ISOLATION OF *STREPTOMYCES* FROM SABAH AND SCREENING FOR THEIR ANTIMICROBIAL ACTIVITIES

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DISERTASI INI DIKEMUKAKAN UNTUK MEMENUHI SEBAHAGIAN DARIPADA SYARAT MEMPEROLEHI IJAZAH SARJANA MUDA SAINS DENGAN KEPUJIAN

PROGRAM BIOTEKNOLOGI
SEKOLAH SAINS DAN TEKNOLOGI
UNIVERSITI MALAYSIA SABAH

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JUDUL: Isolation of Streptomyces from Sabah and Screening for their antimicrobial activities

IJAZAH: IJAZAH SARJANA MUDA DENGAN KEPUJIAN (DALAM PROGRAM BIOTEKNOLOGI)

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"In experimental science,

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-Louis Pasteur

DECLARATION

I hereby declare that this dissertation is my own work except for the quotations and summaries from which the references are fully acknowledge.

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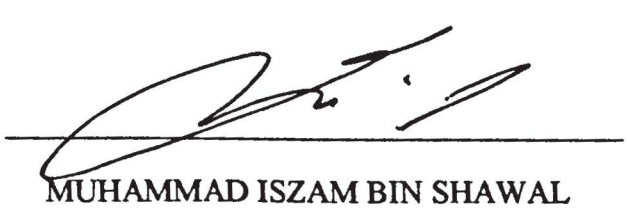

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ACKNOWLEDGEMENT

Firstly, I would like to be thankful to *Allah ta'ala* for his compassion and benevolence in giving me the opportunity, great health, enthusiasm and determination to complete this project as planned.

Secondly, I would like to thank my father, Mr. Shawal bin Majid and my mother, Miss Siti Fatmah bt Hj. Abdul Majid for their indefatigable efforts in raising me into a person that I am today.

Thirdly, I have intellectual debts to Dr. Michael Wong for which I cannot repay. His unrelenting guidance and patience have always become the light at the end of the tunnel for me. Whereas his wide knowledge and experiences in this exciting field of biotechnology really made me stand on the shoulders of giants.

Fourthly, I am deeply grateful to friends and colleagues that I have spent with in the Biotechnology Research Institute, which are Tam, Elaine, Sharon, Chelven, Adrian, Shuhada, Wendy, Alex, Yew and Kenneth for their willingness to help me at critical times though hectic their schedules were. Last but not least, I would like to thank my great friends at the institute, lab assistants Vidarita, Azian, Richard and Rudy that lend me many support in providing materials and chemicals that were used through out the project. Unmistakably, they are the bastions of the friendly and conducive environment that I have gone through in this institute. Lastly, thousand of thanks to Zul'atfi for the camera. Thank You.

ABSTRACT

The objectives of this project were to isolate *Streptomyces* from tropical soil samples in Sabah; to screen for antimicrobial activities of *Streptomyces* against *Escherichia coli* O125, *Vibrio parahaemolyticus* and *Salmonella typhi*; to characterize the *Streptomyces* possessing antimicrobial activities and lastly to identify the *Streptomyces* by 16S rDNA sequencing. The materials and methods involved the sampling of tropical soil samples of SST and FOR samples and were incubated at 37°C for four days after the addition of $CaCO_3$ at 1:1 ratio. The soil were diluted with sterile distilled water and plated onto Streptomycetes agar (SA) containing cycloheximide at 28°C for several days. The *Streptomyces* were then spot-inoculated onto a fresh SA with grid and incubated at 30°C until sporulation. The plate was then being overlayed with soft agar containing target pathogens as stated above and incubated overnight. After the formation of inhibition zones, the *Streptomyces* was purified by restreaking the colony on fresh SA and were Gram-stained, KOH rapid test, and characterized. Then it was grown in broth and later stored in 20% glycerol stock at -80°C. Its temperature, pH, and NaCl growth range were also tested. The genotypic characterization involved the isolation of genomic DNA, the amplification of 16S rDNA gene using PCR, the cloning of the gene using TOPO® TA cloning kit, restriction mapping of plasmid and sequencing of the gene by Sanger chain-termination method. A total of 523 isolates were successfully purified (218 isolates from SST and 305 isolates form FOR samples). Ten isolates exhibited antimicrobial activities (nine from SST samples and one from FOR samples). Four of these isolates were identified as *Streptomyces*, i.e. isolate SST2, No.2; SST4, No.11; SST5, No.14; and SST5, No.17. Further characterizations of the four showed they were different but stop short at identifying the species of the *Streptomyces*. Further genotypic characterization of isolate SST2, No.2 have shown that it has the 16S rDNA gene of 1576bp and similarity searches lead to the *Streptomyces* sp. 10-3 and *Streptomyces* sp. 10-2. It can be concluded that this project has achieved its main objectives while elucidating several new findings for future studies.

