ANTIMYCOBACTERIAL POTENTIAL OF SECONDARY METABOLITES OF ACTINOBACTERIA FROM A TROPICAL MANGROVE IN NORTHERN PENINSULAR MALAYSIA

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS

°C	Degree Celsius
g/L	Gram per litre
µL	Microlitre
°C	Degree Celsius
v/v	Volume concentration of a solution

LIST OF ABBREVIATIONS

ADC	Albumin-dextrose-catalase
AMK	Amikacin
BCG	Bacille Calmette-Guérin
BDQ	Bedaquiline
CFU	Colony forming unit
CM	Capreomycin
CNS	Central nervous system
COVID-19	Coronavirus Disease 2019
DLM	Delamanid
DR-TB	Drug resistance tuberculosis
EACE	Ethyl acetate crude extracts
EMA	European Medicines Agency
EMB	Ethambutol
EPI	Expanded Program on Immunization
EPTB	Extrapulmonary tuberculosis
FDA	Food and Drug Administration
FICI	Fractional inhibitory concentration index
FQs	Fluroquinolones
GFX	Gatifloxacin
GHTB	Generalised hematogenous tuberculosis
HIV	Human immunodeficiency virus
INH	Isoniazid
IZ	Inhibition zone
KAN	Kanamycin

LFX	Levofloxacin
LTBI	Latent tuberculosis infection
LZD	Linezolid
M. bovis	Mycobacterium bovis
M. smegmatis	Mycobacterium smegmatis
M7H10	Middlebrook 7H10 agar
M7H9	Middlebrook 7H9
MDR-TB	Multidrug resistance tuberculosis
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTB	Mycobacterium tuberculosis
MTB H37Ra	Mycobacterium tuberculosis H37Ra
MTT	Thiazolyl blue tetrazolium bromide
MXF	Moxifloxacin
NA	Nutrient agar
NA	Nutrient agar
NB	Nutrient broth
NORD	National Organization for Rare Disorders
NP	Natural products
NTM	Non-tuberculous mycobacteria
OADC	Oleic acid-albumin-dextrose-catalase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMD	Pretomanid
PMDA	Pharmaceuticals Medical Devices Agency
PTB	Pulmonary tuberculosis
PTP1B	Protein tyrosine phosphatase 1B
PZA	Pyrazinamide

RIF	Rifampin
RR-TB	Rifampicin-resistant tuberculosis
SCA	Starch casein agar
SCA	Starch casein agar
STM	Streptomycin
TB	Tuberculosis
TBE	Tris-Borate EDTA
TBM	Tuberculous meningitis
TDR-TB	Totally drug–resistant tuberculosis
TEMA	Tetrazolium microplates assay
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

LIST OF APPENDICES

POTENSI AKTIMIKROB METABOLIT SEKUNDER AKTINOBAKTERIA DARI BAKAU TROPIKA DI SEMENANJUNG UTARA MALAYSIA

ABSTRAK

Aktinobakteria memainkan peranan penting dalam pengembangan antibiotik baru bagi penyakit dengan rintangan antimikrob, seperti pelbagai tuberculosis (TB) tahan ubat, yang disebabkan oleh *Mycobacterium tuberkulosis* (MTB) serta menjadi penghalang utama kawalan TB di seluruh dunia. Dalam kajian ini, metabolit sekunder aktinobakteria baru dengan potensi antimikrob terhadap organisma pengganti MTB dipencilkan dari bakau tropika di bahagian utara Semenanjung Malaysia. Objektif kajian ini adalah untuk: 1) memencil dan menyaring aktinobakteria penghasil-antimikrob dalam sampel sedimen dan air dari persekitaran bakau, 2) melakukan penilaian ekstrak kasar menggunakan ujian asai pencairan mikro tetrazolium (TEMA), ujian asai "checkerboard" dan ujian asai perencatan-masa untuk menentukan perencatan dan kepekatan minimum bakteria (MIC, MBC) dan pecahan indeks kepekatan perencatan (FICI), dan 3) mengenal pasti sebatian melalui gas kromatografi-spektrometri jisim analisis (GCMS). Sebanyak 54 pencilan aktinobakteria bakau dipencilkan dari tiga persekitaran bakau di Balik Pulau, Pulau Pinang dan Merbok, Kedah yang terdiri daripada sampel air dan sedimen dari empat tapak, iaitu lubang ketam, kawasan rhizosfera, kawasan tidak terganggu, dan kawasan terganggu. Saringan awal ke atas pemencilan ini dilakukan terhadap dua spesis organisma pengganti MTB, *M. smegmatis* dan MTB H37Ra, yang mempunyai pertumbuhan-pantas dan pertumbuhan-perlahan dengan menggunakan kaedah silang. Daripada 54 pencilan aktinobakteria bakau, 27 pencilan menunjukkan aktiviti antimikob terhadap salah satu organisma, sementara 12 pencilan menunjukkan

aktiviti antimikob terhadap kedua-dua organisma. SBh dan SBi yang menunjukkan peningkatan aktiviti antimikrob selepas penyimpanan jangka panjang dipilih untuk peningkatan jumlah penghasilan dengan menggunakan kaedah fermentasi permukaan agar. Ekstrak mentah metabolit sekunder aktinobacteria diperoleh dengan pengekstrakan pelarut etil asetat pada nisbah 1: 1. Kedua-dua ekstrak metabolit mentah (EACE) dari SBh dan SBi, yang dikenal pasti sebagai *Streptomyces* sp. berdasarkan pengenalpastian molekul 16S rRNA, menunjukkan interaksi sinergi semasa ujian asai "checkerboard". Kadar perencatan lebih daripada 99% diperhatikan dari asai perencatan-masa ketika EACE SBi etil asetat digabungkan dengan RIF. Pengenalpastian EACE aktinobakteria yang paling aktif dengan kromatografi gas-spektrometri jisim (GC-MS) mendedahkan bahawa sebatian Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3- terdapat dalam kedua-dua EACE SBh dan SBi dengan peratusan kesamaan pada 99 % dan 98 % diuruti tiga lagi sebatian individu dengan persamaan peratusan ≥90 %, dua daripadanya dikesan dalam metabolit SBh: 1) Phenol, 2,4-bis(1,1-dimethylethyl) dan 2) Benzenamine, 4-octyl-N-(4-octylphenyl)-, dan satu daripadanya dikesan dalam metabolit SBi (Hexadecanal). Kesemua sebatian ini masih belum dikaji untuk aktiviti antimikrob terhadap MTB dan dengan itu memerlukan penyelidikan lebih lanjut. Penemuan ini menunjukkan bakau tropika di utara Semenanjung Malaysia mungkin merupakan takungan aktinobakteria penghasil antimikobakteria yang boleh menjadi calon ejen anti-TB, yang akhirnya dapat menyumbang dalam mengurangkan beban TB dan meningkatkan hasil kesihatan awam dengan menyediakan rawatan alternatif dan menangani cabaran rintangan ubat.

