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ACTINOMYCETES FROM BRIS SOIL AND THEIR SECONDARY METABOLITES

ALI ARKAN MAJHOOL

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FACULTY SCIENCE AND MATHEMATICS
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ABSTRACT

A total of 155 actinomycete strains were isolated from Beach Ridges Interspersed with Swales (BRIS) soils. Samples were collected at various depths from the earth surface and subsurface in Setiu district, Terengganu. The isolates were assessed for their antibacterial activity against a panel of microorganisms (*Escherichia coli* DSM30083^T, *Bacillus subtilis* DSM10^T, *Pseudomonas fluorescens* DSM 50090^T, *Klebsiella pneumoniae* DSM30104^T, *Micrococcus luteus* DSM20030^T, and *Saccharomyces cerevisiae*). Results indicated that 65% of all isolates were active against, at least, one of the test organisms. Thirty-three of the isolates were selected and characterized by conventional methods and 16S rRNA gene sequence analysis revealed that most of the isolates belonged to the genus *Streptomyces sp*. (15 isolates) and 8 other rare genera namely *Microtetrapora sp.* (5 isolates), *Streptacidiphilus sp.* (3 isolates), *Actinoplanes sp.* (3 isolates), *Microbispora sp.* (2 isolates), *Rothia sp.* (1 isolate), *Micromonospora sp.* (2 isolates), *Amycolatopsis sp.* (1 isolate), and *Sacharopolyspora sp.* (1 isolate). Isolates that showed antibacterial activities were subjected to metabolite extraction and further analysis. Extracts exhibited a wide range of antimicrobial activities that varied with isolates and concentration of each extract. Thirty-nine compounds were detected both from *Streptomyces sp.* AA13 and *Amycolatopsis sp.* AA12, and thirty-eight compounds from *Micromonospora sp*. AA141, using GC-MS. Whole genome Sequence analysis conducted on isolate *Amycolatopsis sp* AA12 produced a total of 5,304,429 paired-end reads and 51 potential secondary metabolites coding gene clusters. Based on the analysis of metabolites from *Amycolatopsis sp*. (AA12) using NMR spectroscopy, the polyphenolic groups were isolated that including quercetin ($C_{15}H_{10}O_7$), rutoside ($C_{27}H_{30}O_{16}$), isotrifoliin ($C_{21}H_{20}O_{12}$), and 3, 5, 6,7,3,4 heptahydroxy flavone. Most of the polyphenolic compounds exist as sugar conjugates. The biosynthesis of dTDP-4-dehydrorhamnose 3, 5 -epimerase in *Amycolatopsis sp.* (AA12) is influenced by at least ten genes. The DTDP-sugars, derived from glucose 1-phosphate, are involved in the biosynthesis of polyphenolic compounds.

