Yu *et al.*, 2012) isolated two new compounds, which are derivatives of sesquiterpenes, from *S. albospinus*. The purification process involved TLC and column chromatography, while chemical characterization utilized UV, IR, ¹³C NMR, and Distortionless Enhancement by Polarization Transfer (DEPT) spectral analyses. Notably, one of these compounds exhibited weak antibacterial activity.

The reverse HPLC technique facilitated the isolation of two fresh antimicrobial compounds derived from a Saharan soil strain of *Saccharothrix sp.* Their characterization involved analyzing Ultraviolet-Visible (UV-VIS), IR, mass, and both ¹H and ¹⁴C NMR spectra (Boubetra *et al.*, 2012). The identification of bioactive compounds involved analyzing their UV-Vis spectra, which indicated the presence of chromophores within the metabolite. Subsequently, Fourier-transform infrared spectroscopy (FTIR) was conducted to identify the functional groups in these active compounds. The active compounds were separated via TLC, and their bioactivity was determined using Liquid Chromatography Mass Spectrometry (LCMS) (Kesavan *et al.*, 2017).

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To Explore Actinobacteria which were isolated from himalayan region for new antimycobacterial compounds.

3.2 OBJECTIVE

- Subculturing and Cultural characterization.
- *In-vitro* screening against *M.smegmatis*.
- Selection of active culture and production of bio-active metabolites.
- *In-vitro* screening against *mycobacterium tuberculosis*(H37RA).

CHAPTER 4

MATERIALS AND METHODS

4.1 SUB-CULTURING

For this study, about 30 Actinobacterial cultures were obtained from the Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. All the cultures were previously isolated from less explored ecosystems of Himalayan region and maintained in respective agar slants as well as in 30% glycerol broth. (Table 4.1) shows the List of actinobacterial culture used for anti-tuberculosis activity. The given cultures were been subcultured using the streaking technique. Quadrant and full streaking techniques have been done.

Table 4.1: List of actinobacterial culture used for anti-tuberculosis activity

S.NO	CULTURE NAME	MEDIA
1	<i>HS2D-4</i>	NA
2	<i>HS2C-15</i>	SCA
3	<i>HS2C-9</i>	SCA
4	<i>HS2D-13</i>	AIA
5	<i>HS2D-1</i>	NA
6	<i>HS2C-1</i>	NA
7	<i>HS2C-5</i>	NA
8	<i>HS2D-2</i>	NA
9	<i>HS2C-12</i>	SCA
10	<i>HS2Z-19</i>	SCA
11	<i>HS2C-7</i>	NA
12	<i>HS2D-25</i>	SCA
13	<i>HS2C-16</i>	AIA
14	<i>HS2Z-12</i>	ISP-2

15	<i>HS2Z-16</i>	AIA
16	<i>HS2D-35</i>	SCA
17	<i>HS2C-19</i>	AIA
18	<i>HS2D-26</i>	SCA
19	<i>HS2D-14</i>	AIA
20	<i>HS2D-20</i>	AIA
21	<i>HS2D-28</i>	SCA
22	<i>HS2D-3</i>	NA
23	<i>HS2Z-14</i>	AIA
24	<i>HS2D-9</i>	NA
25	<i>HS2D-13</i>	AIA
26	<i>HS2C-18</i>	AIA
27	<i>HS2C-20</i>	AIA
28	<i>HS2D-30</i>	SCA
29	<i>HS2D-33</i>	SCA
30	<i>HS2D-31</i>	SCA

4.2 CULTURAL CHARACTERIZATION

Cultural characterization was done for the 30 actinobacterial culture. Identification of the actinobacteria was done on the basis of macroscopic and micromorphological characterization. For macroscopic characterization, The isolated actinomycetes were observed for color, texture, elevation, form and margin and for micromorphological characterization KOH string test and Microscopic Observation was done by gram staining method. Gram staining is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. First, a loopful of a pure culture is smeared on a slide and allowed to air dry. The culture from a thick suspension of a a pure colony from a plate suspended in water on the microscope slide. Heat fixed the slide by passing it (cell side up) through a flame to

warm the glass. Crystal violet is then added by covering the heat-fixed cells with a prepared solution. Allow to stain for approximately 1 minute. Briefly rinsed the slide with water. Added (Gram's iodine) solution for 1 minute. This acts as a mordant and fixes the dye. Briefly rinsed with water. Decolorized the sample by applying 95% ethanol and alcohol. This can be done in a series of washes. The important aspect is to ensure that all the color has come out that will do so easily. This step washes away unbound crystal violet, leaving Gram-positive organisms stained purple with Gram-negative organisms colorless. The decolorization of the cells is the most "operator-dependent" step of the process and the one that is most likely to be performed incorrectly. Rinsed with water to stop decolorization. Rinsed the slide with a counterstain (safranin) which stains all cells red. The counterstain stains both gram-negative and gram-positive cells. However, the purple gram-positive color is not altered by the presence of the counter-stain, it's effect is only seen in the previously colorless gram-negative cells which now appear pink. gently and allowed the slide to dry.