ANTIMYCOBACTERIAL POTENTIAL OF SECONDARY METABOLITES OF ACTINOBACTERIA FROM A TROPICAL MANGROVE IN NORTHERN PENINSULAR MALAYSIA

ABSTRACT

Actinobacteria play a crucial role in discovery of new antibiotics for diseases with known antimicrobial resistance, such as multidrug-resistant tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB), which is becoming a major impediment to TB control worldwide. In this study, novel actinobacterial secondary metabolites with antimicrobial potential against MTB surrogate organisms were isolated from tropical mangroves in the northern region of Peninsular Malaysia. The objectives of this study were to 1) isolate and screen for antimycobacterial-producing actinobacteria in sediment and water samples from mangrove environments, 2) conduct crude extract evaluation using tetrazolium microplate assay (TEMA), checkerboard assay and time-kill assay to determine minimum inhibition and bactericidal concentration (MIC, MBC) and fractional inhibition concentration index (FICI), and 3) identify compounds through gas chromatography mass spectrometry (GCMS) analysis. A total of 54 mangrove actinobacterial isolates were isolated from three mangrove environments in Balik Pulau, Pulau Pinang, and Merbok, Kedah from water and sediment samples of four sites: crab holes, rhizosphere area, undisturbed area, and disturbed area. Isolates were screened against two MTB surrogate organisms, *M. smegmatis* and MTB H37Ra, which are fast-growing and slow-growing, respectively using the cross-streak method. Of the 54 mangrove actinobacteria isolated, 27 exhibited antimycobacterial activity against either organism, while 12 exhibited antimycobacterial activity against both organisms. Of

these, SBh and SBi, which demonstrated enhanced antimycobacterial activity post-storage, were chosen for larger production using agar surface fermentation. The crude extracts of actinobacteria secondary metabolites were obtained by solvent extraction with ethyl acetate at 1:1 ratio. Both ethyl acetate crude extracts (EACE) from SBh and SBi, which were identified as *Streptomyces* sp. based on their 16S rRNA gene sequences, demonstrated synergistic interaction during the checkerboard assay. A killing rate of more than 99 % was observed in the time kill-assay when RIF and SBi EACE fraction were combined. Identification of the most active actinobacterial EACE by GC-MS revealed that Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3- compound was present in both EACE of SBh and SBi with percentage similarity at 99 and 98 %, respectively, followed by three individual compounds with percentage similarity ≥90 %, two from SBh, 1) Phenol, 2,4-bis(1,1-dimethylethyl) and 2) Benzenamine, 4-octyl-N-(4-octylphenyl)-, and one from SBi (Hexadecanal). These compounds have yet to be investigated for antimycobacterial activity against MTB and thus warrant further research. These findings indicate tropical mangroves may be a good source of antimycobacterial-producing actinobacteria with anti-TB agents, that can ultimately contribute to reducing the burden of TB and improving public health outcomes by providing alternative treatments and addressing the challenge of drug resistance.

CHAPTER 1

INTRODUCTION

1.1 General introduction

According to the World Health Organization's (WHO) Global Tuberculosis Report 2021, tuberculosis (TB) is one of the oldest known human diseases, and it was one of the world's most lethal infectious diseases before the coronavirus disease 2019 (COVID-19) pandemic (WHO, 2021). TB is caused by *Mycobacterium tuberculosis* (MTB) complex, and with an estimated global population of about 7.8 billion in 2019 based on the Population and Vital Statistics Report 2020 (United Nations, 2020), TB claimed more than a million lives every year and infected about one-quarter of the world's population (WHO, 2021). While in Malaysia, in 2020, the TB incidence rate was 92 per 100,000 people, with a death rate of 4 per 100,000 people (Ministry of Health, 2018; The World Bank, 2020). With a population of 32.37 million people, the overall number of TB cases in Malaysia was estimated to be around 300,000, with roughly 13,000 deaths each year (Ministry of Health, 2018; The World Bank, 2020).

Generally, the breakdown of TB case reports is influenced by some demographic factors such as gender, age, and socioeconomic status. Compared to adult females and children, adult men are the most often infected group with TB, accounting for more than half of all reported TB cases in 2019 (WHO, 2020). According to a research done by Abongo *et al.*, in a Kenyan community, the total numbers of adult males who attended for TB screening was fewer than adults females who visited for the screening (Abongo *et al.*,

2020). This research corroborated the findings by Horton *et al.*, that have documented that the prevalence of TB is significantly higher among men than women in low- and middle-income countries, with strong evidence that men face particular barriers to seeking and/or accessing TB treatments (Horton *et al.*, 2016).

Similarly, the demographic factors are exerting the same impact on the number of TB cases reported in Sabah, an East Malaysian state. Sabah has a higher burden of TB than the national figure with a notification rate of 128 cases per 100,000 population and a TB case fatality rate of 8 % between 2012 and 2018, accounting for about 20 % of all TB cases as the state is economically less developed than the states in Peninsular Malaysia, and has a large number of immigrants from the Philippines and Indonesia living in overcrowded settlements (Goroh *et al.,* 2020). These findings were supported by Avoi and Liaw as they observed that majority of most TB deaths in Sabah were mainly involved elderly male patients from low socioeconomic background (Avoi and Liaw, 2021).