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Abstrak

Sebanyak 155 aktinomiset dipencilkan daripada sampel tanah Beach Ridges Interspersed with Swales (BRIS), di Setiu, Terengganu yang diambil dari kedalaman berbeza. Ujikaji ativiti antimikrob terhadap panel mikroorganisma yang terdiri daripada *Escherichia coli* DSM 30083, *Bacillus subtilis* DSM 10^T, *Pseudomonas fluorescens* DSM 50090^T, *Klebsiella pneumoniae* DSM 30104^T, *Micrococcus luteus* DSM 20030^T and *Saccharomyces cerevisiae* telah dijalankan. Dapatan kajian menunjukkan 65% pencilan menghasilkan zon perencatan terhadap sekurang-kurangnya satu mikroorganisma ujian. Analisis filogenetik berdasarkan jujukan gen 16S rRNA telah dilakukan ke atas 33 pencilan dan hasil menunjukkan 15 pencilan merupakan genus *Streptomyces* manakala pencilan lain terdiri daripada genus *Actinoplanes*, *Microbispora*, *Rothia*, *Micromonospora*, *Amycolatopsis* dan *Sacharopolyspora.* Kajian lanjutan melibatkan pengekstrakan metabolit dan alisis kimia ekstrak metabolit dijalankan ke atas pencilan yang menghasilkan banyak aktiviti antimikrob. Hasil menunjukkan kepelbagaian aktiviti antimikrob yang ditunjukkan pada kepekatan ekstrak berbeza. Sebanyak 39 unsur berjaya dikesan daripada pencilan *Streptomyces sp.* AA13 and *Amycolatopsis sp.* AA12, manakala 38 unsur dikesan daripada pencilan *Micromonospora sp*. AA141 berdasarkan analisis kromatografi gas- mass sektrofotometri (GC-MS). Penjujukan Seluruh Genom (WGS) telah dijalankan ke atas pencilan *Amycolatopsis sp.* AA12 yang menghasilkan 5,304,429 bacaan 'paired-end' dan 51 kluster gen yang mengkod metabolit sekunder telah dikesan. Berdasarkan analisis metabolit menggunakan spektroskopi Nuclear Magnetic Resonance (NMR), pencilan *Amycolatopsis sp.* AA12 didapati menghasilkan metabolit dari kumpulan polifenolik yang dikenalpasti sebagai quercetin ($C_{15}H_{10}O_7$), rutoside ($C_{27}H_{30}O_{16}$), isotrifoliin ($C_{21}H_{20}O_{12}$), dan 3,5,6,7,3,4 heptahydroxy flavone.Kebanyakan unsur polifenolik wujud dalam bentuk gula konjugat di mana biosintesis dTDP-4-dehydrorhamnose 3, 5 -epimerase telah didapati dalam 10 gen pencilan *Amycolatopsis sp.* AA12 manakala gula dTDP yang terhasil dari glukosa-1-fosfat terlibat dalam biosintesis tapak jalan polifenolik.

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CHAPTER 1

INTRODUCTION

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1.1 Introduction

The emergence and spread of multidrug-resistant (MDR) pathogens and a lack of treatment options for infections and high mortality rates (Santajit & Indrawattana, 2016). It led to an urgent need to discover new high-effective and productive antimicrobial agents, there has been a weakness in the discoveries of a new antibiotic in the past 30 years despite the scientific development (Lin et al., 2018).

Microorganism's natural products remain the encouraging source of new therapeutic drugs (Kapur et al., 2018). Since the discovery of penicillin, interest has increased in microorganisms for its high ability to produce active compounds including

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antivirals, antimicrobials, and immunosuppressive compounds (Kaur & Teotia, 2019). Actinomycetes have proven as one of the main contributors due to their capacity to produce several types of biologically active natural compounds like herbicides, antibiotics, anti-parasitic, pesticides, and enzymes (Sankar, 2016).

Actinomycetes are a group of Gram-positive bacteria, unicellular filamentous that form a branching network of filaments and produce spores. They have been recognized as sources of odours in drinking water (Mallevialle & Suffet, 1987; Zhou et al., 2017) that spread vastly throughout different environments (Idris et al., 2017). Actinomycetes are particularly abundant in soil (Kinkel et al., 2014) and many species have been isolated from natural caves (Lee, 2006; Belyagoubi et al., 2018), clinical material (Jones et al., 2004; Blyskal et al., 2017), ocean sediments (Weyland, 1969; Kamjam et al., 2017), and plant roots (Araújo et al., 2000; Kuncharoen et al., 2019). They are playing a major role in soil ecology; they produce many enzymes that support the prosperity of organic plant material, chitin, and lignin. Thus their presence is necessary for the formation of compost for plants (Bhatti et al., 2017). Gerber & LeChevallier, (1965) were the first to isolate and to identify the terpenoide geosmin, trans-1, 10-dimethyl-trans-9-decalol, a potent earthy-smelling compound, from actinomycetes cultures.

Actinomycetes are deemed highly valuable for produce several types of biologically natural useful compounds like antibiotics, antivirus, immunosuppressive agents, nutritional materials, enzymes, herbicides, pesticides, anti-parasitic agents, and

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vitamins (El Karkouri & El Hassouni, 2019). At least 70% of the natural compounds are produced from actinomycetes (Pimentel et al., 2010; Sankar, 2016). Actinomycetes have been major stored for the discovery of natural compounds (Choi et al., 2018). *Streptomyces* is the best-characterized genus of actinomycetes, and it is one of the most important types of commercial bacteria depending on its capabilities to producing great secondary metabolites and bioactive natural compounds (Matsumoto & Takahashi, 2017). The rare actinomycetes are deemed as an important source for new bioactive compounds, vitamins, and enzymes with a wide range of biological activities and pharmacological features (Jakubiec et al., 2018).