4.3 IN-VITRO SCREENING AGAINST *M.SMEGMATIS*

Primary screening of actinobacteria was performed by agar plug method, The agar plug method is a technique used to assess the antimicrobial activity of substances, such as actinobacteria, against target micro-organisms like *M.smegmatis*. In this method, agar plugs containing the actinobacterial extracts are placed onto a petri dish swabbed with the target bacteria (*M.smegmatis*) from the 7H9 broth which have been inoculated with *M.smegmatis* from LJ medium, After incubation at 37°C zones of inhibition around the plugs indicate the effectiveness of the actinobacterial extracts against (*M.smegmatis*).

4.4 TIME POINT ASSAY

In this assay, the principle revolves around quantifying the production of antibiotics by the actinobacterial culture at different time points. For this 100ml of Soya bean meal broth have been prepared in 2 conical flask each have been inoculated

with HS2D2 culture, After inoculating the culture in soybean meal broth, incubation was maintained under shaking conditions at room temperature. Beginning from day 1 of post-inoculation and throughout the incubation period, the culture was regularly tested for time point assay. To proceed with the day wise activity of production of antibiotics and inhibition of zone against *M.smegmatis* ,7H10 media plates have been prepared.1000 microlitre is transferred in 1.5ml centrifuge tube from innoculated broth medium.Centrifuged at 10,000 rpm.100 microlitre of *M.smegmatis* added into 1000 microlitre of distilled water and well vortexed.Vortexed *M.smegmatis* swabbed onto the 7H10 media plates.Small wells are made in the agar using a sterile cork borer 100 microlitre of supernatant from the centrifuged tube added into the well,Kept for incubation at 37°C.This steps are repeated throught out the assay to understand the dynamics of the antimicrobial activityand determining the optimal time for harvesting bioactive compounds produced by the HS2D-2 culture.

4.5 SECONDARY SCREENING

Production of crude extract from the *HS2D-2* actinobacterial culture

4.5.1. Preparation of soya bean meal Broth

Prepared 200ml of soya bean meal broth.

4.5.2. Inoculation

Inoculated the prepared soya bean meal broth with the actinobacteria culture *HS2D-2* using aseptic techniques. Incubated the broth culture in shaking conditions at room temperature for actinobacteria growth until it reaches the desired growth phase.

4.5.3. Harvesting

Harvested the actinobacteria culture by centrifugation to separate the biomass from the culture broth. Collected the biomass pellet containing the actinobacteria cells

4.5.4. Extraction with Various Solvents

Divided the culture broth into separate aliquots.Extracted each aliquot with different solvents of varying polarity to obtain a broad spectrum of bioactive compounds. Commonly used solvents include ethyl acetate, methanol, ethanol, chloroform, acetone, and hexane.Added the solvent to culture broth in a suitable

ratio(15ml)and mixed thoroughly. Allowed the mixture to stand for a defined period to facilitate extraction of the bioactive compounds from the actinobacteria cells.

4.5.5.Separation and Collection

After the extraction period, separated the solvent extract from the culture broth. Collected the solvent extract containing the crude bioactive compounds.

4.5.6. Concentration and Drying

Concentrated the collected solvent extracts using techniques such as evaporation to remove the solvent and obtain concentrated crude extracts.

4.5.7. Storage

The obtained crude extract from different solvents is been converted into the ependoff tube,From this extract Minimum Antibiotic Concentration will be determined.

4.6 MICRO PLATE ALAMAR BLUE ASSAY(MABA)

Dilution bioassays offer a significant advantage over diffusion assays due to their ability to accurately determine the minimum inhibitory concentration (MIC) of drugs. With the advent of microtiter plates, these assays have become more efficient, allowing for substantial reductions in test compound concentrations. However, challenges arise when test samples are not fully soluble, potentially interfering with turbidity readings and complicating interpretation. To address this, it is crucial to include negative controls or sterility controls, where extracts are dissolved in blank medium without microorganisms. An alternative approach involves utilizing oxidation/reduction indicator dyes like alamarBlue. These dyes provide a visual indication of growth or inhibition, with the oxidized form appearing blue and non-fluorescent, while the reduced form turns pink and fluorescent. This allows for both visual assessment and quantitative measurement of growth or inhibition using a spectrophotometer, making alamar Blue a valuable tool in non-radioactive assays, particularly in the screening of anti-TB compounds. Overall, dilution methods are suitable for assaying a wide range of extracts or compounds, regardless of polarity, to determine MIC and minimum bactericidal concentrations (MBC). AlamarBlue, with its ability to indicate cellular growth and viability through color changes, offers a versatile and reliable means of assessing antimicrobial activity in various screening assays, including those targeting tuberculosis (Rampersad, S. N. 2012).