Over 95 % of TB-related deaths occur in low- and middle-income countries, where the MTB is the primary pathogen of the disease (Mohajan, 2015; Dlodlo *et al.*, 2019; WHO,2019). Even though TB treatment access has increased from approximately 6 million individuals in 2015 to 7 million individuals in 2018 and 7.1 million individuals in 2019 across the world (WHO, 2020), according to a study conducted by Marahatta *et al.*, in those low- and middle-income countries, the accessibility to health centres is still at a low efficiency due to the inconvenient location of the health centres, which is associated with the travelling cost, poor road conditions, and other factors (Marahatta *et al.,* 2020). TB patients who have limited healthcare access and receive a delayed

diagnosis may develop a more severe form of the TB disease, have a longer period of infectiousness and poor treatment outcomes, including mortality and drug resistance, as well as a wider spread of the TB disease among family members and community as they will serve as reservoirs for TB transmission (Saifodine *et al.,* 2013; Makwakwa *et al.,* 2014; Virenfeldt *et al.,* 2014; Law *et al.,* 2017; Paramasivam *et al.,* 2017; Tefera *et al.,* 2019). Furthermore, sequences from delayed diagnosis that led in the development of more severe forms of TB disease, such as drug-resistant TB (DR-TB), would force the patient to have longer treatment durations that are more complex and toxic regimens that are likely to cause a lot of side effects (WHO, 2020; Ausi *et al.,* 2021).

As drug/multidrug resistance among MTB isolates (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) emerge, TB control and treatment become more challenging. XDR-TB is defined as MDR-TB with additional resistance to fluoroquinolones and one of the second-line injectable agents as a result of inadequate dosing and incomplete treatment regimens, coupled with the ability of TB bacilli to cause latent infections tolerant to currently used drugs, and long-term therapy with drug combinations for treatment (Manikkam *et al.,* 2014; Mohajan, 2015; Hoagland *et al.,* 2016). The totally drug–resistant tuberculosis (TDR-TB) cases have also been detected in Italy, China, India, Africa, Iran, and Eastern Europe, with strains resistant to a broader spectrum of drugs than those categorized as XDR-RB (Parida *et al., 2015;* Hoagland *et al.,* 2016). Therefore, new therapeutic agents are urgently needed to address the limitations in existing drugs and the rise of DR-TB (Hoagland *et al.,* 2016). New anti-TB drugs are expected to have a novel mechanism of action to reduce cross-resistance; to be rapidly bactericidal to shorten treatment duration, with reduced side effects and an

excellent safety profile; and to have improved pharmacokinetic properties and increased potent activity against drug-resistant strains. These ideal criteria are linked with other practical goals such as cost-effective manufacturing, high compound stability, narrow spectrum of activity, and high tolerability.

Novel antibiotics compounds may be found in soil microorganisms such as actinobacteria (Anandan *et al.,* 2016; Elbendary *et al.,* 2018; Liu *et al.,* 2019). Actinobacteria have many characteristics in common with fungus and bacteria, yet many important and interesting features classify them in a category of their own. Given the growing need for new antimycobacterial compounds, as well as the reported diversity and ability of actinobacteria to produce new compounds and metabolites, mangroves harbour actinobacteria with antimycobacterial potential that have yet to be discovered. The constant changes in the mangrove environment influence the adaptation of bacterial metabolic pathways (Shivlata and Satyanarayana, 2015; Bomfim *et al.,* 2018;) and development of specific stress-defence mechanisms. This may result in the emergence of adaptive biosynthetic pathways capable of producing novel biological compounds (Capdeville *et al.,* 2019), which can be investigated through microbial secondary metabolite screening. In fact, actinobacteria produce more than 80 % of the total known microbial antibiotics which are ubiquitously isolated in both aquatic and terrestrial ecosystems, including the first anti-TB drug, Streptomycin, which was discovered in 1944 (Procópio *et al.*, 2012; Shivlata and Satyanarayana, 2015; Jose and Jha, 2016).

The present study examined the potential of actinobacteria isolated from mangrove sediment and water to inhibit the growth of the fast-growing *Mycobacterium smegmatis* (*M. smegmatis*) and the slow-growing *Mycobacterium tuberculosis* H37Ra

(MTB H37Ra) surrogate organisms. The results showed that the mangrove actinobacteria were likely to produce novel antimycobacterial compounds. Comparison of mangrove actinobacteria isolates SBh and SBi after extended storage demonstrated enhancement of antimycobacterial activity with synergistic interaction with the first-line anti-TB drug, rifampicin (RIF) during the checkerboard assay, with the combination of SBi extract and RIF demonstrated ≥99 % killing rate during time kill-assay. These findings suggested that the bioactive compounds in the extracts were sufficiently abundant to qualify these two extracts, SBh and SBi as a possible complementary antimycobacterial drug that merits further investigation.

1.2 Objectives of the study

Considering the urgent need for new anti-TB drugs or drug adjuvants that can improve activity and reduce toxicity effective, cheaper and readily available from the environment, this study was conducted to evaluate the antimycobacterial potential of metabolites from mangrove actinobacteria against surrogate MTB organisms.

Specific objectives are:

1. To isolate and identify actinobacteria and analyze environmental data from different mangrove sampling sites.
2. To evaluate the potential of mangrove actinobacteria to inhibit the growth of the fast-growing surrogate MTB organism, *Mycobacterium smegmatis* (*M. smegmatis*) and the slow-growing surrogate MTB organism, MTB H37Ra by using cross-streak and broth microdilution methods.
3. To determine the potential interactions of the ethyl acetate mangrove actinobacterial metabolite extracts with first-line anti-TB drugs using checkerboard and time-kill assay methods.
4. To identify active compounds from the extracts with high antimycobacterial and/or drug synergistic potential using gas chromatography-mass spectrometry (GC-MS).

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

2.1.1 Overview

As one of the oldest known human diseases, TB, caused by MTB, is an infectious respiratory disease that continues to be the world's major cause of mortality *(Bloom et al.,* 2017). The pathogen, MTB, which is transmitted by airborne droplets may infect those who have come into contact with infected individuals, those who live in crowded conditions with poor ventilation, or those who are immunocompromised due to illnesses and diseases such as malnutrition, diabetes, smoking, or excessive alcohol consumption (Mohajan, 2015; Bloom *et al.,* 2017; Erawati and Andriany, 2020). In addition to a chronic cough and chest pain, individuals with active pulmonary TB often have another symptoms such as weight loss, a high fever, and night sweats (Fogel, 2015; Pai *et al.*, 2018).