ABSTRAK

Objektif utama projek ini ialah untuk memencilkan bakteria *Streptomyces* daripada sampel tanah beriklim tropika; menyaring *Streptomyces* dengan aktiviti antimikrob terhadap patogen *Escherichia coli* O125, *Vibrio parahaemolyticus* dan *Salmonella typhi*; mencirikan *Streptomyces* dengan aktiviti antimikrob dan mengenalpasti *Streptomyces* dengan menjujuk gen 16S rDNA. Bahan dan kaedah termasuklah mengambil sampel tanah di tapak SST dan FOR yang diadun dengan $CaCO_3$ pada nisbah 1:1 dan dieram pada 37°C selama empat hari. Sampel dicairkan dengan air suling dan diplatkan pada *Streptomycetes agar* (SA) berserta *cycloheximide* dan dieram pada 30°C untuk beberapa hari. *Streptomyces* kemudian diinokulat pada SA yang baru dan dieram selama 30°C hingga sporulasi. Plat kemudian dilitupi dengan *soft agar* mengandungi patogen seperti dinyatakan di atas dan dieram semalaman. Apabila halo terlangsung pada plat, *Streptomyces* dipindahkan ke SA yang baru dan ujian Gram dan ujian KOH dijalankan; dan fenotip *Streptomyces* juga dikategorikan. Kemudian, ia juga diinokulat ke dalam medium cecair dan disimpan di dalam stok gliserol 20% pada -80°C. Julat pertumbuhan *Streptomyces* pada suhu, pH dan NaCl yang berbeza turut dicatatkan. Penentuan genotip SST2, No.2 dilakukan dengan mengasingkan genom dari bakteria, tindakbalas polymerase berantai (PCR) ke atas gen 16S rDNA, pengklonan gen tersebut menggunakan kit pengklonan TOPO® TA, pemetaan plasmid dan penjujukan 16S rDNA. Hasil eksperimen menunjukkan sepuluh bakteria dengan aktiviti antimikrob (sembilan dari SST dan satu dari FOR) daripada 523 bakteria yang dipencilkan (218 dari SST dan 305 dari FOR). Empat daripada bakteria ini dikategorikan sebagai *Streptomyces*, iaitu SST2, No.2; SST4, No.11; SST5, No.14; dan SST5, No.17 disertai ciri-ciri fenotip dan fisiologi masing-masing. Penentuan genotip SST2, No.2 menunjukkan ia bersaiz 1576 bp dan pencarian kesamaaan (*similarity search*) menunjukkan ia memiliki persamaan kepada *Streptomyces* sp. 10-3 dan *Streptomyces* sp. 10-2. Dengan itu, projek ini telah mencapai objektifnya dan beberapa hasil daripada kajian ini memerlukan kajian tambahan yang lebih terperinci.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Microorganisms have been the prime source of antimicrobial agents since the "Golden Age of Antibiotics" (1940-1962) principally in the West (Singh & Barrett, 2006). The medical needs of World War II in Europe provided the momentum for essential antibiotics discovery in these early years such as tetracyclines, cephalosporins, aminoglycosides and macrolides (Bérdy, 2005; Singh & Barrett, 2006). Today, more than 22 000 bioactive secondary metabolites were known, either patented or published in the literature (Bérdy, 2005). The most important of these minuscule animals are the *Streptomyces*. This microbe of the soil produced approximately 80% of early antibiotics, mainly targeting bacteria and fungi (Bérdy, 2005). Needless to say, the discovery of antibiotics since then has resulted in the decrease of infectious diseases and concomitant increase of overall health quality of population in the industrial world (Overbye & Barrett, 2005; Zhang & Demain, 2005).