CHAPTER 5

RESULTS AND DISSCUSSION

5.1 CULTURAL MORPHOLOGY

Cultural characterization was done for the 30 actinobacterial culture. Identification of the actinobacteria was done on the basis of macroscopic characterization, The isolated actinomycetes were observed for color, texture, elevation, form and margin were listed in the table (5.1).

Table 5.1: Morphological Characteristics of actinobacterial strains from Himalayan region

S.NO	CULTURE NAME	COLOR	TEXTURE	ELEVATION	FORM	MARGIN
1	HS2D-4	Pale yellow	Mucoid	Convex	Puncti form	Entire
2	HS2C-15	Half white	Smooth	Flat	Round	Entire
3	HS2C-9	Pale yellow	Viscid	Flat	Ir-regular	Entire
4	HS2D-13	Pale yellow	Mucoid	Raised	Round	Scalloped
5	HS2D-1	Mild yellow	Mucoid	Convex	Puncti form	Entire
6	HS2C-1	Mild yellow	Dry	Flat	Filamentous	Curled
7	HS2C-5	Mild yellow	Mucoid	Convex	Puncti form	Entire
8	HS2D-2	Pale yellow	Mucoid	Smooth	Filamentous	Entire
9	HS2C-12	Pale white	Mucoid	Convex	Round	Curved
10	HS2Z-19	Pale yellow	Smooth	Convex	Puncti form	Curved
11	HS2C-7	Mild yellow	Dry	Flat	Filamentous	Entire
12	HS2D-25	White	Dry	Convex	Puncti form	Entire
13	HS2C-16	Light	Viscid	Flat	Ir-regular	Entire

		yellow				
14	<i>HS2Z-12</i>	Half white	Dry	Convex	Puncti form	Curved
15	<i>HS2Z-16</i>	Grey	Smooth	Flat	Ir-regular	Entire
16	<i>HS2D-35</i>	Pale yellow	Dry	Raised	Ir-regular	Entire
17	<i>HS2C-19</i>	Pale yellow	Viscid	Flat	Ir-regular	Undulat ed
18	<i>HS2D-26</i>	Cream White	Dry	Raised	Ir-regular	Undulat ed
19	<i>HS2D-14</i>	Half white	Dry	Convex	Round	Scallop ed
20	<i>HS2D-20</i>	White	Dry	Convex	Puncti form	Scallop ed
21	<i>HS2D-28</i>	Ash	Smooth	Flat	Ir-regular	Entire
22.	<i>HS2D-3</i>	Ash	Dry	Convex	Round	Scallop ed
23.	<i>HS2Z-14</i>	Grey	Dry	Convex	Puncti form	Scallop ed
24.	<i>HS2D-9</i>	Ash	Dry	Raised	Ir-regular	Entire
25.	<i>HS2D-13</i>	Ash	Dry	Convex	Puncti form	Scallop ed
26.	<i>HS2C-18</i>	Grey	Dry	Flat	Puncti form	Entire
27.	<i>HS2C-20</i>	Pale yellow	Smooth	Flat	Puncti form	Entire
28.	<i>HS2D-30</i>	Half white	Dry	Flat	Ir-regular	Entire
29.	<i>HS2D-33</i>	Grey	Dry	Raised	Round	Scallop ed
30.	<i>HS2D-31</i>	White	Smooth	Flat	Ir-regular	Entire

Similarly the study by (Sengupta *et al.*, 2015) focused on investigating the antimicrobial activities of actinomycetes isolated from the Sundarbans mangrove ecosystem. Initially, 54 pure isolated actinomycete strains were assessed using a preliminary antimicrobial assay via the perpendicular streak method. Among them, only nine isolates demonstrated antimicrobial activity against both bacterial and fungal test organisms. These nine isolates were then selected for further characterization studies.

5.2 AGAR PLUG ASSAY

A total of thirty actinobacterial strains were subjected to agar plug assay against *Mycobacterium smegmatis*, utilizing 7H10 medium plates as the growth substrate. Following incubation at 37°C, the plates were carefully observed to determine the presence and extent of inhibition zones around the agar plugs. Similarity (Ortlieb *et al.*, 2021). Development of an agar-plug cultivation system for bioactivity assays of actinomycete strain collections.