Actinomycete has been taxonomically into a phylum Actinobacteria (Goodfellow et al., 2012a; Buedenbender et al., 2017). Whereas Actinobacteria considered one of the largest taxonomic while the phylum Actinobacteria is described based on its branching region in 16S rRNA gene trees. Nevertheless, rRNA sequences do not separate properly between nearly related species or also genera, which leads to vagueness. Currently, the enormous modern increase in the availability of genome sequence information has presented specific information into genome development and had become possible to classify genes specific to microorganisms at the level of genera and family (Ouchari et al., 2019).

Actinomycetes belong to the order of Actinomycetales and extend to the family Actinomycetaceae and the different suborders that belong to Actinobacteria. Forty-three of the fifty-three families within the phylum Actinobacteria are attached to a

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single class. Actinobacteria, whereas the other five classes together include just ten families (Buedenbender et al., 2017). Actinomycetes had been identified more as a free group of organisms, which is nearly related to the bacteria during some of the morphological patterns, maybe has adopted a fungus-like pattern of growth (Mohammadipanah & Dehhaghi, 2017).

One major problem in drug discovery attempts is the rediscovery of known compounds (Buedenbender et al., 2017). Therefore the right search and discovery strategies are important to look for novel compounds. Actinomycetales are one of the important types to produce new natural compounds, notwithstanding the difficulty of detecting new compounds of actinomycetes. But by relying on the idea that said the new species of actinomycete produce new compound and thus rely on the principle of taxonomic diversity is an alternative to chemical diversity. Figure 1.1 shows the bioprospecting strategies. The first step depends on selective isolation procedures, the identification of target actinomycetes, and the subsequent choice of representative strains for screening. These steps are heavily dependent on developments in actinomycete systematics (Goodfellow, 2010; Mohammadipanah & Dehhaghi, 2017).

The remaining steps are specifically the definition of the wanted characteristics in dereplicated strain archives using whole genome sequence, suitable fermentation conditions, primary screening of fermentation broths using HPLC-diode array screening, the discovery of metabolite novelty practising an in-house HPLC-UV-visual database and structural chemical explanation of active systems. The next steps in the

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method include the complete taxonomic characterization of strains producing exciting successes (Goodfellow & Fiedler, 2010).

