

EXPLORING ACTINOBACTERIA FROM MANIPUR FOREST SOIL AGAINST *NON-TUBERCULOUS MYCOBACTERIA*

PROJECT WORK

Submitted in partial fulfillment of the requirements for the
award of Bachelor of Science degree in Microbiology

By

D. HEMASRI (41300014)



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

SATHYABAMA

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

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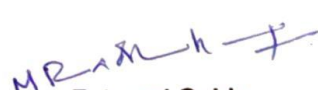
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BONAFIDE CERTIFICATE

This is to certify that this project report is the bonafide work of **D HEMASRI (41300014)** who carried out the project entitled **EXPLORING ACTINOBACTERIA FROM MANIPUR FOREST SOIL AGAINST NON-TUBERCULOSIS MYCOBACTERIUM** under our supervision from January 2024 to April 2024.


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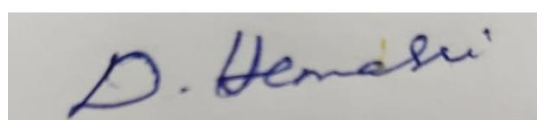

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DECLARATION

I, **D HEMASRI (41300014)** hereby declare that the Project Report entitled **“EXPLORING ACTINOBACTERIA FROM MANIPUR FOREST SOIL AGAINST *NON-TUBERCULOUS MYCOBACTERIA*”** done by me under the guidance of **Dr. Prabhakar Singh** (Internal) and **Dr. M Radhakrishnan** (External) at Center for Drug Discovery and Development (Research Park) is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in **Microbiology**.

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ABSTRACT

The incidence of Non-tuberculous mycobacteria (NTM) infections is rising among individuals with compromised immune systems as well as those with healthy immunity, leading to pulmonary and extra-pulmonary diseases, including disseminated infections in immunocompromised patients. Due to the limited research on NTM infections, there is an urgent need to develop effective treatments. Actinobacteria, a prominent bacterial group found in diverse environments, have recently yielded new genera and species. Our research aims to investigate the bioactive potential of actinomycetes sourced from unexplored Indian ecosystems against non-mycobacterial pathogens. Isolates obtained from soil in Manipur forests were evaluated for their activity against *M. smegmatis*, with promising strains undergoing fermentation to produce extracts. The anti-NTM activity of the extracts will be screened against NTM species through a liquid based assay - Microplate Alamar Blue Assay (MABA). We identified MCD-6 can produce novel metabolite that could contribute to improved management and treatment against NTM infections such as *M. fortitum*.

Keywords: Actinobacteria, Non-Tuberculous Mycobacteria, Microplate Alamar Blue Assay, *Mycobacterium fortitum*, *Mycobacterium smegmatis*.

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LIST OF ABBREVIATION

NTM	Non-tuberculous mycobacteria
MTb	Mycobacterium tuberculosis
LTBI	Latent tuberculosis infection
MAC	Mycobacterium avium complex
COPD	Chronic obstructive pulmonary disease
MDR	MultiDrug resistance
XDR	Extensively Drug-resistant
<i>M.chelonae</i>	<i>Mycobacterium chelonae</i>
<i>M.fortitum</i>	<i>Mycobacterium fortitum</i>
<i>M.gordonae</i>	<i>Mycobacterium gordonae</i>
<i>M.smegmatis</i>	<i>Mycobacterium smegmatis</i>
MABA	Micro plate Alamar blue assay
AIA	Actinomycetes isolation agar
SCA	Starch casein agar
ISP-5	Glycerol asparagine agar base
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CHAPTER 1

INTRODUCTION

1.1 ACTINOBACTERIA

Actinobacteria are a group of aerobic, Gram-positive microorganisms that are predominantly filamentous and saprophytic, constituting one of the largest taxonomic groups within the bacterial kingdom (Ludwig *et al.*, 2012). They exhibit diverse morphological characteristics, ranging from spherical (e.g., *Micrococcus*) and rod-shaped (e.g., *Arthrobacter*) to fragmenting hyphae (e.g., *Nocardiosis*) that form branched mycelium (e.g., *Streptomyces*). Many actinobacteria can form spores (Goodfellow and Williams, 1983; Ventura *et al.*, 2007). While lacking distinct cell walls, actinobacteria produce non-septate and generally slender hyphae. (Barka *et al.*, 2016). Initially, actinobacteria were considered an intermediate group between bacteria and fungi due to their resemblance to fungi in terms of shape and morphology (Rosenberg, 2014). They were previously referred to as "ray fungi," derived from the Greek words "aktin" (ray) and "mukēs" (fungi).

However, some actinobacteria exhibit bacterial-like colony characteristics (Waksman *et al.*, 1940). Certain genera of actinobacteria reproduce through specialized structures known as spores (Passari *et al.*, 2015). These spores are typically spiral and elongated, existing either as single cells or in chains of varying lengths. They play a crucial role in helping actinobacteria survive under harsh and unfavorable environmental conditions (Zothanpuia *et al.*, 2017). The morphological characteristics, colony morphology, and color of actinobacterial isolates exhibit significant diversity on solid culture media and may vary depending on the specific type of media used. Some actinobacterial colonies displayed a powdery texture, while others were observed to be soft and sticky, adhering firmly to the agar surface in culture media (Barka *et al.*, 2016).

The aerial and substrate mycelium of actinobacteria exhibit variability, with the substrate mycelium playing a crucial role in nutrient uptake and absorption to support the growth and development of these microorganisms. The genomes of actinobacteria are typically circular and contain approximately 3,300 coding

sequences. They are characterized by a high guanine and cytosine (G+C) content. Within the actinobacteria phylum, the G+C content varies, ranging from around 50% in certain genera like *Corynebacteria* to $\leq 70\%$ in others such as *Frankia* and *Streptomyces* (Solans *et al.*, 2011; Passari *et al.*, 2015).

Actinobacteria are classified under the Phylum Actinobacteria, Class Actinobacteria, Sub-class Actinobacteridae, Family Actinomycetaceae, and Order Actinomycetales. Actinobacteria are known to produce secondary metabolites such as geosmin and methylisoborneol (MIB), which contribute to the earthy smell often noticed after rainfall (Gerber and Lechevalier, 1965; Gerber 1983). Although actinobacteria are recognized to be significant contributors to the carbon cycle, their precise ecological role and impact on this cycle remain uncertain (Kuznetsov, 1970). Actinobacteria are also known to produce numerous secondary metabolites that play a crucial role in the synthesis of clinically important antibiotics (Sharma *et al.*, 2016). Actinobacteria are also present in symbiotic association with lichens (Parrot *et al.*, 2015).

Actinobacteria have been classified based on their tolerance to various environmental conditions. Mesophilic actinobacteria, which thrive best at temperatures ranging from 27°C to 30°C, constitute most of the group (Edwards *et al.*, 1993). On the other hand, certain actinobacteria such as *Thermomonospora*, *Microbispora*, and others exhibit thermophilic characteristics, enabling them to withstand and thrive at temperatures ranging from 37°C to 65°C, with optimal growth occurring between 50°C and 60°C (Barka *et al.*, 2016). *Streptomyces* remain the primary producers of novel biologically active compounds, exhibiting a diverse array of activities such as antimicrobial and anticancer properties, as well as the production of other valuable compounds for pharmaceutical and biotechnological applications (Berdy, 2005; Wang *et al.*, 2013; 2014; Ser *et al.*, 2015).

The phylum Actinobacteria is renowned for its antibiotic production, contributing to the synthesis of approximately two-thirds of the antibiotics commonly utilized today. These antibiotics encompass various classes, including aminoglycosides, ansamycins, anthracyclines, β -lactams, and macrolides (Zothanpuia *et al.*, 2018). Several widely used antibiotics are derived from actinobacteria, including Kanamycin, Streptomycin, Tetracyclines,

Chloramphenicol, Neomycin, Erythromycin, Vancomycin, Rifamycin, Gentamicin, and Daptomycin. Streptomycin, the first antibiotic obtained from actinobacteria, belongs to the aminoglycoside class and was discovered by Waksman in 1943 from *Streptomyces griseus*. It was initially employed for treating infections caused by *Mycobacterium tuberculosis* (Schatz *et al.*, 2005).