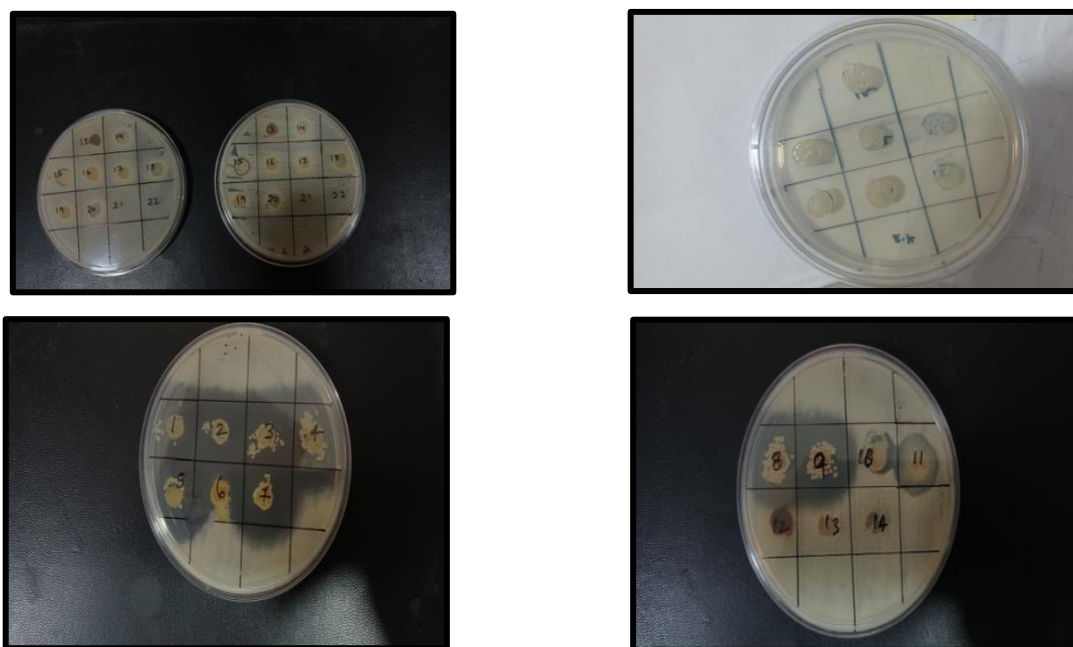


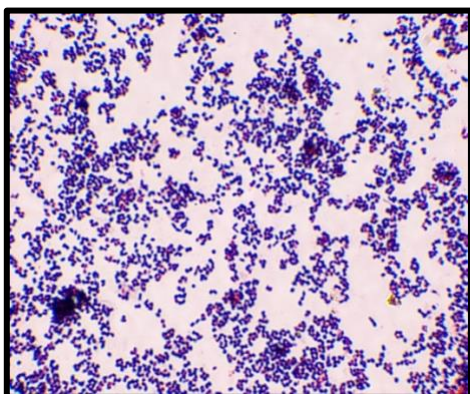
Fig :5.1: The actinobacterial strain exhibited significant inhibition against *M. smegmatis*

**Table 5.2: Antimicrobial Activity of Actinobacterial Strains Against
*M. smegmatis***

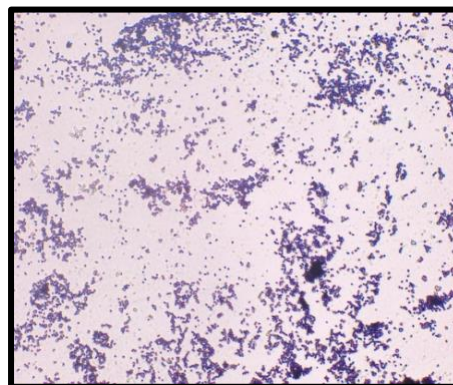
Culture name	Zone (mm)
HS2D-20	11
HS2D-3	20
HS2C-7	22
HS2D-2	25
HS2C-5	24
HS2D-4	20
HS2D-1	18
HS2C-6	22
HS2C-1	21

5.3 MICROSCOPIC EXAMINATION

HS2D-2, have been chosen as potent culture for their potency in inhibiting the growth of *M. smegmatis* have been observed through microscopic examination



HS2D-2(100X)



HS2D-2(40X)

Fig:5.2: Microscopic examination of HS2D-2 actinobacterial strain

5.4 CULTURAL CHARACTERISTICS

The cultural morphology of the strain *HS2D-2* were given in (figure 5.3) the strain showed good growth on ISP3 media with smooth, white coloured colony with mycelium growth of *HS2D-2*. Similarly (Nidhi Srivastava *et al.*, 2019) isolated three actinomycetes from the himalyan region namely *Actinomadura nitrigenes*, *streptomyces niveiscabiei*, and *kitasatospora psammotica*.



Fig:5.3: Cultural colony appearance of HS2D-2 on ISP3 media

5.5 TIME POINT ASSAY

Potent culture *HS2D-2* have been selected for time point assay to observe the dynamics of antimicrobial activity.

