ANTITRYPANOSOMAL SECONDARY METABOLITES FROM SOIL ACTINOMYCETES ISOLATED FROM PENANG NATIONAL PARK

by

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This thesis is dedicated to,

My dear husband, father, mother, lovely daughter, my family and friends

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LIST OF ABBREVIATIONS

BuOH	Butanol
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
DAP	Diaminopimelic acid
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamine tetra acetic acid
EtOH	Ethanol
FACC	FRIM Actinomycetes Culture Collection
FBS	Fetal Bovine serum
HCl	Hydrocloric acid
HPLC	High Performance Liquid Chromatography
ISP2	Yeast extract-malt extract agar
ISP3	Oatmeal agar
ISP4	Inorganic salts-starch
LILIT	Long incubation low inoculation test
M	Molar
MBA	Modified benett's agar
MEM	Minimum Essential Medium
mg	Miligram
ml	Milliliter
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
OD	Optical density
PBS	Phosphate buffer saline
R_f	Retention factor
rpm	Revolution per minute
S.D	Standard deviation
SI	Selectivity Index
sp.	Species
TLC	Thin Layer Chromatography
UV	Ultra violet
Vero	Normal African green monkey kidney cells

v/v	Volume per volume
w/v	Weight per volume
μl	Microliter
μg	Microgram
ηg	Nanogram
ηm	Nanometer

ANTITRIPANOSOM OLEH METABOLIT SEKUNDER DARIPADA AKTINOMISET TANAH YANG DIPENCILKAN DARIPADA TAMAN NEGARA PULAU PINANG

ABSTRAK

Ekosistem Malaysia yang unik menjadikannya sebagai sumber alam yang berpotensi untuk memencilkan pencilan aktinomiset yang dapat menghasilkan bioaktif metabolit sekunder yang menarik. Pelbagai bioaktiviti dapat ditunjukkan oleh metabolit sekunder ini termasuk aktiviti antitripanosom. Penghasilan sebatian pemula yang baharu diperlukan dalam rawatan penyakit ini yang disebabkan oleh parasit trypanosoma, memandangkan ubat yang digunakan pada ketika ini adalah sangat terhad disebabkan rintangan ubat terhadap penyakit tersebut. Dalam kajian ini, sejumlah 83 pencilan aktinomiset telah berjaya dihidupkan semula daripada Koleksi Kultur Aktinomiset FRIM (FACC) yang disimpan didalam tiub krio pada suhu -80^{o}C. Kesemua pencilan aktinomiset ditumbuhkan di dalam tiga media fermentasi (M1, M2, dan M3) dan sejumlah 249 ekstrak telah dihasilkan untuk diuji pada asai antitripanosom secara *in vitro* terhadap parasit *Trypanosoma brucei brucei* strain BS221 melalui asai pertama, kedua dan ketiga. Ekstrak yang menunjukkan aktiviti kuat dan sederhana seterusnya dinilai terhadap aktiviti antitripanosom melalui asai ketiga dan ujian sitotoksisiti untuk menentukan indeks selektif (SI) ekstrak tersebut. Antara ekstrak aktif yang menunjukkan nilai selektif yang bagus terhadap parasit trypanosoma, empat ekstrak iaitu FACC-A032-M3, FACC-A026-M3, FACC-A048-M3 dan FACC-A049-M3 menghasilkan aktiviti antitripanosom yang kuat ($IC_{50} \leq$ 1.56 μg/ml) serta indeks selektif yang tinggi (SI ≥ 20). Kajian seterusnya dijalankan kepada pencilan yang berpotensi ini dengan membuat pemerhatian ke atas kultur,