In the 17th century, TB was often called "phthisis," and was referred to as "the white plague". During the 18th century, a German physician named Johann Schönlein, coined the term "tuberculosis," which refers to the characteristic tubercles or small nodules that develop in the lungs and other organs affected by the disease, and was also referred to as "the captain of all these deaths" since TB was an epidemic in Europe in that century (Barberis *et al.,* 2017). In the early 19th century, the TB vaccine was developed by Albert Calmette and Jean-Marie Camille Guerin and named as Bacille Calmette-Guérin (BCG) vaccine, and has been used as part of WHO's Expanded Program on

Immunization (EPI), often given to infants and children under five to prevent TB meningitis and TB military, especially in countries where TB is common (Galagan, 2014; Li et al., 2020). In the late 19th and early 20th centuries, dedicated hospitals and sanatoriums were established to treat TB patients (Murray *et al.,* 2015). The modern way of treating and controlling of TB was ushered in by the discovery of the first anti-TB agent, streptomycin, in 1944 (Woodruff, 2014; Waters and Tadi, 2021). Given the possibility that a single drug treatment might result in bacterial resistance, effective TB regimens must combine multiple anti-TB drugs; currently, four anti-TB agents are used to treat drug sensitive TB (CDC, 2016; Kerantzas and Jacobs, 2017; Herchline and Amorosa, 2020); which are isoniazid (INH), developed in 1952 (Britannica, 2017), pyrazinamide (PZA) in 1952 (Yeager *et al.*, 1952), ethambutol (EMB) in 1961 (Thomas *et al.,* 1961), and rifampicin (RIF) in 1968 (Sensi, 1983).

2.1.2 Tuberculosis disease

TB can be classified into different types based on the state of infection and the site of infection (**Figure 2.1).**

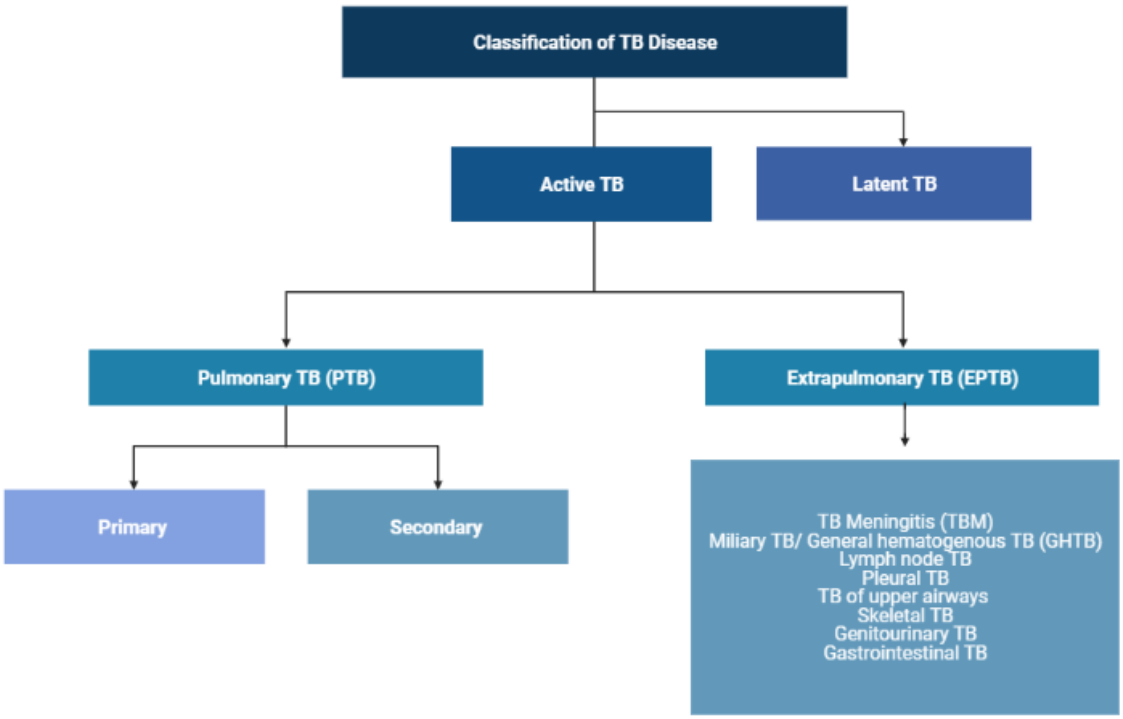

Figure 2.1 Classification of TB disease

Illustration drawn based on the official statement of the American Thoracic Society and CDC on the diagnostic standards and classification of TB in adults and children (2020)

Based on the state of infection, either latent or active TB. Latent TB infection occurs when a person is infected with the MTB but does not exhibit any symptoms or signs of active disease as the bacteria are in an inactive or dormant state. Due to its non-contagious nature, latent tuberculosis infection (LTBI) does not present any clinical signs, radiological abnormalities, or microbiological evidence (Lee, 2016) and is also not infectious because MTB cannot be transmitted from individuals who have LTBI. While, active TB disease refers to the condition where the bacteria MTB multiply and cause symptoms and clinical manifestations. It can affect various organs or systems in the body, with pulmonary TB (PTB) affecting the lungs, which is the most common type of active

TB. PTB is typically infectious and manifests with common symptoms such as cough and chest radiograph abnormality (Bhalla *et al.*, 2015; Bhatt *et al.*, 2012).

Classification based on the site of infection, either PTB or extrapulmonary TB (EPTB). PTB consists of two phases, 1) primary TB and 2) secondary TB. Primary TB mainly affects children, but it may also strike adults in areas where the disease is not as common or less endemic and in this condition, granulomatous inflammation is localized and typically limited to the periphery of the lung (Loddenkemper *et al.*, 2016; Restrepo *et al.*, 2016). It is often associated with ipsilateral lymph node involvement, which is referred to as the Ghon complex. The Ghon complex is a lung lesion seen in the lung that is caused by MTB infection (Basem, 2018). Acute lower respiratory tract infection is the most common sign of TB disease. A history of close interaction with a TB patient is the single most critical indicator of the diagnosis. The secondary TB or post-primary TB, known as reactivation or reinfection, is more common in individuals who have already been infected and whose health has declined (Wetscherek *et al.*, 2022). There is a significant rise in the severity and extent of the granulomatous inflammation, and cavitation is more prevalent in the upper lobe of the lungs (Wetscherek *et al.*, 2022).

EPTB, on the other hand, is a non-infectious disease that affects other parts of the body such as the brain, kidneys, larynx, lymph nodes, bones, or pleura and is not transmitted by the air (EPTB). Individuals with human immunodeficiency virus (HIV) infection have a higher incidence of EPTB as their immune systems are impaired, making them more vulnerable to higher instances of reactivation and spread (Qian *et al.*, 2018).