Tetracyclines, a subgroup of polyketide antibiotics, were first discovered in 1945 from the genus *Streptomyces*. They possess broad-spectrum activity against bacterial infections and have served as a basis for the development of several other effective antibiotics (Mahajan and Balachandran, 2012). In 1949, Chloramphenicol, an antibiotic, was initially isolated from *Streptomyces Venezuela*. Subsequently, it was synthesized to combat various bacterial pathogens by inhibiting their protein synthesis. Actinobacteria have also been a source of other antibiotics, including Neomycin, an aminoglycoside antibiotic derived from *Streptomyces fradiae*; Erythromycin, a macrolide antibiotic from *Streptomyces erythreus*; Vancomycin, a glycopeptide antibiotic from *Amiclotopsis orientalis*; Gentamicin, an aminoglycoside antibiotic derived from *Micromonospora*; and Daptomycin, a lipopeptide antibiotic from *Streptomyces roseosporus* (Mahajan and Balachandran, 2012).

Despite extensive bioprospecting efforts targeting actinobacteria from various habitats, many of these microorganisms have been identified, characterized, and screened for their biosynthetic potential. However, numerous habitats remain unexplored. Given the dwindling discovery of novel Drugs to combat antibiotic resistance, the quest for new compounds must be extended. Recently, there has been growing recognition of the potential value of rare habitats that have been largely overlooked. These habitats could serve as promising sources for isolating pharmacologically valuable secondary metabolites, owing to the ability of microorganisms to thrive in stressful environments.

CHAPTER 2

LITERATURE SURVEY

Actinobacterial research focusing on the production of bioactive secondary metabolites has been conducted across various habitats by researchers worldwide. Several antibiotics have been successfully identified using different techniques and methodologies. However, the emergence of antibiotic-resistant pathogens necessitates the search for additional antibiotics with diverse mechanisms of action on a global scale. Furthermore, infectious diseases remain a leading cause of mortality worldwide, underscoring the importance of exploring untapped habitats such as soil for the discovery of new and potent bioactive compounds, particularly effective antibiotics from actinobacteria, to address current needs. This chapter provides an overview of the research conducted thus far on actinobacteria sourced from different soil environments, encompassing the historical background of actinobacterial research associated with soil, isolation methods and techniques employed, the diversity of actinobacterial isolates obtained, and the bioactive secondary metabolites reported from soil-derived actinobacteria.

2.1 HISTORY OF ACTINOBACTERIAL RESEARCH

The phylum Actinobacteria is widely distributed in nature, being commonly isolated from terrestrial habitats, aquatic environments (particularly marine ecosystems), and soil habitats (Warnecke *et al.*, 2004). In 1969, actinobacteria including *Streptomyces* and *Micromonospora* were initially documented, having been isolated from both soil and river samples obtained from Blelham Tarn (Willoughby, 1971). This event marks a significant milestone in soil actinobacterial research. In the subsequent years, between 1976 and 1977, *Rhodococcus coprophilus* and various other strains of actinobacteria, including *Micromonospora*, *Streptomyces*, *Nocardioform*, *Streptosporangium*, *Actinomadura*, *Actinoplanes*, *Dactyhsporangium*, *Microbispora*, and *Thermomonospora*, were documented from soil and agricultural environments (Johnston and Cross, 1976; Rowbotham and Cross, 1977). Cross (1981) identified actinobacteria from soil habitats as a

potentially rich reservoir of bioactive secondary metabolites, as corroborated by Goodfellow and Haynes (1984). In 1966, Jiang and Xu, researchers from China, successfully isolated and identified six distinct genera of actinobacteria from soil and sediment samples collected from the Middle Plateau in Yunnan, China. These genera comprise *Streptomyces*, *Actinomadura*, *Microbispora*, *Micromonospora*, *Nocardia*, and *Saccharopolyspora* (Jiang and Xu, 1996). Wohl and McArthur (1998) successfully isolated nine diverse genera of actinobacteria, namely *Streptomyces*, *Nocardioides*, *Pseudonocardia*, *Nocardia*, *Streptoalloteichus*, *Actinomadura*, *Microbispora*, *Micromonospora*, and *Actinoplanes*, from soil samples collected in South Carolina. In recent years (2000-2017), there has been a notable increase in published literature pertaining to actinobacterial research conducted in soil environments. Researchers have placed greater emphasis on exploring the diversity of actinobacteria and assessing their antimicrobial activity, particularly considering the escalating issue of antibiotic resistance among pathogens (Deshmukh and Sridhar, 2002; Rifaat, 2003; Chou *et al.*, 2008; de Menezes *et al.*, 2008; Rifaat and El-Sayed, 2008; Sibanda *et al.*, 2010; Cwala *et al.*).

2.2 STREPTOMYCES

Streptomyces, a genus known for its antibiotic production, is the predominant group within the phylum Actinobacteria and ranks among the largest genera within the bacterial domain (Ludwig *et al.*, 2012). *Streptomyces* has emerged as a dominant contributor to the development of numerous Drugs and the pharmaceutical industry, surpassing other microorganisms in its impact (Ser *et al.*, 2015). As previously mentioned, two-thirds of the antibiotics currently in use have been sourced from actinobacteria, with over 70% of these antibiotics originating specifically from the genus *Streptomyces* (Subramani and Aalbersberg, 2012). Among various genera of actinobacteria, *Streptomyces* and *Micromonospora* are commonly isolated from soil habitats. *Micromonospora* has been consistently reported as the most frequently isolated actinobacteria from soil environments, as documented by Cross and Collins (1966), Willoughby (1971), Jiang and Xu (1996), and Rifaat and El-Sayed (2008). *Streptomyces*, on the other hand, typically rank as the second most abundant genus in soil habitats, as supported by Colmer and McCoy (1943) and Willoughby (1971). Recent literature surveys have consistently identified *Streptomyces* as the predominant genus within the phylum Actinobacteria

in soil habitats, as evidenced by numerous researchers (Wohl and McArthur, 1998; Deshmukh and Sridhar, 2002; Ningthoujam *et al.*, 2011; Sanasam *et al.*, 2011; Jami *et al.*, 2015). *Streptomyces* exhibit a wide range of morphological and physiological characteristics that set them apart from other genera of actinobacteria. They are predominantly spore-forming microbes, a feature that enables them to endure adverse environmental conditions (Goodfellow and Williams, 1983). *Streptomyces* play a significant role in the synthesis of numerous antibiotics and remain a subject of considerable interest for antibiotic production. Therefore, ongoing screening for the bioactive secondary metabolites produced by this organism is essential (Ser *et al.*, 2015). The discovery of Streptothricin, the inaugural antibiotic isolated from *Streptomyces* in 1942, followed by *Streptomycin* from the same genus two years later, sparked significant interest (Berdy, 1974; Vandamme, 1984). Additionally, various widely used antibiotics, including Tetracyclines, Chloramphenicol, Neomycin, Erythromycin, Kanamycin, and Daptomycin, among others, have been derived from the genus *Streptomyces*.

2.3 BIOACTIVE SECONDARY METABOLITES FROM SOIL ACTINOBACTERIA

Microbial secondary metabolites, often referred to as "antibiotic factories," have been extensively documented across various organisms and habitats worldwide, as indicated by research conducted by Yuan *et al.* (2014) and Jami *et al.* (2015). Actinobacteria are the primary producers of secondary metabolites among microbial species, particularly prevalent in terrestrial and marine environments. The diverse range of metabolites synthesized by actinobacteria presents a significant area of interest for antibiotic research. However, soil habitats have been relatively underexplored in the quest for actinobacterial secondary metabolites, with most studies focusing on antimicrobial screening and biodiversity. The initial report of actinobacterial secondary metabolites from soil occurred in 2015, featuring diazaquinomycins H and J (DAQH and DAQJ), which demonstrated inhibitory effects against *Mycobacterium tuberculosis* (Mullowney *et al.*, 2015). Deuteromethylactin B, an eight-membered lactone secondary metabolite, was identified for its anti-tumor properties from actinobacteria sourced from soil, specifically *Streptomyces spp.* (Shaikh *et al.*, 2015). The antibacterial secondary metabolites known as nactins (polynactin) were discovered to be produced by *Streptomyces sp.* strain 156A, isolated from Lake Baikal, which is recognized as the

largest freshwater lake in the world (Tatyana *et al.*, 2016). To our knowledge, these are the sole examples of soil-derived actinobacterial secondary metabolites documented in the literature thus far.