However, the search for new antimicrobial agent has been on a decreasing rate between the 1970's until today (Bérdy, 2005). The paucity of new scaffolds was only assisted by only three new antibiotic classes for the last two decades (Peláez, 2006). The antibiotics being produced by the world's giant pharmaceutical companies today were mainly derived from the scaffold of antibiotics discovered decades before. These novel analogs of known compounds were synthesized through new technologies such as High Throughput Screening (HTS) and combinatorial chemistry synthesis. The HTS, based on screening of enzyme inhibitors in cell-free assays was found failing in delivering compounds useful for clinical application (Peláez, 2006). This strategy has been devoid of the most essential of drug discovery, the diversity of a drug structure; procured through evolution of microbes against its natural antagonist (Peláez, 2006). The abandonment of natural products research and development (R&D) by most pharmaceutics were caused by several factors such as the increased in the research cost particularly in large-scale clinical trials; external competition between companies; internal competition against chronic diseases R&D for drug market and the failure of screening paradigm e.g. genomics and HTS (Overbye & Barrett, 2006).

Otherwise, diseases caused by pathogenic microorganisms have been on the rise (Bérdy, 2005; Overbye & Barrett, 2006). Various researches have shown the rise of multi-resistant pathogens (e.g. methicillin-resistant *Staphylococcus aureus, Streptococcus pnuemoniae, Salmonella typhimurium* and *Mycobacterium tuberculosis*) and the re-emergence of pathogenic Gram-negative bacteria (e.g. *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Enterobacter* spp.). The tuberculosis caused by

M. tuberculosis alone will result in the estimated loss of 35 million lives and near a billion left infected between the year 2000 to 2020 (Corbett *et al.*, 2003). The acme of this skulking predicament would be a sudden outbreak of untreatable diseases caused by new pathogenic bacteria.

1.2 Location of study

On the one side, the potential solution for this dilemma lies quite near. The Malaysian state of Sabah, located on the northern part of Borneo, possessed one of the world's hot spots of biodiversity, e.g. 10 000-12 000 species or 5-6% of the world's flowering plants located in Sabah alone (Ho, 2003). Lies within the tropical belt, its rainforest have always been regarded as *terra incognita* by researchers. The highly diverse organisms in the tropics would be one way to maximize biodiversity of *Streptomyces* (Peláez, 2006) and 95-99.9% of existing organisms yet to be characterized (Zhang & Demain, 2005). With its fluid population dynamics, the tropics would be an excellent starting point for the discovery of unknown *Streptomyces* with new antimicrobial agents.

1.3 Potential of antimicrobial study

The world market for antimicrobial compounds recently have reached US$ 55 billion (approx. RM 192 billion), inclusive of antibiotics, synthetics and antiviral agents (Zhang & Demain, 2005). The trend of the market is currently increasing and will be determined by several factors such as the worldwide aging population; increasing number of

immune-compromised patients, infected with HIV that requires longer courses of anti-infective treatment; and increasing microbial resistance worldwide (Zhang & Demain, 2005).

The exploration for new antimicrobial compounds still possessed huge unexplored resources in term of microbial diversity, silent pathways waiting for the right cultivation conditions and the molecular targets yet to be exploited (Pelaez, 2006). The investment of time and resources in this arena will ensure structural novelty as a guarantee (Peláez, 2006). Likewise, the challenge that must be met now by scientists is to optimize the isolation and screening effort for antimicrobial *Streptomyces* through new lenses of genomics, bioinformatics, microbial physiology and other related fields.