TB meningitis (TBM) is another prevalent type of TB disease of the central nervous system (CNS), which develops when the tissues surrounding the brain and spinal

cord get infected with TB (Marx and Chan, 2011). National Organization for Rare Disorders (NORD) reports that it often affects children between the ages of one and five, but it can affect anyone at any age (NORD, 2021). In addition to TBM, military TB, which is an exceedingly rare type of TB that may be fatal if left untreated, and generalized hematogenous TB (GHTB) is a type of TB infection that occurs when the bacteria MTB spread through the bloodstream to various organs and tissues in the body. GHTB can affect multiple organs simultaneously, leading to a systemic infection and was more prevalent before the advent of the BCG vaccination (Ray *et al.,* 2013).

2.1.3 Current epidemiology of tuberculosis infection

TB is declining by approximately 2 % per year, with a cumulative decline of 9 % between 2015 and 2019 (WHO, 2019). However, the COVID-19 outbreak has halted the progress that had been accomplished in recent years in providing essential TB care and reducing the global burden of TB disease. According to the WHO's Global Tuberculosis Report 2021, the number of newly diagnosed and reported TB patients decreased from 7.1 million in 2019 to 5.8 million in 2020, indicating that fewer individuals have access to TB diagnosis and treatment, which has ultimately led to a rise in the number of TB deaths (Chakaya *et al.,* 2021; WHO, 2021). In 2019, TB caused death worldwide with a total of 1.4 million deaths, with approximately 1.2 million deaths in HIV-negative people and 209 000 deaths in HIV-positive people. However, it is estimated that TB deaths among HIV-negative people would reach 1.3 million in 2020, with an additional 214 000 deaths among HIV-positive people, up from 209 000 in 2019 (WHO, 2021).

In 2019, nearly half a million people worldwide developed rifampicin-resistant TB (RR-TB), of whom 78 % had MDR-TB. Of the total reported cases of MDR/RR, 3.3 % are new TB cases and 18 % are previously treated MDR/RR (WHO, 2020). Aside from the significant number of unreported and untreated TB cases in 2020, a bigger number of people with MDR-TB are missing with estimation of only 41 % of people out of half of million people that developed RR-TB and MDR-TB were identified (Chakaya *et al.*, 2021). Consequently, the emergence of DR-TB strains of MTB worldwide is increasing the burden and incidence of TB, making control and treatment more challenging and to date a public health crisis.

Malaysia is not included within the top 30 countries with high burden TB incidence listed by WHO, but according to the Ministry of Health Malaysia's Annual Report 2018, the number of TB cases reported and the mortality rate due to TB are among the highest in Malaysia when compared to other infectious diseases (MOH, 2018). Malaysia recorded 92 TB cases per 100,000 people in 2022, with a mortality rate of 4 cases per 100,000 population, putting Malaysia in the category of countries with moderate TB burden (Ministry of Health, 2018; The World Bank, 2020).

2.1.4 Current treatment and diagnosis of tuberculosis

TB is an airborne disease that can easily spread from person to person and become contagious when a person with active TB coughs, sneezes, or talks, aerosoling saliva containing MTB. The released respiratory droplets into the air may contain MTB, and if inhaled by individuals in close proximity, they can become infected (Turner *et al.*,

2017; Patterson and Wood, 2019). A person with TB disease shows symptoms that vary, depending on where the TB bacteria are growing and in most cases, the bacteria attack the lungs, which are rich in oxygen where they begin to multiply (Erawati and Andriany, 2020). TB disease occurs when first-line defenses, consisting of airway epithelial cells and phagocytes, including neutrophils, monocytes, and dendritic cells, which are basically responsible for defending against invading pathogens, are unable to rapidly eliminate MTB upon inhalation, resulting in phagocytes becoming infected with MTB and actively multiplying within the cells leading to TB disease (Middleton *et al.,* 2002; Günther and Seyfert, 2018; Martino *et al.,* 2019). Currently, all types of TB are diagnosed mostly on the basis of clinical suspicion and radiographic evidence, such as chest radiography (Loddenkemper *et al.,* 2016).

TB diagnostic tests are divided into two groups: those that directly detect MTB, and those that measure the immunological response to MTB. Ziehl-Neelsen staining, acid-fast staining, and sputum culture are all direct methods for detecting MTB. Interferon gamma release tests and serodiagnostic assessment of MTB-specific antibodies are examples of indirect detection approaches. The Mantoux tuberculin skin test evaluates the degree of hypersensitivity to tuberculin by measuring the host reaction to MTB exposure.(Chan *et al.,* 2000; MOH, 2018; Méchaï *et al.,* 2020).

By taking multiple drugs for six to nine months, there is high probability of treating TB disease (Adisa *et al.,* 2021). INH, RIF, EMB, and PZA are the approved drugs by U.S. Food and Drug Administration (FDA) that form the core of treatment regimens as first-line anti-TB agents (Ben *et al.,* 2015). However, incomplete treatment can lead to disease relapse and promotes the development of resistance against available

drugs, making future treatment more difficult, more toxic, less promising, and more expensive.

There are three types of drug resistant TB, which are designated as MDR, XDR and TDR-TB strains. Second-line anti-TB agents are used to treat TB that has become resistant to first-line anti-TB agents. The approved FDA anti-TB second-line agents include kanamycin (KAN), streptomycin (STM), capreomycin (CM), amikacin (AMK), bedaquiline (BDQ), delamanid (DLM), linezolid (LZD), pretomanid (PMD) and fluroquinolones (FQs) such as levofloxacin (LFX), moxifloxacin (MFX) and gatifloxacin (GFX). KAN, CM or AMK which are injectable and FQs are the common second-line anti-TB agents in treating MDR-TB (Padda and Muralidhara, 2021). The type of TB infection of the patients, whether active or latent, dictates the drug combination and treatment length required to treat the disease (Parekh and Schluger, 2013).

MDR-TB develop resistance to at least one of the first-line anti-TB drugs such as INH or RIF, MTB strains with XDR develop resistance to both INH and RIF, FQs, and to at least one of the three second-line injectable anti-TB drugs such as AMK, CM or KAN; or they may develop resistance to both the first-line and second-line anti-TB drugs, while TDR develops stronger resistance compared to XDR as it becomes resistant to all first and second-line anti-TB drugs (Gupta *et al.,* 2010; Galagan, 2014; Parida *et al.*, 2015).