2.4 NON-TUBERCULOUS MYCOBACTERIA (NTM)

Tuberculosis (TB) is a prominent infectious disease in humans caused by *Mycobacterium tuberculosis* (Mtb), primarily impacting the lungs and posing a significant mortality risk. From a pathological perspective, TB manifests as necrotizing granulomatous inflammation affecting the infected organs. The spread of Mtb infection within the population primarily happens through the inhalation of contaminated aerosols from individuals with active pulmonary disease. Upon exposure to Mtb, humans may develop either primary active disease or asymptomatic latent infection (LTBI) (Gopalaswamy *et al.*, 2020b). Around a third to a quarter of the global population is estimated to have latent tuberculosis infection (LTBI). Individuals with LTBI can progress to symptomatic tuberculosis (TB) when their immune system is compromised. The risk of LTBI cases developing active TB is approximately 5% within the first 18 months of infection, after which the likelihood of relapse decreases to around 5% for the rest of their lives (AnDrews *et al.*, 2012). There are approximately 170 species of mycobacteria belonging to the family of *non-tuberculous mycobacteria* (NTM). However, the most common causes of pulmonary diseases in humans are species within the *M. avium complex* (MAC), *M. kansasii*, and *M. abscessus* (Johnson & Odell, 2014b). Human infections caused by NTM are mainly acquired from environmental sources, although the exact mode of transmission is not fully understood. Besides affecting the lungs, NTM infections commonly involve the lymphatic system, skin, and soft tissues. Additionally, various underlying health conditions increase the risk of NTM infections, including chronic obstructive pulmonary disease (COPD), pneumoconiosis, bronchiectasis, previous tuberculosis (TB), fibrosis following radiotherapy, chronic pulmonary aspiration, cystic fibrosis (CF), immune deficiencies, HIV infection, alcoholism, cancer, and diabetes mellitus (DM). Distinguishing between Mtb and NTM species in clinical samples presents a significant challenge, as both can yield positive results with conventional acid-fast staining methods, leading to the potential underestimation of NTM infections in TB-endemic regions (Griffith *et al.*, 2007). The standard antibiotic regimen for Drug-sensitive TB typically includes isoniazid (INH), rifampicin (RIF),

pyrazinamide (PZA), and ethambutol (ETH) administered for at least 6 months. However, the treatment of multi- and extensively-Drug resistant (MDR and XDR) TB cases may require additional antibiotics for an extended duration. Newer Drugs like bedaquiline and delamanid have enabled the development of novel regimens with shorter treatment periods for MDR-TB cases. Unlike TB, NTM diseases do not respond to anti-TB Drugs.

Treatment of NTM diseases is guided by specific guidelines tailored to the infecting bacterial species and requires species identification. Unlike the relatively shorter treatment duration for TB, NTM disease treatment typically spans at least 18 months, with a requirement for a 12-month sputum-negative period (Wu *et al.*, 2018). Both TB and NTM pulmonary diseases are influenced by bacterial characteristics and host factors, which impact susceptibility, the presentation of infection, and treatment outcomes. Our knowledge of the epidemiology, risk factors, and pathophysiology of pulmonary TB in humans has significantly advanced over the past 50 years. However, these areas remain less developed for NTM diseases (Chan & Iseman, 2013). Additionally, there are more diagnostic and treatment options available for TB compared to NTM diseases. Nevertheless, promising new diagnostic methods and treatment modalities are being developed for both TB and NTM diseases. This review assesses the progress made in the areas of Mtb and NTM infections in humans, focusing primarily on epidemiology, diagnosis, and treatment (Weiss & Schaible, 2015). (Table 2.1)

TABLE 2.1: Summary of key features of pulmonary TB and NTM diseases

Category	Tuberculosis (TB)	Pulmonary Non-tuberculosis mycobacteria (NTM) infections
1. Causative agent	<i>M. tuberculosis</i> complex organisms	<i>M. avium</i> complex, <i>M. abscessus</i> , <i>M. kansasii</i> , <i>M. malmoense</i> , <i>M. xenopi</i>
2. Mode of transmission	Inhalation of contaminated aerosols from patients with pulmonary TB	Primarily acquired from the environment -lack of person-to-person transmission

3. Sex with higher disease burden	Male	Female
4. Predisposition/Co-morbidities	HIV, DM, Immunosuppression	Bronchiectasis, Previous history of TB, CF, COPD
5. Diagnosis	Lung involvement, alveolar infiltration, cavitation, lymphadenopathy and pleural effusion	Nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules
5.2 Radiological	X-ray; rarely CT	X-ray; PET/CT or HRCT
5.3 Samples for the diagnostic test	Sputum samples, bronchial or bronchioalveolar lavage aspirates, or tracheal aspirates; Gastric aspirate	Sputum, bronchial wash, or lavage; Gastric aspirate not preferred due to failure to indicate active NTM disease
5.3 Microbiological test		
5.3.1 Staining	Acid fast staining; Ziehl Neelson staining	Acid fast staining; Ziehl Neelson staining
5.3.2 Culture	Solid, Liquid-MGIT 960 system; the VersaTREK system; MB/BacT Alert 3D	Solid or liquid including MGIT but with PNB
5.4 Molecular biological test	TB Ag MPT64 RAPID Nucleic acid amplification test (NAAT) – Amplified <i>Mycobacterium tuberculosis</i> direct (MTD); Amplicor <i>Mycobacterium tuberculosis</i> Test; Xpert MTB-Rif system; Xpert MTB-Rif Ultra	TB Ag MPT64 RAPID – to differentiate <i>M. tb</i> complex from NTM HPLC PCR-RFLP; PCR sequencing NAATs–Accuprobe analysis Anyplex MTB/NTM MDR-TB EZplex MTBC/NTM;

	Loop-mediated isothermal amplification (LAMP)-based MTB detection system; Cross-priming amplification (CPA)-based TB diagnostic system; CE-IVD GeneDrive; Anyplex II MTB/MDR and MTB/XDR; Anyplex MTB/NTM MDR-TB kit; EZplex MTBC/NTM; VereMTB Detection Kit Line probe assays (LiPA) – Inno-LiPA Mycobacteria assay; Genotype Mycobacterium CM and AS assays Others -MALDI-TOF Next gen sequencing For Latent TB - Tuberculin skin test IGRA test - QuantiFERON-TB Gold, QFT-GIT, QFT-Plus and T-SPOT.TB	Genedia MTB/NTM Detection Kit LiPAs-Inno-LiPA Mycobacteria assay; GenoType Mycobacterium CM -AS Others -MALDI-TOF Next gen sequencing
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2.4.1 Epidemiology and transmission of TB and NTM

In 2018, approximately 1.5 million individuals succumbed to TB, with nearly 10 million people worldwide contracting Mtb infection. However, only 6.4 million cases were officially diagnosed and reported. The burden of TB is higher among males, accounting for 57% of cases, compared to females at 32% (Gopalaswamy *et al.*, 2020c). Globally, an estimated 1.7 billion individuals have latent Mtb infection (LTBI) without evident disease symptoms. Those with LTBI typically develop active TB within the first 12 to 18 months, although reactivation can occur decades after the initial infection. In 2018, the global incidence of multidrug-resistant (MDR) or rifampicin-resistant (RR) TB was estimated at 3.4% among new cases and 18% among individuals previously treated for TB. Additionally, the proportion of

extensively Drug-resistant (XDR) TB cases among MDR-TB cases was estimated to be 6.2%. The majority of Drug-sensitive, multidrug-resistant (MDR), and extensively Drug-resistant (XDR) TB cases are documented in Asian countries, particularly India and China. The emergence of Drug resistant *Mtb* strains is often linked to inappropriate clinical administration of anti-TB medications and inadequate patient adherence to treatment regimens, which necessitate prolonged multi-Drug therapies. Furthermore, molecular epidemiological evidence indicates that the transmission of multidrug-resistant (MDR) and extensively Drug-resistant (XDR) *Mtb* strains within the community is the primary mode of dissemination in numerous countries where TB is endemic. Hence, it is crucial to have a comprehensive understanding of *Mtb* transmission and the acquisition of new infections to inform the development of effective TB control strategies (Gopalaswamy *et al.*, 2020c). Like Drug-sensitive TB cases, there is an urgent need to enhance the diagnosis and treatment approaches for MDR- and XDR-TB cases, aligning with one of the primary objectives of the END-TB policy established by the WHO.

