# ISOLATION AND PURIFICATION OF ACTINOMYCETES THAT SERVE AS POTENTIAL INHIBITOR OF GSK3

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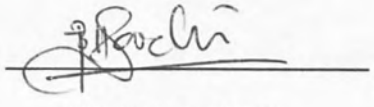

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## ABSTRACT

The main objective of the project was to isolate and purify actinomycetes from primary lowland rainforest in Danum Valley that might serve as potential inhibitor against GSK-3β through screening system using transformed yeasts. A total of 12 soil samples have been collected. The isolation of actinomycetes was achieved through the use of humic acid-vitamin agar media. From the isolation media, a total of 31 actinomycetes were successfully purified using Oatmeal media agar. Glycerol stocks were prepared from these purified actinomycetes for storage purpose using 20% glycerol. These 31 strains from the Oatmeal media agar were then grown in 10ml mannitol-peptone-glucose liquid media separately to obtain the secondary metabolites produced by them. After duration of 120 hours in shaking incubator, 10ml acetone was added into each liquid culture to lyse the cells and release the secondary metabolites. These acetone extracts were used for screening against GSK-3 using yeast strains H10, 075 and H10, 079, with the former transformed with pKT10-gsk3β and the latter transformed with YEP24-MCK1. The screening system is based on temperature sensitivity, in which these two transformed yeast strains are able suppress temperature sensitivity phenotype. The diameter of the halos formed around the discs at the 2 different temperatures (37°C and 25°C) determined the activity of the extract on the yeast. The formation of halos with bigger diameter at 37°C than that of 25°C indicated that the extract has potential inhibitory activity on the yeast. On the other hand, smaller halo at 37°C showed that the extract was toxic to the yeast. From a total of 115 extracts that were screened against GSK-3, only three (H11462, H11490 and H11647) of them showed potential inhibitory activity.

## ABSTRAK

Objektif utama projek ini ialah pemencilan and pengasingan aktinomiset dari hutan tropika primer Lembah Danum yang berpotensi sebagai perencat GSK-3 melalui sistem penyaringan perencatan menggunakan yis yang telah ditransformasi. Sebanyak 12 sampel tanah diperolehi. Pengasingan aktinomiset dilakukan menggunakan agar media humic asid-vitamin. Dari media tersebut, sebanyak 31 aktinomiset berjaya dipencilkan dalam media oatmeal. Penyediaan stok gliserol dari media oatmeal untuk tujuan penyimpanan dicapai dengan menggunakan 20% gliserol. 31 strain aktinomiset tersebut ditumbuh di dalam 10ml media mannitol-pepton-glukos secara berasingan untuk mendapatkan metabolit sekunder. Selepas 120 jam dalam incubator bergerak, 10ml aseton ditambah ke dalam kultur untuk memecahkan sel dan membebaskan metabolit sekunder. Ekstrak-estrak aseton tersebut digunakan dalam sistem penyaringan GSK-3 menggunakan strain yis H10, 075 dan H10, 079, yang masing-masing telah ditransformasi dengan pKT10-gsk3β dan YEp24-MCK1. Sistem penyaringan ini berdasarkan sensitiviti yis terhadap suhu tinggi, di mana kedua-dua yis tersebut boleh mengatasi fenotip sensitif terhadap suhu. Diameter bulatan yang terhasil di sekitar cakera kertas pada 2 suhu yang berlainan (37°C dan 25°C) menentukan sama ada ekstrak yang diuji mempunyai potensi perencatan terhadap yis. Pembentukan bulatan yang lebih besar di suhu 37°C menunjukkan bahawa ekstrak yang dikaji merupakan perencat yang berpotensi untuk merencat pertumbuhan yis. Sebaliknya, diameter yang lebih besar pada suhu 25°C merupakan toksid kepada yis.Dari 115 ekstrak yang diuji terhadap GSK-3, hanya tiga (H11462, H11490 dan H11647) yang menunjukkan potensi sebagai perencat.