In the combat against MDR-TB, treatment options are constantly evolving (Bloom *et al.*, 2017). Patients with MDR-TB strains are eligible for treatment with a shorter regimen which typically lasts around 9-12 months, but not patients with XDR-TB strains. It is important to test patients for susceptibility or resistance to fluoroquinolones and second-line injectable agents before initiating the shorter MDR-TB regimen. Patients

confirmed to have XDR-TB strains are not eligible for the shorter MDR-TB regimen due to the extent of drug resistance. Instead, they are typically switched to a conventional MDR-TB regimen, which includes a longer duration of treatment and the use of second-line or reserve drugs. This helps ensure that patients are not treated with drugs to which they are already resistant, optimizing the effectiveness of the treatment. Treatment of MDR-TB and XDR-TB has always been more difficult as it requires the use of second-line or reserve drugs, which are more expensive and can cause more side effects. In addition, the prescribed courses of the drugs are much longer and can last up to two years (Kanabus, 2020).

2.1.5 *Mycobacterium tuberculosis* test organisms

MTB, the etiological agent of TB, is highly virulent and requires biosafety level 3 (BSL-3) facilities, which are not available in most/non-clinical laboratories. A number of surrogate organisms such as attenuated MTB or non-tuberculous mycobacteria (NTM), which are less pathogenic and safer to handle in biosafety level 2 (BSL-2) laboratories, are often used as alternatives to replace the MTB in drug discovery research based on their comparable drug response and genetic profiles.

2.1.5a *Mycobacterium smegmatis (M. smegmatis)*

Mycobacterium smegmatis is a non-pathogenic-fast-growing-mycobacterium, as it has a generation time of three hours and lives mainly in the layers cells that join together to form a group known as biofilm which has been commonly found in soil, water and

plants (T *et al.,* 2020). *Mycobacterium smegmatis* has a high efficiency for transformation because it has many traits and similar genetic sequences with pathogenic MTB. which enables *M. smegmatis* to imitate the characteristics of MTB (Wallace *et al.,* 1988; Wang *et al.,* 2005; Yu *et al.,* 2006). Moreover, *M. smegmatis* is an ideal surrogate TB, as it has a similar profile to MDR-MTB due to its high resistance ratios against INH and RIF, which is similar to resistance ratio of MDR-MTB (Chaturvedi *et al.,* 2007), although the mechanism of resistance may differ. In fact, as reported by Lelovic *et al., M. smegmatis* strains demonstrate drug sensitivity comparable to the virulent laboratory strain, MTB H37Rv, with susceptibility to 20 antibacterial agents, including first- and second-line anti-TB drugs. (Lelovic *et al.,* 2020).

2.1.5b *Mycobacterium tuberculosis* H37Ra (MTB H37Ra)

MTB H37Ra is the avirulent counterpart of the virulent laboratory strain of MTB H37Rv. It is a slow-growing attenuated strain of tubercle bacilli that is most commonly used as a surrogate for studying the pathogenesis and virulence of MTB and in the study of new anti-TB drugs because it is non-pathogenic and can be cultured in Biosafety Level 2 facilities (Brosch *et al.,* 2002; Soto *et al.,* 2002). Since the 1940s, the MTB H37Ra strain has been extensively used as a reference strain in research of the pathogenesis of MTB and has been employed in vaccination as an adjuvant to boost the immunogenicity of the vaccine (Zheng *et al.,* 2008). Both the MTB H37Ra and MTB H37Rv strains are descended from their highly infectious parent strain, MTB H37, which was first identified from a patient suffering from chronic pulmonary TB (Zheng *et al.,* 2008). Mutations in

protein expression and structure are the underlying cause of the difference in virulence that may be seen between MTB H37Ra and MTB H37Rv (Jena *et al.,* 2013). In comparison to MTB H37Rv, the MTB H37Ra strain was found to include a total of 53 insertions and 21 deletions, in addition to the 198 single nucleotide variations (SNVs), 102 transitions, and 96 transversions (Zheng *et al.,* 2008).

2.1.6 New anti-tubercular drug candidates

For the first time in over half a century, the US FDA authorised a new anti-TB drug for human use in 2012, BDQ (FDA, 2012). In 2014, another new anti-TB drug, DLM obtaining the approval from the European Medicines Agency (EMA) and the Pharmaceuticals Medical Devices Agency (PMDA) in Japan (EMA, 2014; Otsuka Pharmaceutical Co., 2014), as well as conditional approval by the WHO based on WHO Consolidate Guidelines on Drug-Resistant Tuberculosis Treatment published in 2014. DLM may be included in the TB treatment of MDR-TB/ RR-TB in children 3 years old or older (WHO, 2020a). However, both anti-TB drugs, on the other hand, are associated with adverse effects and are only suggested for individuals who do not have access to other treatment options. Given the constraints on BDQ usage and the fact that XDR and TDR strains can only be treated with anti-TB drugs, there must be many more compounds discovered in developing new anti-TB drugs in order to completely eradicate the TB epidemic (Quan *et al.,* 2017).

Ideal characteristics for a new anti-TB drug are 1) to have a novel mechanism of action, 2) be promptly bactericidal, 3) have fewer side effects and an outstanding safety

profile; and 4) have enhanced pharmacokinetic features and greater potency against drug resistant strains. As currently available anti-TB drugs have resulted in many side effects, many patients with chronic diseases seek for complementary drugs such as herbs or vitamin supplements as an alternative (Yang *et al.*, 2018). For examples the first-line anti-TB drugs, INH and STM have been associated to liver problems, as well as hepatic reactions, skin reactions and digestive problems (Castro *et al.*, 2015; Cojutti *et al.*, 2016). Other practical aims like cost-effective manufacture and high compound stability, as well as a restricted range of action and excellent tolerability, are also associated with these ideal characteristics. A new actinobacteria-derived anti-TB drug is expected to meet those ideal criteria, since natural products (NP) have been extensively utilized in addressing various human diseases (Girão *et al.*, 2019). In fact, active compounds of actinobacteria NP have been reported to have the ability to inhibit the growth of microorganisms, viruses, cancer cells and tumors (Dharmaraj, 2010; Manivasagan *et al.*, 2014; Hassan and Shaikh, 2017).