2.4.2 Epidemiology of NTM diseases

Initially, human infections caused by NTM were thought to primarily originate from contaminated environmental sources transmitted through aerosols (Bhalla *et al.*, 2018). However, recent studies suggest that person-to-person transmission may also contribute to NTM infections. Numerous clinical reports of NTM cases have highlighted similarities in disease symptoms between NTM infections and TB (Bryant *et al.*, 2016). Research on population genomics has demonstrated that genetically clustered NTM organisms are responsible for the majority of infections globally. Furthermore, this study has unveiled the recent emergence of predominant clones of *M. abscessus* that have disseminated across continents. The occurrence and frequency of NTM cases, as well as the distribution of strains, exhibit significant variability across different geographical regions. A worldwide examination of NTM species obtained from human samples revealed that approximately half of them is classified under the *M. avium* complex (MAC) (Hoza *et al.*, 2016). Nevertheless, the proportion of MAC isolates varies considerably by geographic area, with figures ranging from 31% in South America to 52% in North America and 71% in Australia. In a clinical investigation involving cystic fibrosis (CF) patients afflicted with NTM infection, MAC was identified in 61% of cases, *M. abscessus* in 39%, and other NTM

species in 21% of cases from at least one specimen (Jain *et al.*, 2014). Among these patients, approximately 19% exhibited the isolation of multiple NTM species. Although the distribution of NTM species varies widely across different regions, resulting in a range of diseases, pulmonary NTM infections represent a significant and frequently underestimated burden of illness in human populations. Moreover, pulmonary NTM infections can manifest in individuals without concurrent chronic conditions like cystic fibrosis (CF) (Hoefsloot *et al.*, 2013). A study conducted by Marras *et al.* indicates that the prevalence of NTM cases has risen in North America, from 9.1 to 14.1 per 100,000 persons per year, during the period from 1997 to 2003 (Orme & Collins, 1985). Prior research has noted a decline in the incidence of both pulmonary NTM and TB cases in various nations following the introduction of national Bacillus Calmette-Guérin (BCG) vaccination policies.

This implies that BCG vaccination may offer cross-protection against NTM infections. Consequently, in countries lacking a widespread BCG immunization initiative, an increase in NTM cases is anticipated. Significantly, in contrast to tuberculosis, pulmonary *non-tuberculous mycobacteria* (NTM) infections exhibit a higher prevalence among women (59%) and older individuals (with a median age of 66) compared to younger men. Moreover, *Mycobacterium avium complex* (MAC) is identified as the predominant species causing NTM infections. (Prevots *et al*) observed an uptick in the prevalence of pulmonary NTM cases among individuals aged over 60 years, escalating from 19.6 cases per 100,000 person-years during the period between 1994 and 1996 to 26.7 cases per 100,000 person-years from 2004 to 2006. Within this study cohort, *Mycobacterium avium complex* (MAC) emerged as the most frequently isolated species among patients diagnosed with definitive disease, accounting for 80.1% of cases, followed by *M. chelonae* and *M. abscessus* (12.1%), *M. fortuitum* (5.6%), and *M. kansasii* (5.5%). Likewise, an epidemiological investigation into the prevalence of pulmonary non-tuberculous mycobacterial (NTM) diseases in Australia revealed a rise in pulmonary NTM cases from 5.5 to 10.2 per 100,000 individuals over a six-year period spanning from 1999 to 2005. This increase was notably observed among individuals aged over 60 years, with a predominance of cases among women. (Degner *et al.*, 2017). Moreover, there has been an increase in the prevalence of pulmonary non-tuberculous mycobacterial (NTM) diseases in Asia, with rates rising from 1.3 to 7.9 cases per

100,000 population (Banaschewski *et al.*, 2019). The majority of these cases were attributed to *Mycobacterium avium complex* (MAC) and *M. abscessus* (Baldwin *et al.*, 2019).

In Europe, the prevalence of NTM cases has risen from 0.9 to 2.9 cases per 100,000 individuals between 1995 and 2006. Collectively, these studies suggest an increasing trend in the occurrence and frequency of pulmonary NTM infections globally, which are linked to various underlying health factors including immunosuppression, age, gender, and prior lung conditions (Marras *et al.*, 2007).

2.4.3 NTM diseases in HIV and diabetes mellitus cases

NTM infections manifest differently in patients with HIV/AIDS compared to those without the virus. Additionally, there is a notable risk of NTM infections being overlooked in HIV-positive individuals, as tuberculosis is often prioritized as the primary concern when symptoms of pulmonary mycobacterial disease are observed in these patients (Falkinham, 1996).

In individuals with HIV co-infection, localized NTM pulmonary disease is uncommon, and disseminated forms of the disease are more prevalent. This contrasts with tuberculosis, where both localized and disseminated forms can affect the respiratory system in humans. While diabetes mellitus (DM) is known to increase susceptibility to tuberculosis (TB), the relationship between NTM infections and DM is not well understood (Lu *et al.*, 2018). Some studies have suggested that DM may be a co-morbid condition in cases of soft tissue and pulmonary NTM infections during baseline assessments (Pande *et al.*, 2015). Therefore, there is a need for further research to enhance our understanding of how diabetes mellitus affects host immunity and disease progression during NTM infections, as this remains an area requiring significant improvement (Lai *et al.*, 2011).

2.4.4 Diagnosis of TB and NTM diseases

Distinguishing between colonization of NTM species in individuals, where they act as commensals or colonizers, and NTM as a causal agent of disease is essential but challenging. Many suspected NTM cases show colonization without pulmonary disease symptoms, raising questions about the presence of low-grade NTM infection or disease, or if the specimen is contaminated with environmental

NTM species (Kostera *et al.*, 2018). The American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) have established criteria to diagnose pulmonary NTM disease. These criteria encompass clinical observations like lung involvement seen on chest radiographs or PET/CT scans, microbiological testing, and strain identification to confirm the presence of NTM disease (Huang *et al.*, 2013). In contrast to tuberculosis (TB), the clinical manifestation of *non-tuberculous mycobacterial* (NTM) infections is diverse and can be complicated by underlying comorbidities like diabetes mellitus (DM), chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) (Kontturi *et al.*, 2018). Symptoms observed clinically, alongside the detection of pulmonary nodules or cavities on radiographs, or the presence of multifocal bronchiectasis and dispersed multiple small nodules on high-resolution computed tomography (HRCT) scans, suggest the presence of active NTM disease (Stephenson *et al.*, 2019). While acid-fast bacilli (AFB) staining can detect mycobacteria, it cannot differentiate between NTM and *Mtb*. Therefore, it is advised to culture NTM from clinical specimens like sputum, bronchial wash, or lavage on solid and/or liquid media (Gopalaswamy *et al.*, 2020d). Culturing mycobacteria on growth media is the preferred method for identifying both rapid and slow-growing species and is considered the "gold standard" diagnostic approach.

2.4.5 Molecular biological diagnosis of NTM diseases

Another technique for NTM species identification involves using high-performance liquid chromatography (HPLC), which analyzes mycolic acids, a unique type of cell wall fatty acids found in *mycobacteria*. This method is highly specific to species and has demonstrated the ability to differentiate 99.5% of mycobacterial cultured from clinical specimens and 95.6% of NTM species (Chemlal & Portaels, 2003). Furthermore, Plikaytis *et al.* have devised a two-step assay that combines gene amplification and restriction fragment length polymorphism (RFLP) analysis to distinguish among slow-growing mycobacteria. This method has demonstrated the capability to differentiate over 90% of potentially pathogenic *mycobacteria* isolates and more than 86% of all strains in clinical specimens, including *Mtb*, *M. bovis*, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. goodii*. Subsequent advancements in this technique have expanded its applicability, allowing PCR and PRA for nearly 20 NTM strains with distinct patterns established for each of them. PCR-based sequencing has emerged as the preferred method for

identifying mycobacterial species, regarded as the gold standard in diagnostics. This approach typically targets the gene responsible for encoding 16S ribosomal RNA, which encompasses both conserved and variable regions and is ubiquitous across bacterial species (Stephenson *et al.*, 2019b). Amplification and sequencing of this gene enable accurate identification of mycobacterial species. Accuprobe analysis is a widely utilized method that employs nucleic acid hybridization assay for rapid identification of various mycobacterial species, including the Mtb complex, MAC, *M. intracellulare*, *M. goodii*, and *M. kansasii*.

2.4.6 Treatment of pulmonary NTM diseases

While the Mtb complex family consists of only a few member species, pathogenic NTM encompasses approximately 170 species. Each of these NTM species has been associated with various diseases, such as pulmonary disease, lymphadenitis, disseminated diseases, as well as skin and soft tissue infections (Zha & Nahid, 2019). The majority of pulmonary NTM disease cases are attributed to five NTM species, namely MAC, *M. abscessus*, *M. kansasii*, *M. xenopi*, and *M. malmoense*. The incidence of each strain varies across different geographical regions worldwide (Xu *et al.*, 2012). Achieving sputum conversion, transitioning from a positive to a negative presence of bacteria, poses challenges in NTM cases despite the administration of various antibiotics, particularly in individuals infected with macrolide resistant NTM species (Van Ingen & Kuijper, 2014). In certain cases, involving focal disease or persistent symptoms, resection surgery has been contemplated as an additional treatment option for severe pulmonary NTM disease (Sarkola *et al.*, 2004). Illnesses caused by *M. kansasii* are typically manageable because they share similar disease presentation and pathology with pulmonary TB, marked by fibro-cavitary lesions predominantly found in the upper lung lobes (Theel *et al.*, 2018). Moreover, *M. kansasii* infections are usually susceptible to anti-TB medications, although resistant strains are frequently encountered. According to the ATS/IDSA guidelines, treatment for Drug-sensitive *M. kansasii* infections typically involves a daily regimen of rifampicin (RIF), ethambutol (ETB), isoniazid (INH), and pyridoxine (George *et al.*, 2017). For strains resistant to rifampicin, the recommended regimen consists of a combination of clarithromycin or azithromycin, moxifloxacin, ethambutol, sulfamethoxazole, or streptomycin. Treatment duration

for *M. kansasii* lung diseases should also include 12 months after negative sputum culture result (Hu *et al.*, 2019) (Table 2.2).