**CONTENTS**

# LIST OF ABBREVIATION AND SYMBOLS

| | |
|---|---|
| % | Percentage |
| °C | degree Celsius |
| µg | microgram |
| A | Absorbance |
| cm | centimeter |
| g | gram |
| M | Molarity |
| m | meter |
| mg | milligram |
| mm | milimeter |
| ml | milliliter |
| µm | micrometer |
| NaOH | Sodium hydroxide |
| nm | nanometer |
| rpm | rotation per minute |
| v/v | volume over volume |
| w/v | weight over volume |
| YEp-24 | Yeast episomal plasmid-24 |

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# CHAPTER 1

# INTRODUCTION

Actinomycetes are gram-positive bacteria and produce secondary metabolites that are useful to human. Actinomycetes are well-known antibiotic producers as well as agents of diseases such as pneumonia and tuberculosis (Lechevalier and Lechevalier, 1981). Majority of actinomycetes live as free-living soil organisms. The role of actinomycetes as an important producer of antibiotics has emerged from the discoveries of various antibiotics produced from these organisms. The antibiotics are produced for self-survival of the actinomyctes in the competitive soil ecosystem by eliminating other bacterial or fungi, as these antibiotics are toxic to them. The development of antibiotics has been the most important breakthrough in medicine as many antitumour and anticancer drugs have been discovered from the researches performed on the actinomycetes. *Streptomycetes*, a member of this Order, is the major producer of the present antibiotics as anti-viral, anti-tumour, and various enzyme inhibitors (Thompson *et al.*, 2002). The actinomycetes were isolated from soil samples collected at lowland

dipterocarp forest in Danum Valley. Lowland dipterocarp forest is the most extensive forest type found in Borneo (Newbery *et al.*, 1992).

All organisms react to their environment. Signals are transmitted from cell surface into the cell through the signal transduction pathway. In the eukaryotic cell, GSK-3, an evolutionary conserved serine/threonine protein kinase, functions in the dephosphorylation of glycogen synthase (Ali *et al*, 2000; Dozza *et al*, 2004; Frame and Cohen, 2001). It was first implicated in the muscle energy storage and metabolism. After its cloning, its participation have grown to a more generalized role in cellular regulation, mostly contributed by the substrates regulated by this enzyme that includes cytoplasmic proteins and nuclear transcription factors (Ali *et al*, 2000; Wojtaszewski *et al*, 2002). Most of the substrates for GSK3 encompassed protein implicated in diabetes, Alzheimer's Disease, neurological disorder, and cancer (Ali et al., 2000; Doble and Woodgett, 2003). In the budding yeast, there are 4 GSK3 homologs, namely *MCK1*, *MDS1*, *MRK1* and *YOL128c*. The yeast GSK-3s are important in the mitotic and meiotic cell cycle (Andoh *et al.*, 2000).

There are 4 objectives to be achieved in the project. The first one is to collect soil samples from the area of low land Dipterocarpaceae in Danum Valley. Isolation of actinomycetes from the soil samples collected would be the second objective. Following the isolation of actinomycetes, secondary metabolites produced by the actinomycetes particularly from the Genus Streptomyces would be extracted for screening system as potential inhibitor of GSK-3β.

Mutant yeasts (gsk-3 null mutant) inserted with plasmid carrying specific homologs of the GSK-3, the mammalian gsk-3β and yeast homolog MCK1 are used in the screening system to identify the inhibition of the targeted protein kinase, GSK-3β.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Characteristic Of Actinomycetes

Actinomycetes are gram-positive bacteria with high G+C content, which can grow on ordinary laboratory media, but their growth is slower than other Orders (Lechevalier and Lechevalier, 1981). The word actinomycetes derived from Greek 'aktis', which means 'a ray' and 'mykes', fungus (Pandey *et al.*, 2004). Some of them are present in a more evolved form possess a fungal like appearance. Some of the members of Actinomycetales are *Streptomyces, Nocardia, Arachnia, Micromonospora, Microtetraspora, Frankia*, and *Mycobacterium*. Majority of its member are free-living, saprophytic bacteria found widely distributed in soil, water and colonizing plants (Pandey *et al.*, 2004). Actinomycetes are bacteria that form branching filaments with aerial hypae and spores. The branching network of hypae is developed by actinomycetes on the surface of a solid substratum to form substrate mycelium. The

hypae is divided to long cells containing several nucleotides by septa. Majority of the actinomycetes also possesses aerial mycelium that extends above the substratum forming asexual, thin-walled spores, known as conidia. The actinomycetes are classified in various ways based on their physiology, chemical, morphology and also physical qualities (Lechevalier and Lechevalier, 1981).