makromorfologi, ciri-ciri fisiologi dan biokimia serta jenis rantaian spora. Pencilan FACC-A032 dan FACC-A026 dikenalpasti menyerupai *Streptomyces* sp. dan pencilan FACC-A048 dan FACC-A049 dikategorikan dalam kumpulan bukan *Streptomyces.* Kajian seterusnya melalui analisis terhadap isomer diaminopimelic acid (DAP) bagi hydrolysates keseluruhan sel mengesahkan bahawa pencilan FACC-A032 dan FACC-A026 yang mengandungi isomer LL-DAP sebagai spesis dari kepunyaan genus *Streptomyces*. Manakala, pencilan FACC-A048 dan FACC-A049 yang mengandungi isomer meso-DAP telah dikenalpasti kepunyaan genera bukan *Streptomyces*. Kajian terhadap profil pertumbuhan pencilan FACC-A032 dan FACC-A049 di dalam media M3 menunjukkan aktiviti antitripanosom maksimum oleh ekstrak kultur dicapai selepas lapan hari fermentasi pada keadaan 28 ± 2^{o}C dan 200 rpm. Pencilan FACC-A026 dan FACC-A048 di dalam media M3 menunjukkan aktiviti maksimum selepas hari ketujuh pada keadaan yang sama. Daripada empat pencilan yang dipilih, hanya pencilan FACC-A032 masih menunjukkan aktiviti kuat ($IC_{50} \leq 1.56$ μg/ml) semasa penghasilan ekstrak menggunakan fermentasi kelalang goncang (1L). Pemencilan berpandukan bioasai terhadap ekstrak daripada *Streptomyces* sp. FACC-A032 menghasilkan dua kolum fraksi yang aktif (2Sn-A032-M3-F8 dan My-A032-M3-F5). Berdasarkan maklumat yang diperolehi dari kajian terdahulu, puncak spektrum UV-nampak yang dikenalpasti pada dua fraksi aktif ini mempunyai ciri UV-nampak yang sama dengan sebatian staurosporine. Ini menunjukkan bahawa kehadiran sebatian yang berkemungkinan seperti staurosporine menyumbang kepada aktiviti antitripanosom yang kuat pada pencilan FACC-A032.

ANTITRYPANOSOMAL SECONDARY METABOLITES FROM SOIL ACTINOMYCETES ISOLATED FROM PENANG NATIONAL PARK

ABSTRACT

The unique ecosystems in Malaysia can be utilized as potential sources for isolating actinomycetes isolates that produce interesting bioactive secondary metabolites. This secondary metabolite exhibits a wide range of bioactivity including antitrypanosomal activity. The option of new lead compound is needed for the disease treatment that caused by the trypanosome parasite since the existing drugs to cure are very limited due to drug resistance. In this study, a total of 83 actinomycetes isolates were successfully revived from cryovials stored at -80^{o}C in FRIM Actinomycetes Culture Collection (FACC). The isolates were grown in three different fermentation media (M1, M2, and M3) and a total of 249 culture broth extracts obtained were evaluated for *in vitro* antitrypanosomal assay against *Trypanosoma brucei brucei* strain BS221 via primary, secondary and tertiary assays. Extracts that showed strong and moderate activities were evaluated for antitrypanosomal activity in tertiary assay and cytotoxicity to determine their selectivity index (SI) values. Among the active extracts that showed good selectivity toward the trypanosoma parasite, four extracts FACC-A032-M3, FACC-A026-M3, FACC-A048-M3 and FACC-A049-M3 produced strong antitrypanosomal activity (IC_{50} value $\leq$ 1.56 μg/ml) and high selectivity (SI $\geq$ 20). The potential isolates were further studied for their cultural, macromorphological, physiological and biochemical characteristics and spore chain type. Isolates FACC-A032 and FACC-A026 were tentatively identified as *Streptomyces* sp. and isolates FACC-A048 and FACC-A049 were categorized into the non-*Streptomyces* group. Analysis of diaminopimelic acid