**TABLE 2.2: Description of Drugs used in the treatment of adults with TB
based on the ATS/ CDC/ IDSA Guidelines**

Drug	Group	Dosage
First-line	Isoniazid (Oral or IM or IV)	5 mg/kg (~ 300 mg) Pyridoxine 25–50 mg/day, is given with INH to persons at risk of neuropathy
	Rifampicin (Oral or IV)	10 mg/kg (~ 600 mg)
	Pyrazinamide (Oral)	1000 mg (40 - 55 kg weight) 1500 mg (56 – 75 kg weight) 2000 mg (76 – 90 kg weight)
	Ethambutol (Oral)	800 mg (40 - 55 kg weight) 1200 mg (56 – 75 kg weight) 1600 mg (76 – 90 kg weight)
Second-line	Cycloserine (Oral)	10–15 mg/kg (usually 250–500 mg once or twice daily)
	Ethionamide (Oral)	10–15 mg/kg (usually 250–500 mg once or twice daily)
	Streptomycin (IM or IV)	15 mg/kg daily. Some clinicians prefer 25 mg/kg 3 times weekly or the 15 mg/kg dose 3 times weekly for patients with poor renal function
	Amikacin/kanamycin (IM or IV)	15 mg/kg daily. Some clinicians prefer 25 mg/kg 3 times weekly or the 15 mg/kg dose 3 times weekly for patients with poor renal function
	Capreomycin (IM or IV)	15 mg/kg daily. Some clinicians prefer 25 mg/kg 3 times weekly or the 15 mg/kg dose 3 times weekly for patients with poor renal function

	Para-amino salicylic acid (Granules or tablets or IV)	200–300 mg/kg total (usually divided 100 mg/kg given 2 to 3 times daily)
	Levofloxacin (Oral or IV)	500–1000 mg daily
Anti-TB Drugs with limited data available on safety and effectiveness	Bedaquiline (Oral)	400 mg daily for 14 days followed by 200 mg 3 times/week
	Linezolid (Oral or IV)	600 mg daily
	Clofazimine (Oral)	100 mg daily
	Delamanid (Oral)	100 mg twice daily
	Meropenem (IV)	1,000 mg 3 times/day
	High-dose isoniazid (Oral or IV)	15 mg/kg daily

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

To isolate anti-nontuberculous compounds from actinobacteria isolated from rare ecosystem.

3.2 OBJECTIVES

- Isolation and Culture characterization of actinobacteria from Manipur Forest soil
- *In-vitro* screening against *M. smegmatis*
- Screening for the production of metabolites from potential isolates
- Microplate Alamar blue assay (MABA) against NTM pathogen (*M. fortitum*)

CHAPTER 4

MATERIALS AND METHODS

4.1 ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FROM MANIPUR FOREST SOIL

4.1.1 Collection of samples

About 20 actinobacterial cultures from the Center for Drug Discovery and Development culture consortium Sathyabama Institute of Science and Technology in Chennai, Tamilnadu were used in this investigation. All of the cultures were kept in Actinomycetes Isolation Agar (AIA), Glycerol Asparagine Agar Base (ISP5), and Starch Casein Agar medium (SCA) after being previously isolated from terrestrial samples obtained from various understudied ecosystems in the Manipur Forest, India.

4.1.2 Characterization of actinobacteria

Actinobacterial strains cultural characteristics was investigated through subculturing them on Oat meal agar (ISP3). ISP3 agar plates were made, sterilized, and poured into sterile petri plates under aseptic circumstances. They were then autoclaved at 120°C for 15 minutes to solidify. Then, after being inoculated into ISP3 agar plates, all of the actinobacterial cultures were incubated for 6 days at 28°C. After incubation the morphological traits to be compared among the isolates encompass their color or presence of soluble pigment, surface morphology, type of aerial hyphae, and the production of both aerial and substrate mycelia employed to isolate actinobacteria, including SCA, AIA, ISP2, ISP3, and ISP5(Li *et al.*, 2015).

4.2 PRIMARY SCREENING OF ACTINOBATERIA AGAINST *M. SMEGMATIS*

4.2.1 Inoculum Preparation

The glycerol stock of *M. smegmatis* underwent thawing within a refrigerated environment set at 4°C. The glycerol stock was then transferred into a 1.5ml centrifuged tube under pivotal circumstances and subjected to centrifugation at

14,000rpm at a temperature of 15°C. Following centrifugation, the supernatant was discarded, and the pellet underwent washing with phosphate buffered saline. Autoclaved McCartney bottles containing glass beads were sterilized under UV light for a specified duration. Subsequently, 5ml of Middlebrook 7H9 broth transferred in McCartney bottle. The pellet was then suspended in 7H9 broth and vigorously vortexed for 2-3 minutes. A 200ul of suspension of this mixture was transferred into 7H9 broth media within the McCartney bottles and were maintained at 37°C for 2 days in shaking.

4.2.2 Production of Bioactive Compounds

The production of bioactive compounds from actinobacteria cultures was achieved through agar surface fermentation (Radhakrishnan *et al.*, 2014). The 20 actinobacterial cultures were inoculated into ISP3 agar plates and subjected to 6 days incubation period at 28C to induce the synthesis of secondary metabolites. Through this incubation phase, extracellular metabolites were exuded into the agar medium.

4.2.3 Testing for Antimycobacterial Activity

The antimycobacterial activity of actinobacterial cultures was evaluated through the adoption of the agar plug method. Actinobacterial cultures cultivated on ISP3 agar plates were utilized for this purpose. A standardized test pathogen *M. smegmatis*, was the swabbed onto Middlebrook 7H10 plates using a sterile cotton swab. Agar plugs, measuring 7mm in diameter, were excised from ISP3 agar were actinobacterial cultures had proliferated and placed over Middlebrook 7H10 agar plates swabbed with *M. smegmatis*. Following this, all plates underwent incubation at 28c for a duration of 48 hours. The resulting zone of inhibition was quantified in terms of millimeters(mm) in diameter.

4.3 EFFECT OF LIQUID FERMENTATION METHOD ON ANTIMYCOBACTERIAL COMPOUND PRODUCTION

The impact of submerged fermentation on the production of antimycobacterial compounds by the strain MCD-6 was investigated. Spores of the actinobacterial strain MCD-6 were introduced into Oatmeal broth (ISP-3) and subjected to

incubation in a rotary shaker operating at 120rpm for a duration of 6 days. Subsequently, aliquots of 2ml from the ISP-3 broth were extracted and subjected to centrifugation at 10,000 rpm for 20 minutes. The resulting cell- free supernatant was then assessed for its antimycobacterial activity against *M. smegmatis* species using the well diffusion method. This assessment was conducted at intervals of every 24 hours throughout the entire 6 days incubation period.

4.4 PRODUCTION AND EXTRACTION OF ANTIMYCOBACTERIAL METABOLITES

As the strain MCD-6 demonstrated activity in both the agar plug and well diffusion methods, the production of actinobacterial compounds was sustained in both solid and liquid cultures until the 6th day of fermentation. Consequently, to facilitate the subsequent large-scale production of the compounds derived from strain MCD-6, submerged fermentation methods were implemented.

4.4.1 Submerged Fermentation

The highly potent strain MCD-6 were introduced into an ISP-3 broth and allowed to incubate for 6 days at 28°C within a rotary shaker operating at 120 rpm. After incubation, the broth underwent centrifugation at 10,000 rpm for 20 minutes, yielding a supernatant which was carefully collected. Equal volumes of various solvents, including ethyl acetate, methanol, n-hexane, dichloromethane (DCM), and chloroform, were then added to the supernatants, thoroughly mixed and left undisturbed. The resulting solvent portion was isolated and subsequently concentrated using an Eppendorf concentrator plus system before quantification.