### 2.1.1 *Streptomycetes*

*Streptomycetes* are mycelial, saprophytic soil bacteria that undergo complex morphological differentiation that is coordinated with the excretion of coloured bioactive compounds and possess similar appearances to fungi. It is the most extensively studied actinomycetes as over 500 species of *Streptomycetes* are recognized, where the GC base ratios clustered between 69-73% (Madigan *et al.*, 2003). The life cycle begins with spore germination, followed by the development of branching hyphae that grows on and into the substrate. The substrate mycelium produces aerial hyphae, which undergoes septation as a response to complex but weakly defined signal. The septation process will yield chains of unigenomic spores. As the aerial mycelium grows, the substrate mycelium produces various antibiotics that are believed linked to the sporulation (triggered by nutrient depletion) process. *Streptomycetes* have been endowed for 'chemical warfare', which allow them to eradicate bacterial and fungal rivals in the soil ecosystem (Hayakawa *et al.*, 2004; Hesketh *et al.*, 2002; Madigan *et al.*, 2003; Thompson *et al.*, 2002). *Streptomycetes* can

be isolated from almost any rich soil sample. The growth of *Streptomycetes* is more favorable in alkaline and neutral soil. It requires lower water potential for growth than other soil bacteria. (Hesketh *et al.*, 2002; Madigan *et al.*, 2003).

### 2.1.2 Roles of actinomycetes

Actinomycetes play a very important role in medical point of view especially as agents of infectious diseases such as tuberculosis and pneumonia (Lechevalier and Lechevalier, 1981). However, the role of actinomycetes is now shifting to serve as main antibiotic producers for human, veterinary, agriculture and biochemistry field. Lately, they are produced as commercially important bioactive compounds of antitumour agents and enzymes of industrial interest (Gharaibeh *et al.*, 2003). *Streptomycetes* have been well studied amongst the member of actinomycetes for the biochemical structure, biosynthesis and the application of its natural bioactive compounds.

There are thousands of antibiotics produced by this genus have been well described, but those represent only a small fraction of the range of bioactive compound produced by *Streptomycetes*. Moreover, emperical screening using various assays have been performed on these compounds have revealed that these compounds are pharmaceutically active compounds such as anti-cancer, anti-viral, enzyme inhibitors and modulators of immune system. Some of the classic products include tetramycin and vancomycin, amphotericin and doxorubicin (Thompson *et al.*, 2002). Besides

*Streptomycetes*, other actinomycetes are also known to be producing antibiotic applicable to the human. The role of actinomycetes as antibiotic producer could not be under-estimated as it produces approximately three quarter of all known antibiotics (Pandey *et al.*, 2004). The quest for new actinomycete antibiotics continues because many infectious diseases are still not adequately controlled by existing antibiotics. It is believed that there is a possibility of actinomycetes to inhibit GSK3 as early investigation performed on them has shown a promising prospect.

## 2.2 Secondary Metabolites

Secondary metabolites are products formed during the late or near the end of the growth phase, usually at the stationary phase of growth (Madigan *et al.*, 2003). Microorganisms have invented many devious ways to thwart their foes and survive in adverse environments. Non-motile microbes, particularly those inhibiting soil and marine environments, are notably able to fabricate a wide range of chemicals, the secondary metabolites, which is named after their seeming dispensability for the organism's ontogeny (Hutchinson, 2003). These secondary metabolites involve unusual biochemistry and biosynthesis as well as complex genetics devoted to the property of self-defense, intercellular communication and other aspects of microbial life. These secondary metabolites, which is also known as antibiotics appear to be toxic to other microorganisms.

In *Streptomycetes*, the biosyntheses of these natural bioactive metabolites are believed to be directed by 20 gene clusters and involve numerous secreted proteins, sigma factors, Ser/Thr kinase homologs, and transcription regulators (Hesketh *et al.*, 2002). From the high-throughput chromatography that has been performed on thousands of *Streptomycetes* strains, the analyses revealed that each strain possesses its own unique profile of secondary metabolites, mostly compounds that have not been synthetically produced (Thompson *et al.*, 2002).

In the last few decades, it was discovered that these secondary metabolites have particular importance in human medicine, veterinary, biochemistry and also agriculture. Of the products derived from secondary metabolites, probably the most important are the antibiotics as in the 1950s, it was considered as the Golden era of antibiotics. The development of antibiotics as agents for treatment or infectious diseases is believed to poise more impact on the medicine field than any other single development (Gharaibeh *et al.*, 2003; Hakala, 2003; Madigan *et al.*, 2003). Most of the drug discoveries are performed through screening. In this particular approach, a vast number of possible antibiotic producing microorganisms are obtained from nature in pure culture. Then, these isolates are tested for antibiotic production through observation of production of diffusible materials that inhibits the growth of test bacteria, which are selected to represent bacterial pathogens (Madigan *et al.*, 2003). The screening of diverse microbial strains is a viable strategy, as the producers have themselves optimized the structures for antibiotic activity during an evolutionary process with effective antibiotics. Hence,

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