(DAP) isomer of whole-cell hydrolysates further confirmed the isolates FACC-A032 and FACC-A026 that contained LL-DAP isomer as species belonging to the genus *Streptomyces*. Whereas, isolates FACC-A048 and FACC-A049 that possess the meso-DAP isomer were confirmed to belong to non-*Streptomyces* genera. Growth profile study of isolates FACC-A032 and FACC-A049 in medium M3 showed that maximum antitrypanosomal activity in culture extracts was achieved after eight days of fermentation at 28 ± 2^{o}C and 200 rpm. Isolates FACC-A026 and FACC-A048 in medium M3 showed a maximum activity after seven days of fermentation under the same conditions. Among the four selected isolates, it was observed that only isolate FACC-A032 maintained a strong activity (IC_{50} value $\leq$ 1.56 μg/ml) in extracts produced using 1L flask fermentation conditions. Bioassay-guided isolation of crude extract from *Streptomyces* sp. FACC-A032 afforded two active column fractions (2Sn-A032-M3-F8 and My-A032-M3-F5). Based on information from the literature, the UV visible spectra of two peaks identified in the active fractions were identical and they matched with UV-vis characteristics of the compound staurosporine. This indicates the possible presence of staurosporine-like compounds which contributed to the strong antitrypanosomal activity in isolate FACC-A032.

CHAPTER 1

INTRODUCTION

1.0 Introduction of the study

Human African Trypanosomiasis (HAT), also known as sleeping sickness in humans and nagana in cattle is caused by protozoan parasites of the genus *Trypanosoma*. HAT disease still remains as a serious health problem to human and farm animals on the WHO list of neglected tropical diseases and a major problem to the poorer countries in the world especially throughout sub-Saharan Africa (WHO 2010). Even though HAT is an endemic disease, the growth in an international travel and immigration which is currently taking place is a well-known phenomenon, therefore HAT is no longer geographically-restricted as both immigrants and travelers in the western world may be present with these infections (Norman *et al.*, 2010). Chemotherapy, together with the vector control, remains one of the most important elements in the control of trypanosomatid disease as at the moment, there are no vaccines to prevent the trypanosome infection. Several drugs such as suramin, pentamidine, melarsoprol and eflornithine were currently registered to treat the disease. However, these drugs are limited and all the current treatment suffers from significant drawbacks (Abdel Sattar *et al*., 2009). Thus, there is an urgent need for more effective and safer trypanocidal drugs to treat sleeping sickness.

Trypanosome parasites (*Trypanosoma brucei*) transmitted by tsetse fly vector (*Glossina* spp.) to human can also infects animals. Trypanosomes are transmitted by

the bloodsucking male and female tsetse flies from one mammalian host to another (Salem *et al*., 2006). The transmission to human is caused by the injection of metacyclic trypanosomes in saliva of the tsetse fly. After the bite of trypanosome-infected tsetse fly, the life cycle of *T. brucei* subspecies begun with metacyclic forms differentiate into the bloodstream, subcutaneous tissues and lymph. In some but not all infections, a skin reaction occurs at the site of inoculation, which is caused by a local inflammatory response to the parasites (Sternberg, 2004). Following the first stage, the disease develops rapidly into a second stage when parasites cross the blood-brain barrier, invading the central nervous system.

Actinomycetes are a group of morphologically diverse gram-positive bacteria. The growth of actinomycetes is made up of branching filament, producing mycelium that may be of two kinds, substrate mycelium and aerial mycelium. Actinomycetes can be isolated from soil and marine sediments. Soil, in particular is an intensively exploited ecological niche for isolation of actinomycetes that produce many useful biologically active natural products, including clinically important antibiotics. A large collection of actinomycetes isolated from various locations throughout Peninsula Malaysia has been maintained in FRIM Actinomycetes Culture Collection (FACC). Besides preparing stock cultures for various research activities, the FACC also maintains a database containing information on the location of soil collection, name of collector, morphological and taxonomical characteristics and biochemical and gene typing profiles of the isolates were also documented (Getha *et al*., 2007).