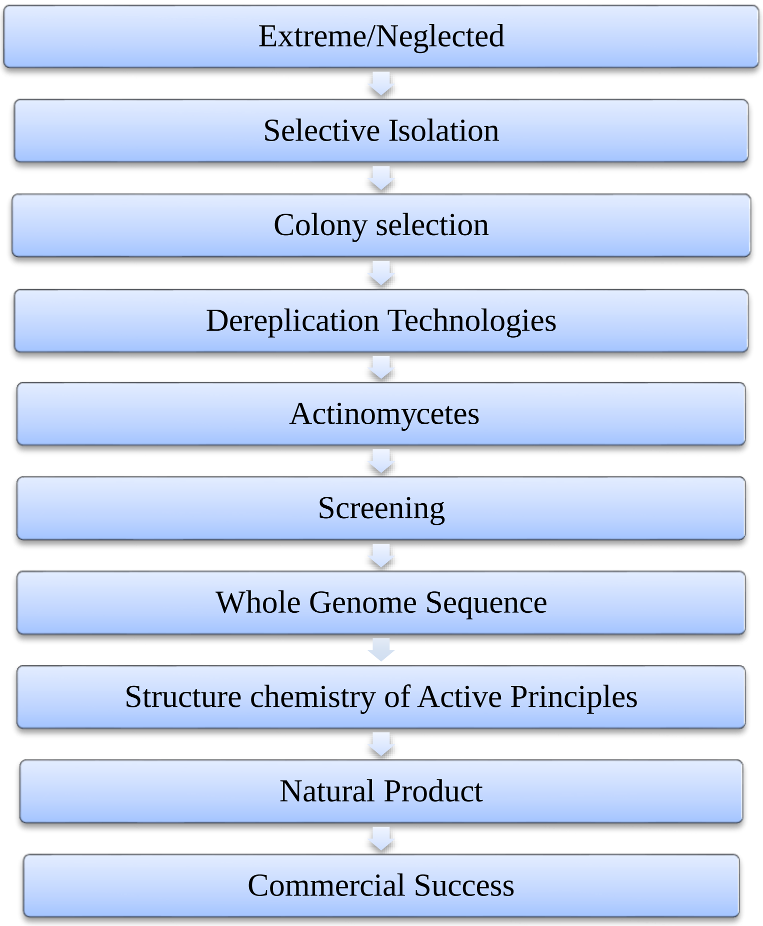

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Figure 1.1 Culture-dependent bioprospecting strategies (Goodfellow & Fiedler, 2010; Idris, 2016).

Beach soils had been reported to signify a habitat for organisms, especially actinomycetes (Ariffin et al., 2017). Numbers of potential actinomycetes and their novel metabolite compounds have been discovered (Newman, 2017) which proves that beaches soil provide unique habitat that harbours rare actinomycetes. One of the extreme environments around the coastal area consists of BRIS (Beach Ridges Interspersed with Swales) soil, which arises from sand and some of the sediment of the

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sea currents that expanded from the broken of layers of cliffs through the sea currents during the tidal and monsoon (Mustapha et al., 2017).

BRIS soil is characterized by a decrease in its physical and chemical properties that why it's not good for the production of crops. This soil is considered very dry and its nutrients are lost quickly as a result of the high temperature and lowering water retention ability. This condition can dangerously affect plants. Further, this high temperature for soil may cause the fast vaporization of moisture and nitrogen on soil surfaces that severely affect plant growth (Mustapha et al., 2017).

This study was aiming to determine the presence of actinomycetes in BRIS soil to selective the antimicrobial isolates and divided depend on phylogenetic approach and focused on potential compounds against antibiotic resistant microorganisms using whole genome sequencing and chemical analyses, where 155 strains were isolated and identified 33 isolates belong to 9 genera based on their 16S rRNA gene sequences. The antimicrobial screening of all isolates was conducted and the results were astounding. Complete genome sequencing of *Amycolatopsis sp.* AA12, revealed the number of genes predicted to encode proteins and the chemistry of biosynthetic gene clusters of secondary metabolites. Secondary metabolite extracts showed biological activity against a panel of microorganisms. The chemical profiles for extracts were detected using Gas Chromatography-Mass Spectrophotometry (GCMS) approach. The results indicating the secondary metabolites are rich in bioactive compounds. The ability to produce Rifampin was detected using HPLC-UV based on comparison against the

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standard. Six compounds with antimicrobial activity of the AA12 strain were isolated and the structure of these compounds were elucidated using NMR spectroscopy. Results indicate that *Amycolatopsis sp.* and other actinomycetes isolate from the extreme environments can exhibit a high variety of biological activities compounds that are used in pharma and medicinal products, suggesting that the actinomycetes are an untapped resource for future natural product discovery.

1.2 Problem Statement

Discovery of new actinomycetes is of significant interest to drug discovery due to a growing need for the development of new and potent therapeutic agents. Antimicrobial resistance (AMR) is now a global threat. Its emergence rests on antimicrobial overuse in humans and food-producing animals. Modern molecular technologies are adding strength to the target-directed search for the detection and isolation of bioactive actinomycetes. They are known for the important role they play in soil ecology and as a source of effective drugs (Ghosh & Cheeptham, 2017). There was no report on the actinomycetes, and its biological activity in BRIS soil that collected from Setiu districts. There is a possibility to get rare actinomycetes and new biological activity to be selected as potential drug leads.

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Whole Genome Sequencing (WGS) approach out from single cells has made a scientific breakthrough, many genomes of Actinobacteria have been successfully

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sequenced, and about 56,168 bacterial genomes have been sequenced out of which 6997 belong to the class Actinobacteria. Amongst these, only 486 Actinobacterial genomes have been completely sequenced and annotated till date with the majority of them representing organisms that are a source of commercially important drugs or are contagious and infectious to humans and animals. The constantly increasing number of drug-resistant bacteria and the urgent need for discovering new antibiotics has emerged as a major scientific challenge this requires to focus on the evaluation of the genetic capacity of organisms for metabolite production (WGS) and activation of cryptic gene clusters.