1.4 Objectives of study

With the background of this study being explained, it is imperative to outline the research's objectives of which being stated below:

i. To isolate *Streptomyces* from tropical soil samples in Sabah.

ii. To screen for antimicrobial activities of *Streptomyces* against *Escherichia coli* O125, *Vibrio parahaemolyticus* and *Salmonella typhi*

iii. To characterize the *Streptomyces* possessing antimicrobial activities.

iv. To identify the *Streptomyces* by 16S rDNA sequencing.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotic

Classical definition of antibiotic is secondary metabolite isolated from microorganism that exhibits activities of antimicrobe (i.e. antibacteria, antifungus or antiprotozoa); antitumour; or antiviral (Bérdy, 2005). On a broader definition, antibiotics are all microbial secondary metabolites that function as regulators of growth processes and replications, that create responding action to life cycle of prokaryotic or eukaryotic cells, e.g. to regulate, inhibit and stimulate; at the biochemical level with a minimal concentration (Bérdy, 2005).

The sources for antibiotics can be divided into two, first is the natural products and secondly is the synthetic compounds (Singh & Barrett, 2006). The natural products can be defined as carbon-containing compounds isolated from diverse living things, be it primary (polysaccharides, protein, nucleic acids and fatty acids) or secondary metabolites (Bérdy, 2005). Natural products have been the main source of antibiotics, i.e. all but three

antibiotics classes are of natural products origin (Singh & Barrett, 2006). It ranges from minute molecular weight compounds such as penicillins to large peptides such as teicoplanin, with most compounds having structurally complex scaffold and high number of functional groups (Singh & Barrett, 2006).

The productions of antibiotics were thought to be caused by the restricted growth of the producers in soil; intense competition between floras; morphological differentiation such as spore's formation; and as a natural selective advantage for the producer (Williams & Vickers, 1986). However, it is important to note that antibiotics were not detected in the natural soil; with the possible reasons such as it was not produced, rapidly inactivated, adsorbed by soil colloids, less sensitive detection method or the growth of producer is halted by insufficient nutrients (Williams & Vickers, 1986).

The origin of antibiotics from the natural products can be rather diverse. It is distributed in almost all living things, prokaryotes and eukaryotes alike but the distribution is uneven with some produce a high number of it and *vice versa* (Bérdy, 2005). According to Bérdy (2005), the filamentous actinomycetes species account for over 10 000 of known bioactive compounds where 7600 are *Streptomyces*-originated and 2500 rare actinomycetes-originated, representing 45% of the overall bioactive microbial metabolites. Other important antibiotic-producing organisms are prokaryotes (e.g. *Bacillus* sp., *Pseudomonas* sp., Myxobacteria, Cyanobacteria), fungi, plants, algae, lichens and vascular plants, terrestrial and marine animals. Although antibiotics are very diverse in nature, it can be classified according to its chemical structure, mechanisms of

action and/or spectrum of antimicrobial activity (Madigan & Martinko, 2006; Sweetham, 2005) as shown in Table 2.1.

2.2 *Streptomyces*

The name *Streptomyces* was first proposed by S.A. Waksman and A.T. Henrici in a 1943 paper entitled *"The Nomenclature and Classification of Actinomycetes"* (Anderson & Wellington, 2001). This genus is classified under the family of Streptomycetacae; order of Actinomycetales and phylum of Actinobacteria. Today *Streptomyces* is the sole member of the family *Streptomycetacae* after long periods of reclassification using various taxonomic methods (Anderson & Wellington, 2001). It amounted to more than 450 species, covering a tenth of the whole bacteria genera; the largest in eubacteria (Hain *et al.*, 1997; Ho, 2003). It is a Gram-positive obligate aerobic bacterium with DNA G+C content of 69-78%. It produces extensive branching of primary (substrate) mycelium and more or less abundant secondary (aerial) mycelium. The diameter of substrate hyphae are approximately 0.5-1.0 μm and with no cross-walls during the vegetative phase.

The lifecycle of *Streptomyces* is of "spore to mycelium to spore" (Fig. 2.1). It starts by growth at the hyphal apices and eventually branching that produces a complex of tightly woven matrix of hyphae during the vegetative growth phase. As time passes,

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