2.2 Tropical mangrove actinobacteria as a source of new antimycobacterial agents

2.2.1 Overview

New bioactive compounds are urgent due to the emergence of antibiotic resistant pathogens such as MDR, XDR and TDR strains of MTB. Actinobacteria, the well-known bioactive compounds producer particularly the genus *Streptomyces* has significantly contributed to the development of antibiotics, with approximately 45% and more than

80% of currently available antibiotics (Das *et al.,* 2018; Elbendary *et al.,* 2018). It is also becoming increasingly evident that natural and unexplored habitats are rich with new sources of actinobacteria for interesting novel bioactive metabolites (Das *et al.,* 2018; Jiang *et al.,* 2018). Hence, many researchers have expanded their research to investigate new and extreme environments such as mangroves for the discovery of novel pharmaceutical compounds. Mangroves are a unique ecosystem that thrives in intertidal zones, typically between the sea and land, and experiences daily tidal cycles, resulting in regular changes in water levels which leads to fluctuations in salinity, moisture, and oxygen availability (Xu *et al.,* 2014; Liu *et al.,* 2019). Mangroves possess specific characteristics that are only habitable to microorganisms and plants that can withstand the unique and challenging conditions of the habitat, which include frequent large fluctuations of conditions. Compared to many other environments or ecosystems, mangroves offer a distinct set of conditions that only certain organisms have adapted to survive and thrive in (Zenova *et al.,* 2011; Shivlata and Satyanarayana, 2015; Jiang *et al.,* 2018).

Due to constant changes in environmental factors in the mangrove ecosystem, such as pH, salinity, temperature, moisture, nutrients and tidal gradient, microbes living in mangroves drive the enhanced adaptations of metabolic pathways in the microbes residing in mangroves (Zenova *et al.,* 2011; Shivlata and Satyanarayana, 2015; Jiang *et al.,* 2018). As a result, there is a high potential for isolation of new bioactive compounds from microorganisms living in mangrove forests. Actinobacteria, in particular, are known for their ability to thrive in diverse habitats, including mangroves (Zenova *et al.,* 2011; Shivlata and Satyanarayana, 2015; Hassan and Shaikh, 2017). About ten to twenty

secondary metabolites are potentially produced by each actinobacteria strain (Bentley *et al.,* 2002; Sosio *et al.,* 2000) due to their ability to produce various chemical and physiological structures, leading to diverse biological activities (Janardhan *et al.,* 2014; Zenova *et al.,* 2011). The production of secondary metabolites is thought to be an adaptive mechanism that helps actinobacteria survive in hostile and adverse conditions.

2.2.2 Actinobacteria

Phylum Actinobacteria is regarded as one of the most significant taxonomic units among the lineages that comprise the Bacteria domain (Ludwig *et al.,* 2012). Actinobacteria are Gram positive bacteria with high guanosine-cytosine (GC) DNA content, and they exhibit a filamentous, fungi-like morphology and have a filamentous fungi-like type of bacteria (Barka *et al.,* 2015; Chamikara, 2016; Jose and Jha, 2016). Actinobacteria, despite their filamentous appearance resembling fungi, are classified as bacteria due to their distinct features and differences from fungi. One of the key distinguishing factors is the composition of their cell walls, as Actinobacteria have cell walls that do not contain chitin and cellulose, which are characteristic components of fungal cell walls (Chamikara, 2016). Instead, the cell walls of Actinobacteria are composed of peptidoglycan, a key feature of bacterial cell walls (Rahlwes *et al.,* 2019).

Actinobacteria, also known as actinomycetes, were derived from Greek that means “ray fungi” and were firstly discovered through the discovery of a filamentous organism in a concretion from a human lacrimal duct and named as *Streptothrix foersteri* by Ferdinand Cohn in 1875 (Sowani *et al.,* 2017). More than 80 % of all known

microbial antibiotics are derived from actinobacteria, including the first anti-TB drug Streptomycin which was discovered in 1944 (Procópio *et al.,* 2012; Shivlata and Satyanarayana, 2015; Jose and Jha, 2016). The extensive secondary metabolism shown by actinobacteria are closely related to their ability to thrive in unique environments and under challenging conditions (Meklat *et al.,* 2011; Bull and Goodfellow, 2019), making actinobacteria continue to be a rich source of potential drug candidates and have contributed significantly to the field of antibiotic discovery and development (Shivlata and Satyanarayana, 2015; Jose and Jha, 2016), which are still widely used in the treatment of human diseases (Dharmaraj, 2010; Manivasagan *et al.,* 2014; Hassan and Shaikh, 2017). Since the discovery of streptomycin, natural product research on actinobacteria has captivated researchers and led to the development of a slew of life-saving antibiotics (Jose and Jha, 2016).

2.2.3 Taxonomy and classification of actinobacteria

The "Bergey's Manual of Systematic Bacteriology" is a widely recognized reference in the field of bacterial taxonomy and classification (Whitman *et al.,* 2012). Volume 5 of the manual divides Phylum Actinobacteria into six classes namely Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria and Thermoleophilia which is further divided into 16 orders (**Figure 2.2**).