Natural products have been studied as sources of traditional and modern drugs for years. The interest in this field remains relevant as new potential therapeutic drugs have been emerging continuously. the chemical profiles of actinomycetes showed a various group of chemical constituents that have served as scaffolds for important drugs such as anticancer (Rajivgandhi et al., 2018), antibacterial (Gurovic & Olivera, 2017), antifungal (Jakubiec-Krzesniak et al., 2018) and antioxidant (Abdel-Aziz et al., 2019). Therefore, the chemical components of this family must be further examined and present the potential compounds using modern technologies.

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The studies indicate that *Amycolatopsis spp*. and other microorganisms isolated from the extreme environments can exhibit a high variety of biological activities compounds that are used in pharmacy and medicinal products. Suggesting that *Amycolatopsis spp*. is an untapped resource for future natural product discovery. Based

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on a literature study on actinomycetes, the chemical and biological activities of *Amycolatopsis samaneae* strain RM287 have yet to be established and remain to be investigated, there is a possibility to isolate new chemical compounds that may have various biological activities such as anticancer and antimicrobial from *Amycolatopsis samaneae*.

The public health officials consider the current state of available antibiotics to be perilous and some of the organisms are close to having complete resistance to all commercially available antibiotics. Fortunately, new antibiotics are constantly being discovered from microorganisms and actinomycetes represent the biggest possibility to obtain further medically, agriculturally and industrially valuable compounds which may serve as direct or indirect drugs leading compounds for structural modifications and templates for the rational drug design and other derivatives.

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1.3 Research Hypothesis

1. BRIS Soil collected from Setiu District, Terengganu, Malaysia is the habitat of actinomycetes.
2. Actinomycetes can produce bioactive compounds.
3. Identification of actinomycetes based on 16s rRNA.
4. Analysis the *Amycolatopsis sp.* AA12 strain by whole genomic sequencing (WGS).
5. Potential the biological activity compounds using HPLC –UV and GC-MS.
6. Isolation natural compounds from *Amycolatopsis sp.* AA12.

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1.4 Research Objectives

1. To isolate and identify actinomycetes from BRIS soil samples based on 16S rRNA gene sequences.
2. To screen antimicrobial activity of actinomycetes isolates and its secondary metabolites against a panel of microorganisms.
3. To identify secondary metabolite syntheses by whole genomic sequencing (WGS).
4. To determine the secondary metabolites present in the crude extracts and characterize spectroscopically.

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1.5 Research Questions

1. Which Actinomycete genera present in BRIS soil?
2. What are the antimicrobial activities of Actinomycete isolates and their metabolite extracts?
3. What are the secondary metabolite synthase genes identified from Actinomycete whole genome sequence?
4. What are the secondary metabolites produced by Actinomycete isolate?

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1.6 Significance of study

Actinomycetes are the foundation of antibiotics and therefore are an important role in human health. About 70 % of the natural compounds have been separated from actinomycetes often from the genera belonging to *Streptomyces sp*. and *Micromonospora sp.* Therefore, these natural products are a source of the development of new drugs to search for new antibiotics that given better effectiveness on resistant organisms for drugs, and it currently takes an important area in medical research.

This study is significant to the research development by expanding the information and knowledge base about the actinomycetes in BRIS soil and the characterization of the chemical compounds of actinomycete species that lead to the discovery of new compounds and expand information. Besides, the bioactivity investigation useful to provide source knowledge about a biological activity to actinomycetes. The outcomes of this study will serve as future references and be resources for knowledge on the therapeutic discovery that can lead to the development of potential and safe drugs in the medical field.

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The availability of genome information will provide useful insights to infer the presence of molecular structures as well as numbers of secondary metabolite in this gene. It will also help in ascertaining the mechanisms involved in the regulation of secondary metabolite biosynthesis and aid the search for novel secondary metabolites through genetic engineering. Therefore the genomic information will help in the

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identification of key molecular targets to achieve industrial strain improvement. This is the first complete genome report of the use of NGS to produce high quality and non-fragmented genome sequence of *Amycolatopsis sp.* AA12, an essential prerequisite for efficient genome mining for natural product discovery in these GC-rich bacteria.

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