Natural product compounds could be derived from either primary or secondary metabolism of living organisms where the primary such as polysaccharides, nucleic and fatty acid are common in all biological system. However, the secondary metabolites are chemically and taxonomically extreme diverse compounds with indistinguishable function (Berdy, 2005). The secondary metabolites are important part of natural products where it usually exhibits some kind of biological activities or bioactivity such as antibacterial, antifungal, antiparasitic, antiviral, anti-inflammatory, anticancer and others. Secondary metabolites are synthesized by the pathways which are often connected and influenced by primary metabolism. The concept of secondary metabolites include products of overflow metabolism because of nutrient limitation, force of metabolism produced during idiophase or defense mechanism regulator molecules (Sarker *et al*., 2006). The composition of the culture medium in actinomycetes growth influenced the biosynthesis of the bioactive molecules (Tortora *et al.*, 2001). Hence by using a different type of culture medium, it is assumed that different bioactive molecules will be produced from the actinomycetes isolates. These active molecules are generally extracellular and their isolation in a highest purity from the complex fermentation broth needs the application of a combination of various separation steps (Raoudha *et al*., 2006).

Studies have shown that the unique ecosystem in Malaysia can be potential source for isolating bioactive secondary metabolite from actinomycetes (Getha *et al.*, 2008) and previous study also discovered that metabolites from soil microorganisms has potential antitrypanosomal activity in vitro (Otoguro et al., 2008). Therefore, in the present study, actinomycetes isolated from Malaysian soil in the FACC

collections are studied for activity against the trypanosome parasite *Trypanosoma brucei brucei* strain BS221 and bioassay-guided studies are conducted to isolate the active fraction/s from the active extract/s. The optimized antitrypanosomal assay methods provided by Kitasato Institute, Japan was used in this study (Otoguro *et al*., 2008). However, the strain of test organism which is *T. b. brucei* used in the current study differs from that used by Otoguro *et al*. (2008). Kitasato Institute used the *T. b. brucei* strain GUTat 3.1 while the *T. b. brucei* strain BS221 was used in this study. Therefore, the inoculum size of test organism needs to improvise before screening a total of 249 actinomycetes extracts for *in vitro* antitrypanosomal activity. The potential isolate/s with strong antitrypanosomal activity will be characterized based on their morphological, physiological and biochemical characteristics. Bioassay-guided fractionation of the culture extracts from potential isolate/s will lead to the isolation of active fraction/s. During the bioassay-guided fractionation, the process of fractionation of actinomycetes extract/s and biological testing of the fraction/s is repeated until the active fraction/s is obtained (Rimando *et al.*, 2001). Followed by high performance liquid chromatography (HPLC) analysis of the active fraction/s to get their chemical profile.

1.1 Objectives

The objectives of the study are:

i. To optimize the inoculum size of *Trypanosoma brucei brucei* in the *in vitro* antitrypanosomal assay to test crude extracts from actinomycetes.

ii. To characterize the potential actinomycetes that produce extracts with high antitrypanosomal activity.

iii. To determine the optimum fermentation period for maximum antitrypanosomal metabolites productions to carry out bioassay-guided isolation of active fraction/s from the potential extract.

CHAPTER 2

LITERATURE REVIEW

2.1 General overview of actinomycetes

One particular family of microbe that grabs the attention of scientists was actinomycetes. Some scientists considered the actinomycetes as bacteria while others categorized them as fungi because they have filamentous form. Actinomycetes are intermediate in character between bacteria and fungi as they share some properties with true bacteria and some with fungi. Finally, research investigation has considered the actinomycetes as prokaryotic organism because they have a cell wall structure characteristic of bacteria (Goodfellow and Williams, 1986).

According to National Center for Biotechnology Information (NCBI) on taxonomy browser, actinomycetes and actinobacteria are synonyms. Actinomycetes are Gram-positive filamentous bacteria with high DNA guanine-plus-cytosine (G+C) contents. They belong to the order *Actinomycetales* (Superkingdom: Bacteria, Phylum: Firmicutes, Class: Actinomycetes, Subclass: Actinobacteridae), sub-order of Actinomycineae and family of Actinomycetaceae (Schaal *et al*., 2006). Some actinomycetes form a branching filament and produce mycelium. The mycelium can be a substrate or both substrate and aerial. Actinomycetes are also a group of physiologically diverse bacteria where the diversity can be seen in the production of extracellular enzymes and in the thousands kinds of metabolic products which they

synthesize and excrete (Ensign, 1992). Most of these products are antibiotics with the ability to inhibit growth of other bacteria, fungi, viruses and protozoa.