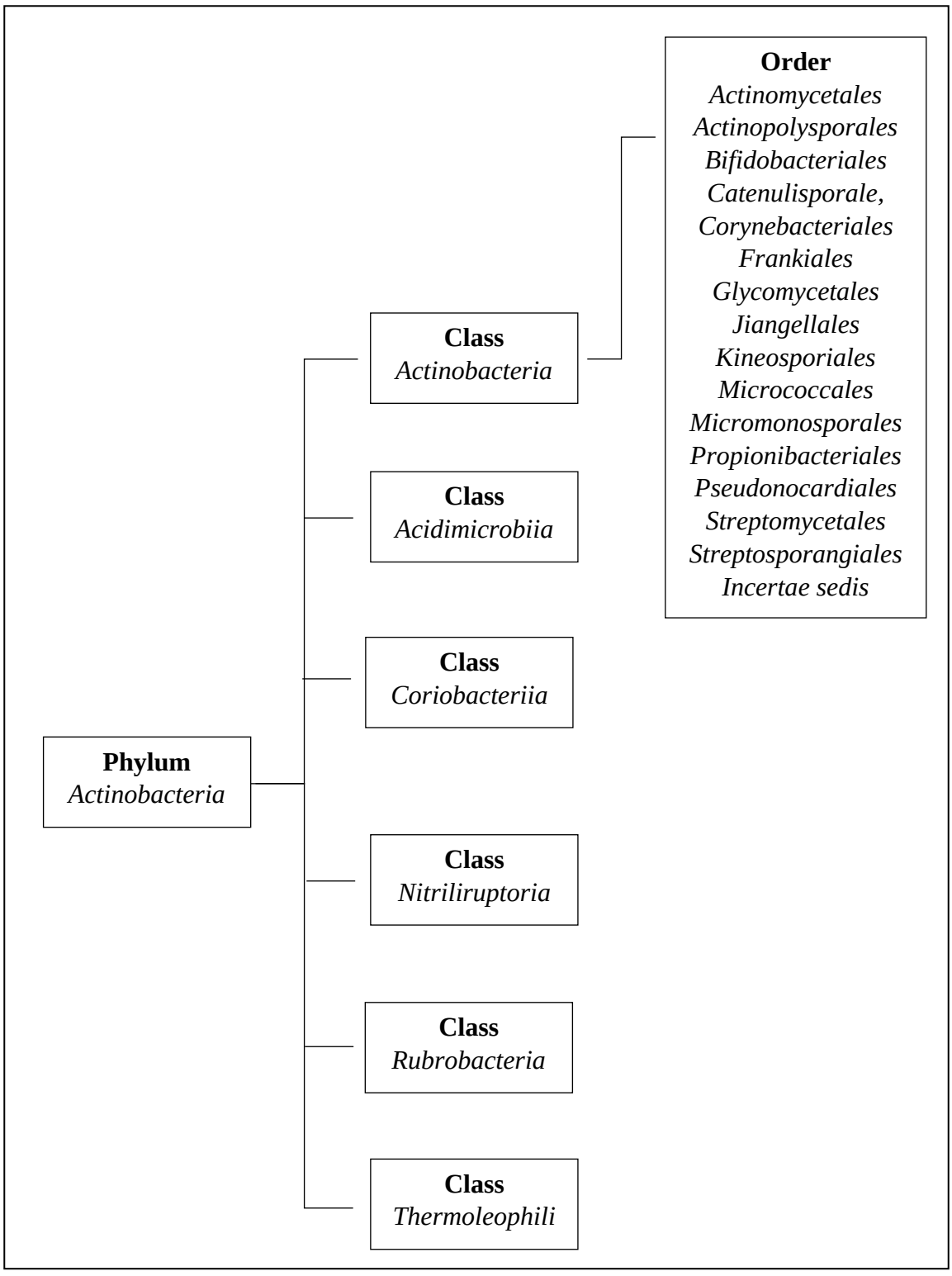

Figure 2.2: Proposed taxonomic branching for actinobacteria in the Bergey's Manual of Systematic Bacteriology (Whitman *et al.*, 2012).

2.2.3a Growth characteristics of actinobacteria

The growth of actinobacteria is strongly influenced by the availability of nutrients. In contrast to other bacterial species, actinobacteria exhibit a versatile metabolic capacity and can utilize a wide range of including starch, glucose, amino acids, and proteins, for their growth and energy production (van Bergeijk *et al.*, 2020). Study by van Bergeijk et al. (2020) has highlighted the broad range of substrates that utilized by actinobacteria as energy sources have allowed actinobacteria to adapt to different nutrient conditions and thrive in diverse environments.

2.2.3b Laboratory cultivation of actinobacteria

It is essential to choose an appropriate medium while isolating bacteria from their respective environments as isolation media are often associated a specific genus or species of bacteria (Schneegurt and Vreeland, 2012; El Karkouri *et al.*, 2019). Various isolation media have been suggested for the isolation of actinobacteria, including Kuster's agar, starch casein agar, actinomycetes isolation agar, and starch nitrate agar, as well as ISP medium No.2 and No.4 with addition of cycloheximide and nalidixic acid as antifungal and antibacterial substances (Baskaran *et al.*, 2011; Lee *et al.*, 2014; Kumar and Jadeja, 2016). These media often contain specific carbon and nitrogen sources, as well as minerals necessary for the growth and isolation of actinobacteria. According to a previous study, starch casein agar (SCA) has been shown to be a favorable medium for the isolation of actinobacteria due to its high carbon-to-nitrogen ratio. This is contrasted with glycerol asparagine agar, humic acid agar, and glucose yeast extract malt extract

agar, which all produced less favorable conditions for actinobacteria isolates (Yu *et al.*, 2015; El Karkouri *et al.*, 2019). SCA, which is required to isolate actinobacteria, consist of glucose, glycerol, or starch as carbon sources; nitrate or casein as nitrogen sources; and minerals such as sodium chloride (NaCl), dipotassium phosphate (K2HPO4), magnesium sulphate heptahydrate (MgSO4.7H2O), calcium carbonate (CaCO3) and iron (II) sulphate heptahydrate (FeSO4.7H2O). To avoid the growth of bacterial and fungal contaminants in isolation media, numerous actinobacteria isolation-related publications recommend adding antibacterial and antifungal agents as well as fungicides (Corke and Chase, 1956).

As actinobacteria are slow-growing bacteria, pretreatment is crucial to suppress or eradicate undesirable microorganisms and facilitate the separation of certain actinobacteria species (Tiwari and Gupta, 2012; Subramani and Aalbersberg, 2013; Jiang *et al.*, 2016a). Pretreatments help to enhance the isolation and separation of actinobacteria from soil or water samples. Chemical or physical pretreatments may be used since actinobacteria spores are resistant to desiccation. Physical treatments include dry heat, wet heat, air dry, moist incubation with radiation, glycerol, and centrifugation; chemical treatments include phenol, SDS, calcium chloride, calcium carbonate and chitin treatment, yeast extract, germicide, chemotactic agents, and chloramine-T. Actinobacteria may be isolated by heating dry soil or soil suspensions. Dry heat at 100 °C has been shown to be an ideal treatment since it eliminates a significant number of contaminating bacteria and fungi, thereby enhancing the growth of actinobacteria while inhibiting the growth of other microorganisms, especially Gram-negative bacteria that have a thinner peptidoglycan layer in their cell walls, making them more susceptible to desiccation and other environmental stresses (Jiang *et al.*, 2016a).