4.4.2 Quantification of The Crude Extract

The microcentrifuge tubes were initially weighed to obtain their weight (W1). Subsequently, the crude extract was added to the microcentrifuge tubes, and their combined weight (W2) was determined. The quantity of the crude extract was then computed utilizing the following intricate formula:

$$\text{Quantity of the crude extract} = W2 - W1$$

4.5 CHARACTERIZATION AND IDENTIFICATION OF POTENTIAL STRAINS

4.5.1 Micromorphological Characterization

The micromorphological characterization of the strains were analysed using the Gram staining. The smear was created by applying the spore of culture onto a glass slide and then heat fixed. Crystal violet was applied to the smear for 30-60 seconds and rinsed off with water. Gram's iodine was then applied to the smear for 30-60 seconds, followed by decolorization with alcohol and another rinse with water. The smear was then stained with safranin counterstain for 2 minutes. After washing and Drying, the slides were observed under a phase-contrast microscope at 100x magnification.

4.5.2 Cultural Characterization

The Actinobacterial strains were inoculated into Oatmeal agar (ISP-3), Glycerol Asparagine Agar (ISP-5), Starch Casein Agar (SCA), and Actinomycete Isolation Agar (AIA) in order to study the culture properties. The plates were then incubated for six days at 28°C.

4.5.3 Physiological Characterization

For optimal metabolite production, strain MCD-6 were chosen based on several characteristics and appropriate media. The fermentation medium utilized for optimization is composed of 0.4% yeast extract, 1% NaCl, 0.4% glucose, and 2% agar-agar, with a pH of 7 maintained.

4.5.4 Effect of Carbon Source

To study the effect of carbon source a loopful of potential bacterial inoculum was inoculated into a 12 well plate containing 3 ml of minimal broth medium (replacing with carbon source) with 1% different carbon sources (glucose, fructose, sucrose, mannitol and starch). Then 12 well plate was incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.5.5 Effect of Nitrogen source

To study the effect of nitrogen source about a loopful of potent bacterial inoculums were inoculated into 12 well plate containing 3 ml of Minimal broth medium (replacing with nitrogen source) with 1% different nitrogen sources (yeast

extract, malt extract, beef extract, peptone, urea). Then 12 well plate was incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.5.6 Effect of Mineral source

To study the effect of nitrogen source about a loopful of potent bacterial inoculums was inoculated into a 12 well plate containing 3ml of Minimal broth medium (replacing with mineral source) with 1% different nitrogen sources (CaCl₂, MnCl₂, FeSO₄, MgSO₄, No mineral source). Then 12 well plate was incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.5.7 Effect of pH

To study the effect of pH a loopful of inoculums was inoculated into 12 well plate containing 3 ml of minimal broth medium with different pH (5, 6, 7, 8 and 9). Then 12 well plate was incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.5.8 Molecular characterization

Sanger sequencing is a method for DNA sequencing, providing insight into the genetic makeup of organisms. Meanwhile, 16S rRNA sequencing is a culture-independent approach used to analyze bacterial diversity in complex environments. By comparing 16S rRNA gene sequences, one can identify bacteria down to genus or species levels, facilitating classification across bacterial phyla and even distinguishing between strains.

4.5.9 Phylogenetic Analysis

The 16S rRNA gene sequences from the isolates were compared to those in the NCBI's GenBank database using BLAST search. Subsequently, the 16S rRNA sequences of the MCD-6 strain was deposited into the GenBank database.

4.6 ANTIMYCOBACTERIAL ACTIVITY BY MICROBROTH DILUTION METHOD (MINIMUM INHIBITORY CONCENTRATION- MIC)

4.6.1 Microplate Alamar Blue Assay (MABA)

A McFarland standard 1 was used as the reference, and 100 ul of fresh glycerol stock of *M. smegmatis*, *M. fortitum*, was used to prepare the inoculum. The inoculum

was diluted 1:20 and resuspended in Middlebrook 7H9 medium. The Drug stock solutions were defrosted and diluted in Middlebrook 7H9 stock at a ratio of four times the highest concentration that was ultimately evaluated. Using 100 μ l Middlebrook 7H9 stock, serial 2-fold dilutions of crude pigment were made directly in a sterile 96-well microtiter plate at concentrations ranging from 125 to 500 μ g/mL. On each plate, a sterile control and a growth control devoid of antibiotics were also created. To prevent evaporation over the incubation period, sterile water was added to each perimeter well. After covering and sealing the plate in plastic bags, it was incubated at 37°C. The plate was re-incubated for an additional night after addition of 30 μ l of Alamar blue solution had been added to each well following a 7-day incubation period. The growth of bacteria was indicated by change in colour from blue (oxidized state) to pink (reduced state), and the minimum inhibitory concentration (MIC) was determined as the lowest Drug concentration that prevented this colour shift (Radhakrishnan *et al.*, 2015).

CHAPTER 5

RESULT AND DISCUSSION

5.1 ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FROM MANIPUR FOREST SOIL

Total 30 actinobacterial isolates were obtained from the Actinobacterial Research Lab at the Centre for Drug Discovery and Development Consortium, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. These cultures were isolated from soil samples collected from less explored ecosystem of Manipur Forest, India.

5.2 CHARACTERIZATION OF COLLECTED ACTINOBACTERIA

The ISP3 agar medium facilitated excellent growth for all 20 actinobacterial cultures. Majority of the bacterial isolates were pigmented colonies on ISP3 agar plates. Of the 12 were pigmented colonies and 10 were non-pigmented colonies. Pigments include brown, ash, dark yellow and reddish brown. (Table 5.1). In Previous study (Kumar, 2018) isolated bacteria from rare ecosystem soil samples.

Table 5.1: Morphological characteristics of actinobacterial cultures

S. No	Culture name	Colour	Texture	Elevation	Form	Margin	Pigment
1	MCD-1	Ash	Dry	Umbonate	Punctiform	Undulate	Ash
2	MCD-2	White	Dry	Convex	Punctiform	Entire	No Pigment
3	MCD-3	Pale White	Dry	Convex	Convex	Entire	Dark Yellow
4	MCD-4	Ash & White	Dry	Convex	Convex	Entire	Dark Brown
5	MCD-5	Ash	Dry	Flat	Circular	Entire	No Pigment

6	MCD-6	White	Dry	Umbonate	Round	Scalloped	No Pigment
7	MCD-7	Ash	Dry	Flat	Round	Entire	No Pigment
8	MCD-8	White	Dry	Flat	Irregular	Entire	No Pigment
9	MCD-9	Pale White	Dry	Convex	Punctiform	Entire	Reddish Brown
10	MCD-10	White	Dry	Flat	Irregular	Entire	Brown
11	MCD-11	White	Dry	Convex	Punctiform	Serrate	Brown
12	MCD-16	White	Dry	Convex	Punctiform	Scalloped	No Pigment
13	MCD-17	Pale White	Dry	Raised	Round	Scalloped	No Pigment
14	MCD-19	Pale White	Dry	Raised	Round	Scalloped	No Pigment
15	MCD-20	White	Dry	Raised	Round	Scalloped	Ash
16	MCD-21	Ash	Dry	Umbonate	Round	Scalloped	Ash
17	MML-1	Ash	Dry	Umbonate	Punctiform	Entire	Light Brown
18	MML-2	Ash	Dry	Umbonate	Round	Scalloped	Black
19	MML-3	Ash	Dry	Umbonate	Round	Scalloped	No Pigment
20	MML-8	Grey	Dry	Raised	Round	Scalloped	No Pigment