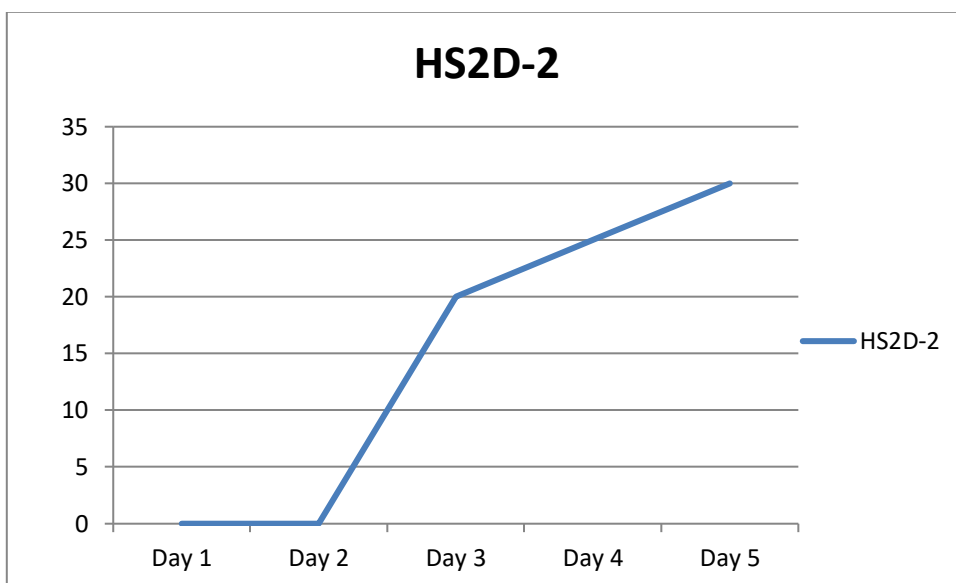
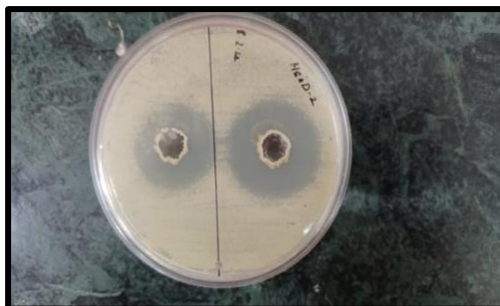


Fig:5.4: Dynamics of antimicrobial activity of HS2D-2 actinobacterial strain



HS2D-2:(30mm)

Fig:5.5: The maximum inhibition zone of the HS2D-2 strain was observed through a time point assay on day 5

5.6 PRODUCTION AND EXTRACTION OF ANTIMYCOBACTERIAL COMPOUNDS

Production and extraction of antimycobacterial compounds from *HS2D-2* culture, In a similar vein,(Sengupta *et al.*,2015) conducted extraction of secondary metabolites and preparation of crude extracts for three specific strains, namely *SMS_SU21*, *SMS_SU13*, and *SMS_7*, originating from unexplored regions of the Sundarbans mangrove ecosystem.

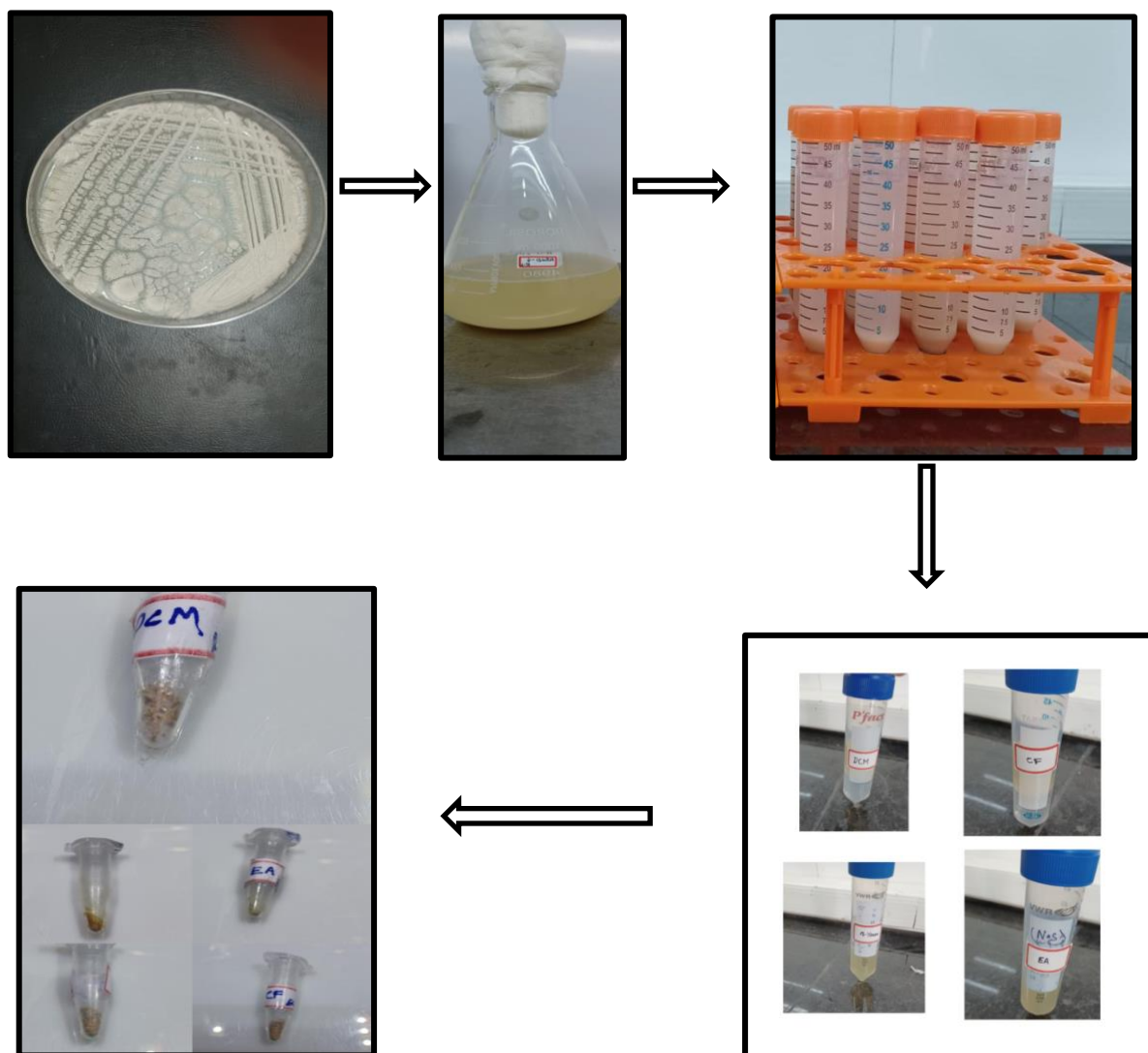
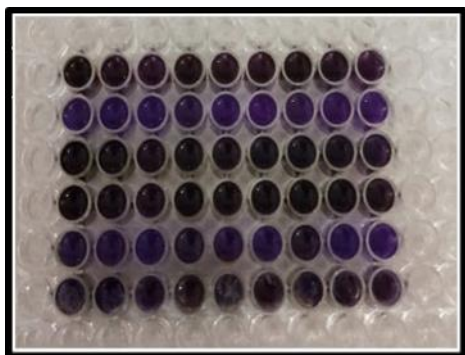