Actinomycetes are widely distributed in various habitats including soil, water, marine sediment, plant and extreme environment such as desert region, antarctic soil, hot spring and highly saline area (Sharma *et al*., 2012). However, the largest population of actinomycetes have come from soil (Iwai and Takahashi, 1992). In natural soil habitats the genus *Streptomyces* is common and usually a major component of the total actinomycetes population (Hayakawa, 2008). Isolation of actinomycetes from soils of a tropical rainforest has been reported by Xu *et al.* (1996); Lo *et al.* (2002); Thakur *et al.* (2007) and Van *et al.* (2011). In each of the study, *Streptomyces* was found to be the most abundant genus isolated, followed by non-*Streptomyces*. Besides that, in Malaysia, the *Streptomyces* genus was also found to be the major group of actinomycetes isolated from soil samples collected from coastal mangrove areas (Getha *et al*., 2004) and from around roots of medicinal and forest plant species (Getha *et al*., 2008). Actinomycetes collected from Penang National Park were generally grouped into the following genera: *Streptomyces* (33.3% of total isolates), *Micromonospora* (11.8%), *Actinoplanes* (2.9%), *Nocardioform* (10.8%). About 27.5% of the isolates were grouped as oligosporic-type. Isolates that do not fall under these groups were categorized as others (Getha *et al*., 2008).

A numbers of studies have shown that the unique ecosystem in Malaysia can be a potential source for isolating bioactive actinomycetes. Vikineswary *et al.* (1997) reported on the isolation and screening for antifungal activity of indigenous

actinomycetes from the mangrove stands of Morib, Selangor. Among the selected strains, 33% exhibited antifungal activity against all the fungi tested. They concluded that actinomycetes isolates from the tropical mangrove mud might be a potential source for the discovery of antifungal compounds. Ismet *et al.* (2002) reported on the diversity of *Micromonospora* spp. in Malaysian mangrove rhizosphere soil. Seventy putative *Micromonospora* isolates were selected and assigned to twelve single and multi-membered colour group based on their colony colour and spore morphology. However the data were not sufficient for the accurate identification of the species. Further taxonomic characterization, especially data on the molecular systematic is essential for species identification.

Getha and Vikineswary (2002) reported on an antagonistic effect of *Streptomyces violaceusniger* strain g10 isolated from a coastal sand bar on *Fusarium oxysporum* f.sp. *cubense* race 4. Inhibition zones in "cross-plug" assay plates demonstrated the evidence for *in vitro* antibiosis of strain g10. An indirect method also showed that the strain produced diffusible metabolites which have inhibitory effects on the growth of *F. oxysporum* f. sp.*cubense* race 4 in a soil environment. Their study suggested the potential of developing strain g10 as a biocontrol agent for *Fusarium* wilt disease of banana. Subsequent studies on mangrove actinomycetes focused on their antimicrobial activity against pathogenic bateria and fungi (Vikineswary *et al.,* 1997; Vikineswary *et al.*, 1998; Kavithambigai *et al.,* 2001).

Isolation of marine actinomycetes was reported by Tan *et al.* (2005) in which non-*Streptomyces* strains from bryozoan or aquatic organisms and marine sponges of Langkawi island, Malaysia were isolated. This study showed that the marine

organisms hold relatively high diversity of non-*Streptomyces* isolates that could be potential producers of novel bioactive compounds. Broad distribution of actinomycetes from the genera *Micromonospora*, *Nocardia,* or *Streptomyces* from marine sources was identified by Goodfellow and Haynes (1984). Kumar *et al*. (2011) screened marine actinobacteria for antimicrobial compounds. A total of 78 isolates were obtained from marine sediments of Bengal bay where *Streptomyces* is the predominant genus. Twenty eight percent of the isolates exhibited antibacterial activity and others exhibited both antibacterial and antifungal activity.