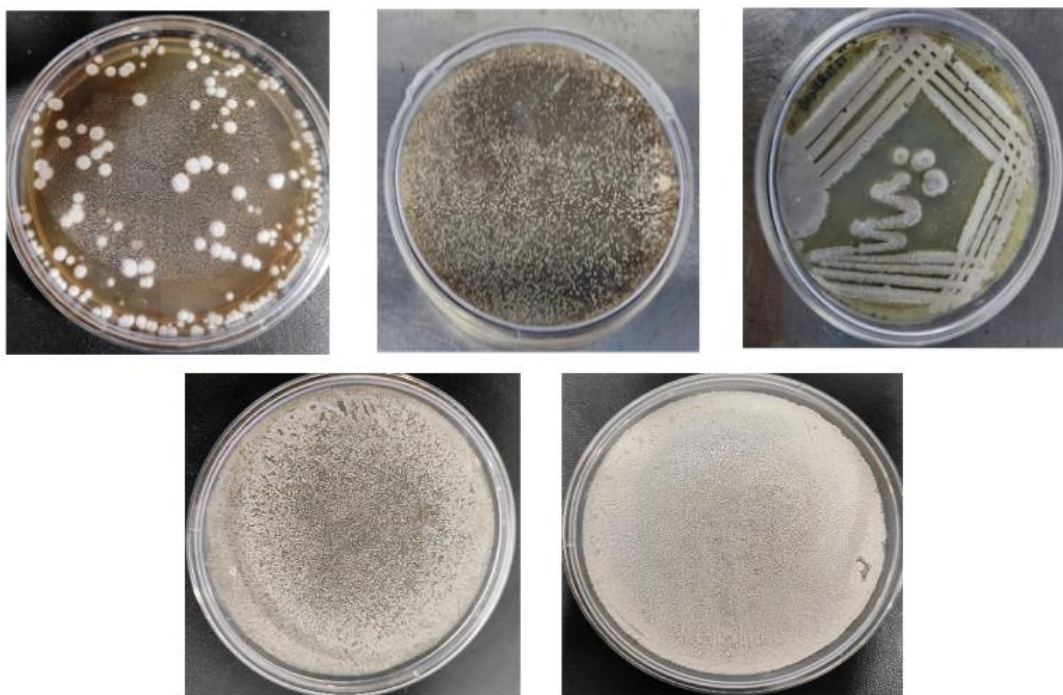


Fig 5.1: Actinobacterial culture from Manipur Forest soil

5.3 PRIMARY SCREENING OF ACTINOBACTERIA AGAINST *M. smegmatis*

In this investigation, all 20 actinobacterial strains underwent screening for antimycobacterial activity targeting *M. smegmatis*. Among these, strain MCD-6, exhibited noteworthy outcomes, demonstrating the highest zone of inhibition against the pathogen *M. smegmatis*, ranging from 7.5 to 12 mm. (Figure 5.2 & Table 5.2)

Table 5.2: Antimycobacterial activity of actinobacterial culture in agar plug
Method

Strain	Zone of Inhibition in Diameter (in mm)
MCD-6	12 mm



Fig 5.2: The agar plug method was performed to examine the antimycobacterial properties of actinobacterial cultures against *M. smegmatis*

5.4 EFFECT OF LIQUID FERMENTATION METHOD ON ANTIMYCOBACTERIAL COMPOUND PRODUCTION

Figure 5.3 illustrates the impact of fermentation methods on the production of antimycobacterial compounds from strain MCD-6. The findings indicate that strain MCD-6 commenced antimycobacterial metabolite production on the third day of fermentation, regardless of whether it was conducted on agar surface or submerged. Upon comparing the outcomes across different fermentation methods, submerged fermentation exhibited the most favorable antimycobacterial activity by the sixth day.

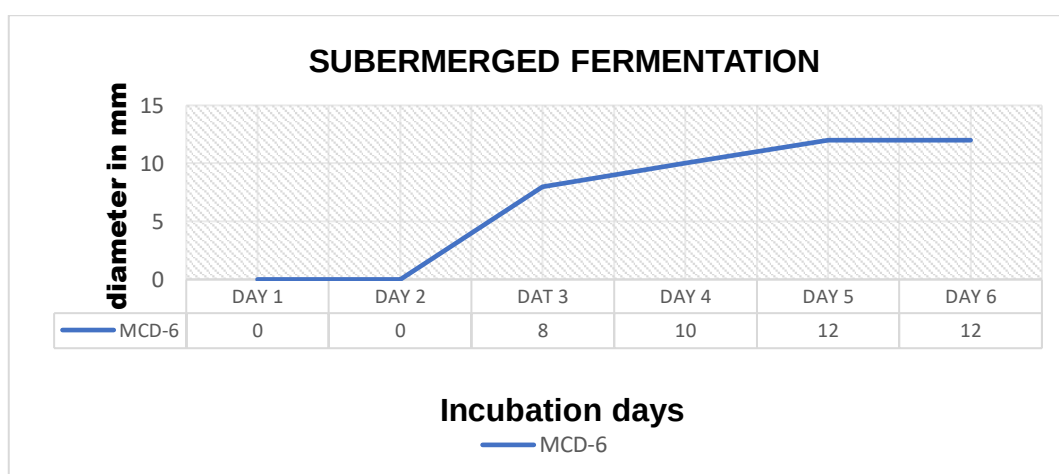


Fig: 5.3: Effect of fermentation method on bioactive compound production against *M. smegmatis*

5.5 PRODUCTION AND EXTRACTION OF ANTIMYCOBACTERIAL METABOLITES

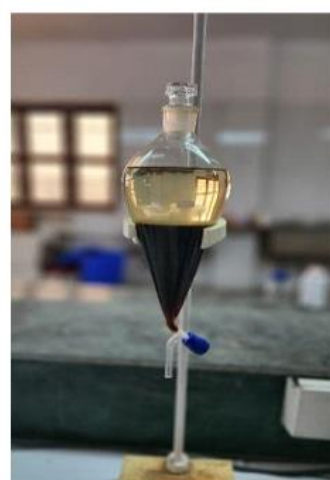
Strain MCD-6 displayed excellent growth in ISP3 broth medium. Subsequently, the broth underwent extraction with various solvents including methanol, ethyl acetate, dichloromethane (DCM), chloroform, and n-hexane. The resulting concentrate was dissolved in 10% DMSO for evaluation of antimycobacterial activity, as detailed in (Figure 5.4).



Mother culture



Liquid-Liquid Extraction



Solvent separation



cell free supernatant



extracted Crude



Powdered Crude

Fig 5.4: Overview of the Liquid Fermentation Process

5.6 CHARACTERIZATION AND TAXONOMY OF POTENT ACTINOBACTERIAL STRAIN

5.6.1 Micromorphology

Under bright-field microscopy, strain MCD-6 exhibited characteristics of Gram-positive bacteria, displaying spores and a cocci shape.

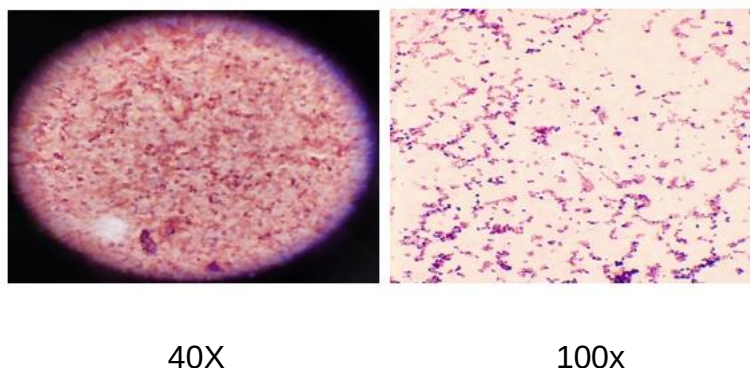


Fig 5.5: Microscopic observation of strain MCD-6

5.6.2 Cultural characteristics

The cultural morphology of strain MCD-6 has exhibited robust growth and produced powdery colonies on the ISP3 medium (fig 5.6).



Fig 5.6: Growth of potential strain MCD-6 on ISP3 medium

5.6.3 Physiological characteristics

From figure 5.7 it is understood that, the Strain MCD-6 has demonstrated robust growth and utilization of a diverse array of carbon, nitrogen, and mineral sources, as well as adaptation to various pH conditions, as indicated in table 5.3.

Table 5.3: Effect of culture conditions on antimycobacterial activity of potential actinobacterial strain MCD-6

Variables		Antimicrobial activity (mm in diameter)
MCD-6		
Different media	ISP2	10mm
	ISP3	18mm
	ISP5	12mm
	ISP7	NI
	AIA	8mm
	NA	NI
	SCA	NI
Carbon source	D-Glucose	13mm
	Lactose	21mm
	Raffinose	NI
	Cellulose	9mm
	Mannitol	9mm
	Starch	12mm
	Fructose	7mm
	Xylose	10mm
	No sugar	11mm
Nitrogen source	Malt	12mm
	Yeast	10mm
	Peptone	13mm

	Soya bean meal	11mm
	KNO ₃	18mm
Mineral source	NaCl	8mm
	MgSO ₄	13mm
	FeSO ₄	NI
	CaCl ₂	11mm
	MnCl ₂	8mm
pH	4	NI
	5	NI
	6	10mm
	7	9mm
	8	12mm
	9	13mm
NaCl (in %)	0.1	11mm
	0.5	10mm
	1	10mm
	2	10mm
	3	11mm
	4	9mm
	5	8mm
	No NaCl	10mm