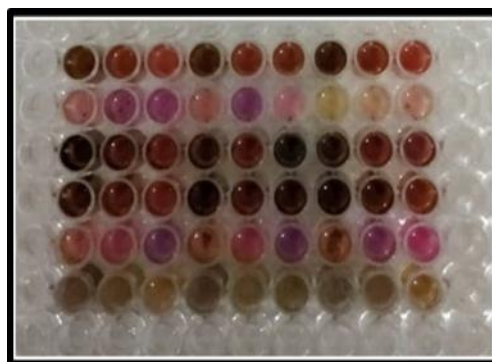
Fig:5.6: production and extraction of antimycobacterial compounds

5.7 MICRO PLATE ALAMAR BLUE ASSAY

Anti-mycobacterial activity of *HS2D-2* culture by microplate alamar blue assay. Similarly (Li, G.,*et al* 2013) performed Antimicrobial susceptibility of standard strains of non-tuberculous mycobacteria by microplate Alamar Blue assay.

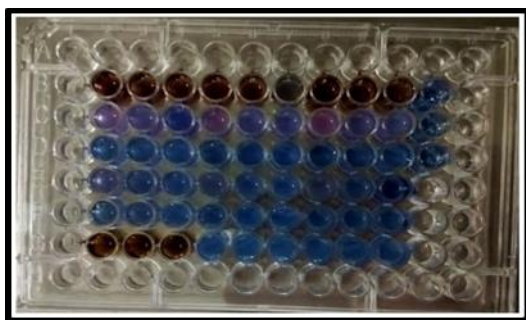


Alamar Blue Dye Addition

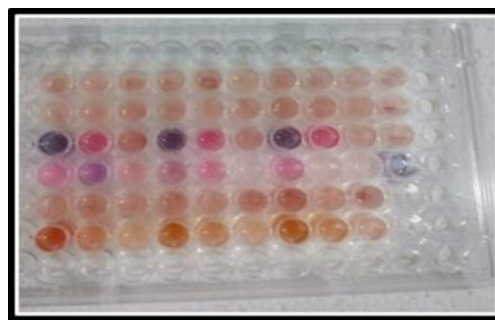


Alamar Blue Dye Incubation

Fig:5.7: MABA analysis of HS2D-2 strain against M.smegmatis



Alamar Blue Dye Addition



Alamar Blue Dye Incubation

Fig:5.8: MABA analysis of HS2D-2 strain against H37RA

Table 5.3: The percentage of crude extract obtained from the *HS2D-2* strain to inhibit *M.smegmatis*

S.NO	CULTURE	500µg/ml	250µg/ml	125µg/ml
1	<i>HS2D-2</i>	63%	25%	5%

Table 5.4: The percentage of crude extract obtained from the *HS2D-2* strain to inhibit *H37RA*

S.NO	CULTURE	500µg/ml	250µg/ml	125µg/ml
1	<i>HS2D-2</i>	78%	33%	12%

5.8 SEQUENCING RESULTS

The strain *HS2D-2* was subjected to 16sRNA analysis. The PCR amplification of *HS2D-2* 16sRNA gene yields 1202 base pairs. NCBI BLAST analysis of *HS2D-2* shows 99% similarity to sequence of *Streptomyces. sp* the evolutionary history was inferred by using the maximum likelihood method based on the Tamura- Nei model (Nandhini *et al.*,2018) which further confirms distinct phylogenetic tree constructed with *Streptomyces. sp HS2D-2* is designated as *Streptomyces rochei* (fig: 5.9). It's coming under rare forms and further studies like DNA-DNA hybridization are need to done.