Apart from the forest soil and marine habitats, actinomycetes have been isolated from other unusual habitats with extreme environment. Li *et al.* (2005) isolated alkaliphilic actinomycetes, strain YIM 80305 from a muddy sample in Chaka salt lake. In the study, optimum pH for growth of the strain was 9.0 to 10.0 with a limited growth at pH 7.0 and the strain was assigned to the genus *Streptomyces*. Another alkaliphilic actinomycetes belonging to a new genus and species, *Borgoriella caseilytica* was obtained from a soda soil sample. This strain has the characteristics of not acid fast, halotolerant and optimal growth occurs at pH values between 9.0 and 10.0 (Groth *et al.,* 1997). According to Yumoto *et al.* (2011) alkaliphilic bacteria can be defined as a bacteria that can grow at above or at pH 10.0 or grow equally better at pH 9.0. Acidophilic actinomycetes have been isolated from acidic forest soils and lowland peaty soil. It was found that acidophilic actinomycetes were present in soils whose actual pH does not exceed 6.8. This study concluded that all soil actinomycetes that are isolated through media with acidic pH are able to grow at pH 5.0 or lower can be considered to be acidophilic (Zakalyukina *et al.*, 2002).

Thermophilic *Streptomyces* can be grown between 28^{o}C and 55^{o}C (Goodfellow *et al.*, 1987). According to Tendler and Burkholder (1961) thermophilic actinomycetes can be divided into two genera which are *Streptomyces* and *Thermoactinomyces*. Results on temperature limits of the thermophilic actinomycetes showed that organisms that grow to fairly narrow ranges of high temperature (65 to 67^{o}C) belong to the genus *Thermoactinomyces* and only a few *Streptomyces* isolates can grow at temperature above 60^{o}C. Studies on a cluster analysis of *Streptomyces* from extreme environments suggested that the thermophilic (growth at 65^{o}C), psychrophilic growth at 0^{o}C; optimum growth at 10 to 15^{o}C; no growth at 28^{o}C, acidophilic (optimum growth at pH3.0 to 4.0) and alkalophilic (optimum growth at pH10.0) strains should be regarded as an independent species-group (Jiang *et al.*, 1989).

It is believed that one efficient way of discovering novel bioactive metabolites is through isolation of novel microorganisms from new niche habitats. Thus, the isolation of new actinomycetes strains for screening of bioactive compounds from the desert soils at Mojave Desert, California was investigated by Takahashi and Omura (2003). In this study, the optimum temperature for growth of actinomycetes isolated from the desert soil was higher than the actinomycetes isolated from the temperate zone. This can be logically understood with the relationship of the high temperature and dryness of the desert environment. Bioactive compound were also discovered from actinomycetes isolated from extreme environments such as Antartic (Spring *et al.*, 2003).

Actinomycetes within healthy plants have also been widely reported. Endophytic microorganisms include bacteria, actinomycetes and fungi that live in the tissue of living plants (Hasegawa *et al.*, 2006). There are about 300,000 plant species on earth and each plant is a host to at least one endophytic microorganism (Strobel and Daisy, 2003). Endophytic actinomycetes from tropical plants were reported to be widespread in roots and uncommon in leaves (Janso and Carter, 2010). In their study, 57% of the isolates obtained were identified as *Streptomyces* sp. and the remaining as *Nocardioides*, *Kitasatosporia*, *Pseudonocardia*, *Actinomadura* and *Kibdelosporangium*. Priya (2012) found that endophytic *Streptomyces* sp. were prolific producers of fungal inhibitory bioactive compounds.

2.2 Characterization of actinomycetes

A polyphasic taxonomic approach involving phenotypic and phylogenetic analysis is adopted in classification and description of actinomycetess. Phenotypic characterization of actinomycetes includes morphological (aerial mass/surface colour, reverse side colour of culture, soluble pigment appearance and structure of spore chain), physiological, and biochemical characterization. While phylogenetic analysis is derived from molecular methods such as 16S rRNA sequencing and DNA hybridization (Vandamme *et al.*, 1996). According to Goodfellow and Haynes (1984), a combination of macromorphological, biochemical and spore characteristics can be used for identification of many genera.