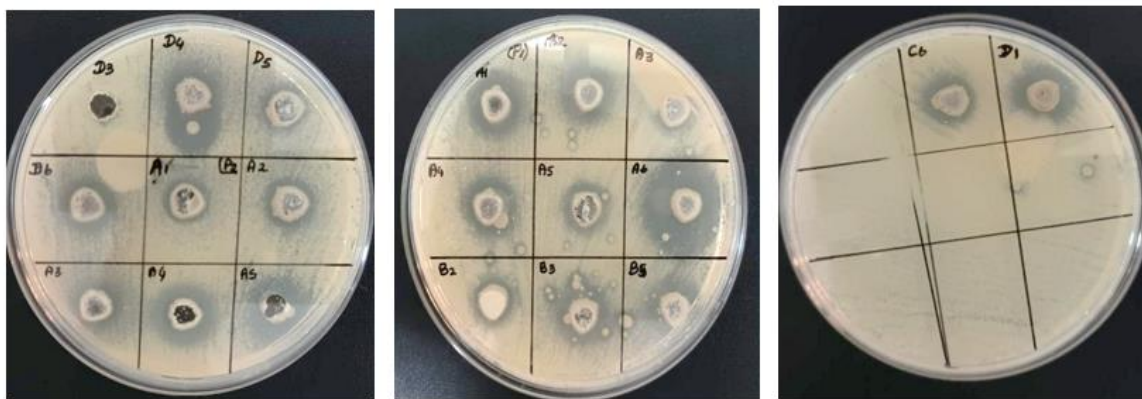


Fig 5.7: Effect of culture condition on antimycobacterial activity of potential actinobacterial strain MCD-6

5.6.4 Molecular characterization

The PCR Amplification of *Streptomyces* sp. MCD-6 16s rRNA gene yields 790 base pairs. NCBI BLAST analysis of MCD-6 shows 85% similarity to sequence of *Streptomyces novaecaesareae*. The evolutionary history which confirms distinct Phylogenetic tree construction with *Streptomyces* sp. MCD-6 designated as *Streptomyces novaecaesareae* (Fig 5.8).

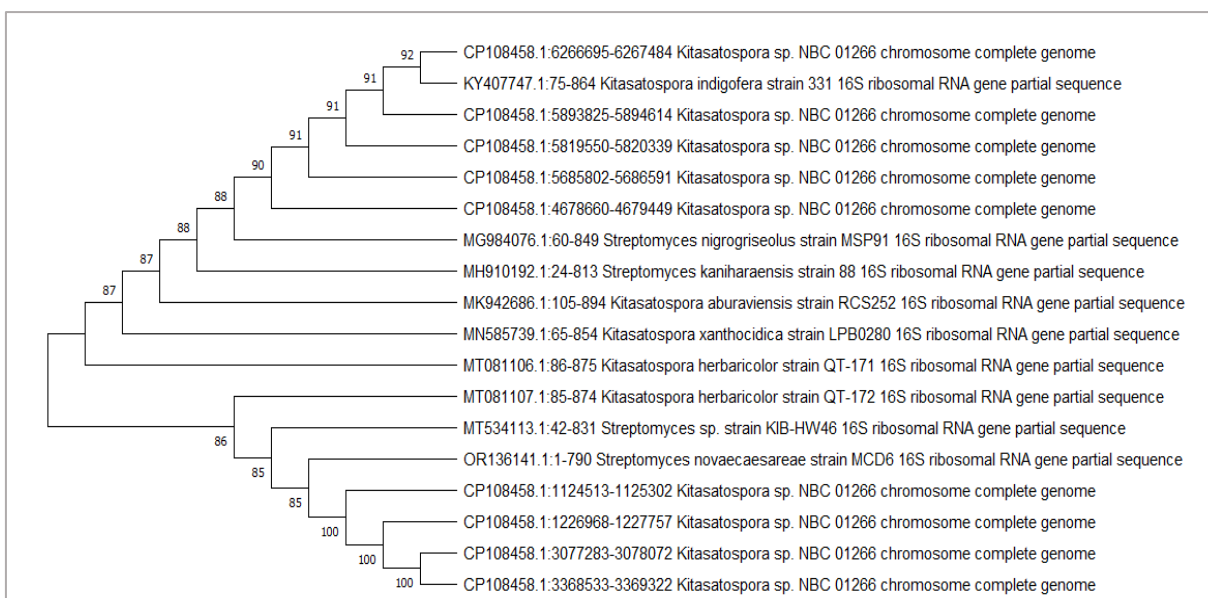


Fig 5.8: Phylogenetic tree of *Streptomyces* sp. MCD-6

5.7 ANTIMYCOBACTERIAL ACTIVITY BY MICROBROTH DILUTION METHOD (MINIMUM INHIBITORY CONCENTRATION- MIC)

Antimycobacterial activity is observed using Microplate Alamar Blue Assay (MABA) method, which is one of the Microbroth dilution method. From figure 5.9, it is understood that the culture MCD-6 shows different inhibition against *M. smegmatis* and *M. Fortitum* which is taken in various ratios. More over table 5.4 and 5.5 depicts the various percentages of crude extract obtained from MCD-6 culture to inhibit against *M. smegmatis* and *M. Fortitum* respectively.

Table 5.4: The percentage of crude extract obtained from the MCD-6 culture to inhibit *M. smegmatis*

Culture	500µg/ml	250µg/ml	125µg/ml
MCD-6	69%	22%	8%

Table 5.5: The percentage of crude extract obtained from the MCD-6 culture to inhibit *M. fortitum* strain

Culture	500µg/ml	250µg/ml	125µg/ml
MCD-6	73%	25%	15%

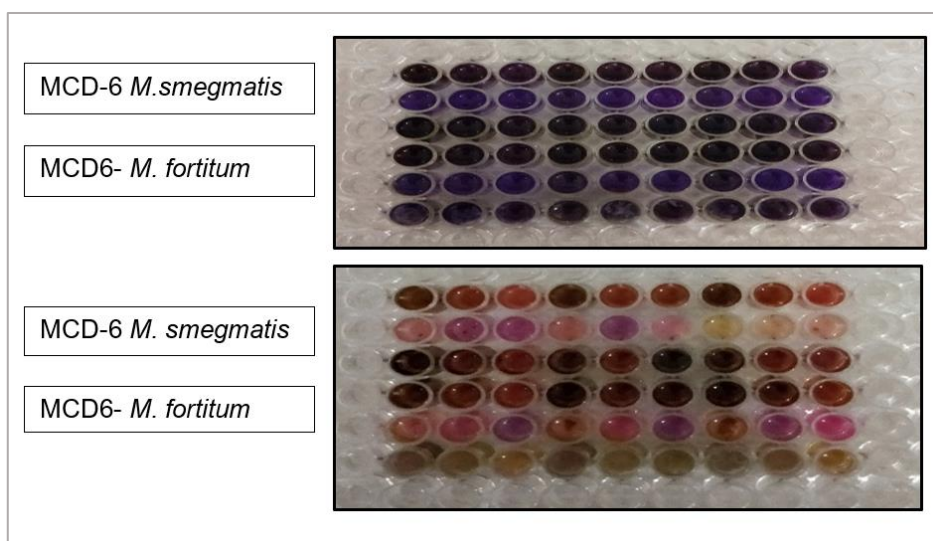


Fig: 5.9: Minimal inhibitory concentration of antimycobacterial on microtiter plates using MABA assay

CHAPTER 6

SUMMARY AND CONCLUSION

The current investigation sought to probe the potential of actinobacteria in combating antimycobacterial resistance among NTM pathogens. A total of 20 actinobacterial colonies were sourced from the Bioprospecting division of the Centre for Drug Discovery and Development, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu. The antimycobacterial efficacy of these strains was evaluated through the agar plug method. Notably, strain MCD-6 exhibited notable activity (inhibition ranging from 7.5 to 12 mm) against *M. smegmatis*. To further explore their bioactive potential, bioactive metabolites were extracted from strain MCD-6 utilizing agar surface fermentation and submerged fermentation with ISP3 agar and ISP3 broth, respectively. The antimycobacterial activity of these extracts was assessed against *M. smegmatis* using agar plug and well diffusion methods over a period of 6 days. Submerged fermentation was identified as the preferred method for subsequent investigations due to its efficacy. Crude antimycobacterial extracts from strain MCD-6 was obtained via submerged fermentation and extracted using methanol and ethyl acetate. Further examination via Minimum Inhibitory Concentration (MIC) assays revealed inhibitory concentrations ranging from 125 to 500 µg/ml against NTM pathogens including, *M. fortitum*, and *M. smegmatis*, using the Microplate Alamar Blue Assay (MABA). Microscopic, cultural, and physiological characteristics of strain MCD-6 was meticulously examined following standard procedures. Based on these phenotypic assessments, strain MCD-6 was identified as members of the *Streptomyces* genus. Moreover, the impact of various medium components on antimycobacterial compound production by strain MCD-6 was investigated utilizing the agar plug method. Noteworthy influences of different sugars, nitrogen compounds, and minerals on antimycobacterial compound production were observed. In summary, the findings of this study suggest that strain MCD-6 hold promising potential as sources for antimycobacterial metabolites against *M. fortitum*.

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