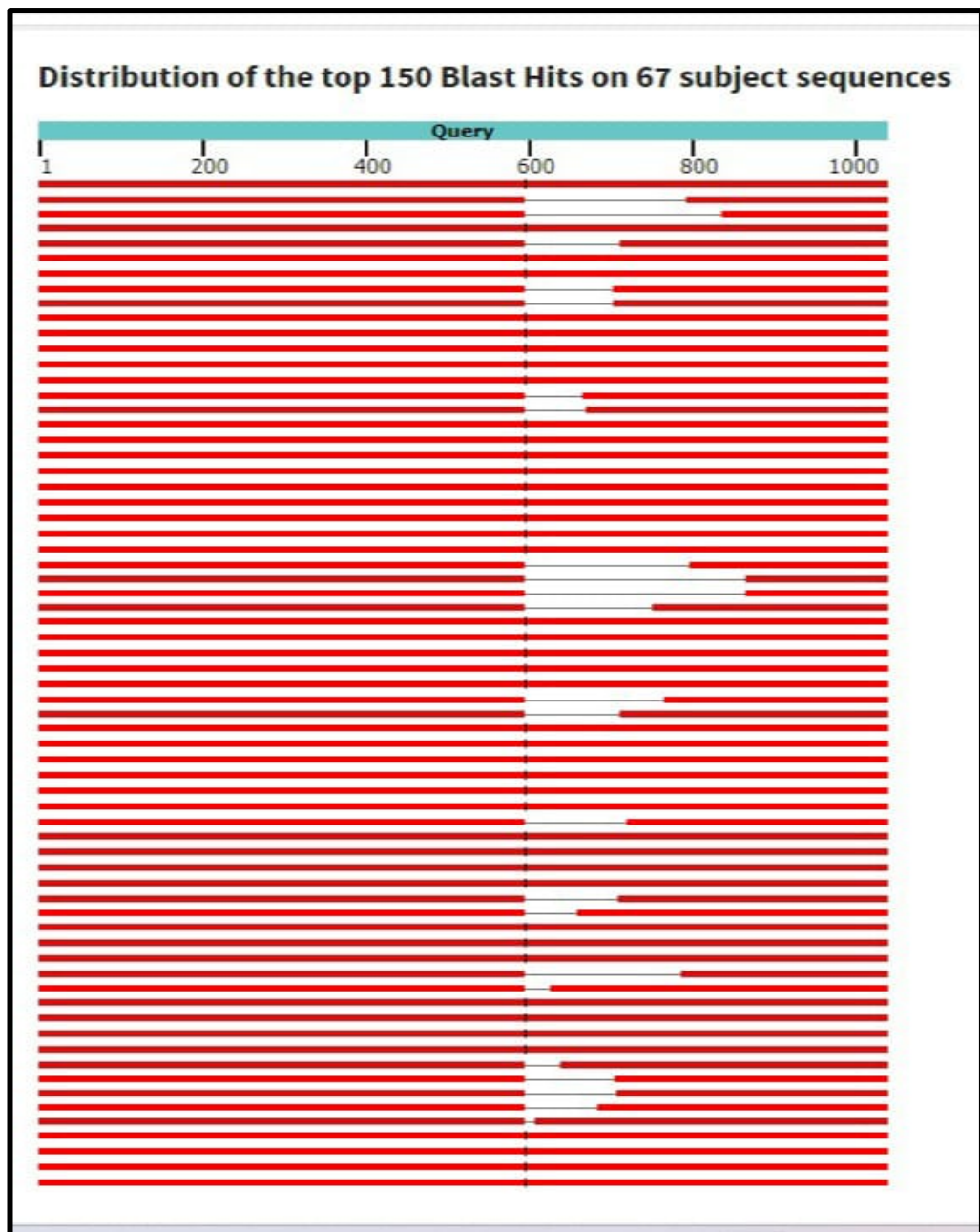


Fig:5.9: Blast results of HS2D-2

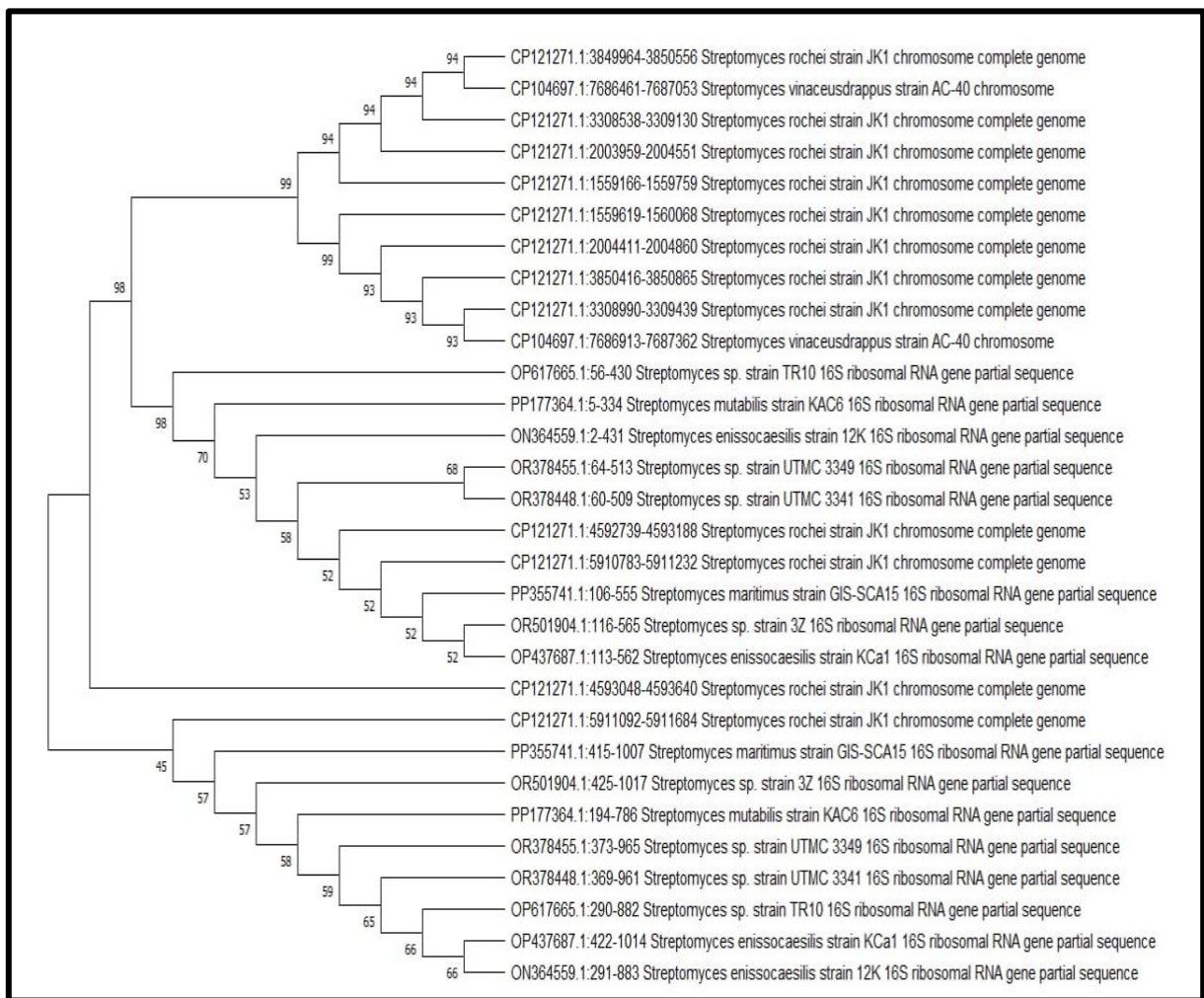


Fig:5.10: Phylogenetic tree

CHAPTER 6

SUMMARY AND CONCLUSION

The study aimed to investigate Actinobacteria from the Himalayan region for novel anti-mycobacterial compounds against the *H37RA* strain. Thirty bacterial strains were obtained from the Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology. Cultural characterization was conducted based on macroscopic and micromorphological features. The strains were further screened for anti-tubercular activity using the agar-plug assay, with nine strains showing significant activity, notably *HS2D-2*. Subsequent time point assays revealed that *HS2D-2* exhibited the highest inhibition zone against *M. smegmatis* on day 5. This strain was selected for further experimentation to determine the optimal time for harvesting secondary metabolites. Secondary screening involved extraction to obtain crude extracts for the microplate Alamar Blue Assay, aimed at determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC).

conclusion, this study investigated the anti-mycobacterial potential of Actinobacteria isolated from the Himalayan region, focusing on the *HS2D-2* strain. Through the microplate Alamar Blue Assay, we found that crude extracts from *HS2D-2* demonstrated significant inhibition against both *M. smegmatis* and *H37RA* strains at varying concentrations. Specifically, at 500 µg/ml concentration, the crude extract inhibited *M. smegmatis* by 63% and *H37RA* by 78%. These findings suggest that the *HS2D-2* strain harbors promising compounds with potent anti-tubercular properties. Further research is warranted to isolate and characterize these bioactive compounds for potential development as anti-tuberculosis therapeutics.

CHAPTER 7

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