2.2.1 Morphological characteristics

The morphological characteristics of actinomycetes are useful in identification of *Streptomyces* spp. as described by Nonomura (1974) and Buchanan and Gibbons (1974).These characteristics are useful in routine identification for taxonomy of actinomycetes for many years. Mature cultures on growth media such as yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts-starch agar (ISP4) (Shirling and Gottlieb, 1966) with heavy spore mass are visually examined to determine aerial spore mass colour, substrate mycelium pigmentation and colour of diffusible pigments or other melanoid pigments (Shirling and Gottlieb, 1966; Williams *et al.,* 1969). According to Korn-Wendisch and Kutzner (1992) morphological criteria such as spore-chain type and spore surface ornamentation are useful for characterization of actinomycetes.

2.2.1.1 Grouping into colour series

The spore mass colour groups were initially developed for the grouping of *Streptomyces* spp. because observation by naked eyes is subjective and prone to human error (Tresner and Backus, 1963). Cultures without aerial mycelium/ non-sporulating aerial mycelium (degenerate strains) are not evaluated using this colour system. Nevertherless, to a certain extent, colour grouping has allowed preliminary differentiation into smaller, manageable groups for further tests when handling a large number of different isolates.

2.2.1.2 Micromorphological characteristics

The spore chain formation and spore bearing-hyphae can be described only with mature cultures and should be determined by using direct microscopic examination of the culture surface (Shirling and Gottlieb, 1966). As the colony become mature, characteristics of aerial filaments known as sporophores are formed. The *Streptomyces* spore called conidia is produced simply by the formation of cross-walls in the multinucleate sporophores followed by separation of the individual cells directly into spores (Figure 2.1). According to Korn-Wendisch and Kutzner (1992) characterization of *Streptomyces* should involve light and electron microscopy because the structure of arthrospores which is one of the taxonomic properties for species identification can only be determined by electron microscopy. In this study, with direct microscopic observation using long working distance lens and adjustment of 300x magnification, the presence or absence of spore chains can be established to observe the nature of sporophores and grouped into different category of "morphological section" based on different sporebearing structures (Figure 2.2).

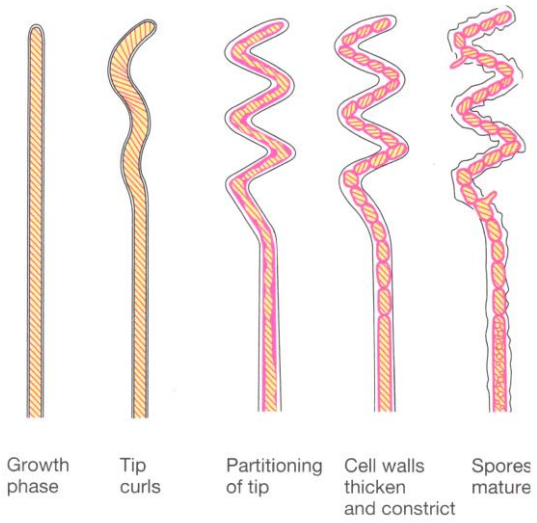

Figure 2.1: Diagram of stages in the conversion of a *Streptomyces* aerial hypha (sporophores) into spores (conidia) (Madigan *et al.*, 2000).

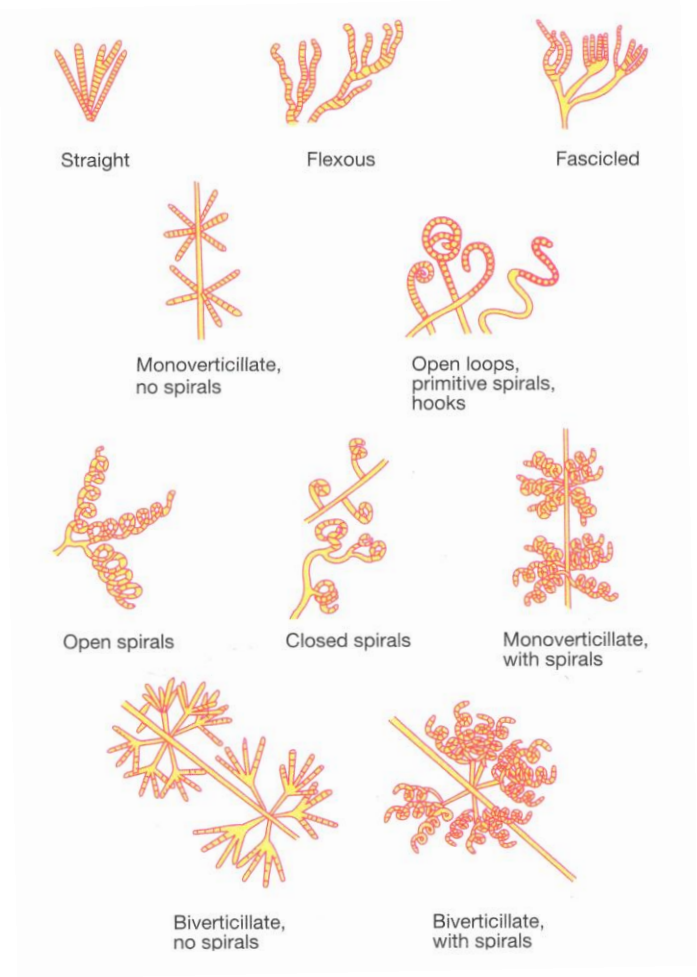

Figure 2.2: Morphological sections of *Streptomyces* based on various types of sporebearing structures (Madigan *et al.*, 2000).

2.2.2 Physiological characteristics

To provide a more reliable characterization of the actinomycetes, a number of useful physiological tests have been utilized that includes the determination of temperature range for growth, pH, utilization of carbohydrate and a similar compounds as a carbon source, formation of melanin, utilization of organic acids, use of nitrogen sources, reduction of nitrate to nitrite, hydrolysis of urea (Korn-Wendisch and Kutzner 1992). Williams *et al.* (1983) had reported that the type of substrate utilized and susceptibility of the isolates to different antibiotics are useful physiological tests in the identification of actinomycetes. As reported by Kavitha and Vijayalakshmi (2007), *Streptomyces rochei* exhibited sensitivity to a number of antibiotics such as amikacin, bacitracin, ciprofloxacin, clindamycin, erythromycin, furoxone, gentamicin, kanamycin, metronidazole, neomycin, oxytetracycline, penicillin-G, polymycin-B, roxithromycin, streptomycin, tetracycline and vancomycin. However, this strain was resistant to ampicilin, deoxycycline hydrochloride, methicillin, nalidixic acid, rifampicin and trimethoprim. Based on this physiological characteristic, the strain was identified as *Streptomyces rochei*.

Other physiological criterion tested is temperature range where optimal growth of the isolate is obtained. Actinomycetes which can grow at low temperatures up to -10^{o}C are called psychrophiles. Mesophilic actinomycetes can grow at medium temperature between 20-45^{o}C and thermophilic actinomycetes at temperature above 45^{o}C. Formation of aerial mycelium is observed at appropriate time intervals which are 5, 10 and 15 days for mesophilic and 2, 5 and 10 days for thermophilic actinomycetes (Korn-Wendisch and Kutzner, 1992).

Other physiological characteristics of actinomycetes studied are NaCl tolerance and pH tolerence. The optimum pH range for growth of actinomycetes is in the range of 6.5 to 8.0. In acidic soils with pH less then 5.0, actinomycetes are almost non-existence. The usefulness of NaCl tolerance as a taxonomic aid for *Streptomyces* has been reported (Tresner *et al.,* 1968). Tolerance up to 13% NaCl was observed in the study. Relationship of NaCl tolerance to various taxonomic features of the test strains was also established. Halo-tolerance test on multiple strains of every species examined in the study showed that in most cases, the responses from strain to strain within each species were uniform. According to Remya and Vijayakumar (2008), the physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physical conditions.

2.2.3 Biochemical characteristics

Actinomycetes contain 2,6-diaminopemelic acid (DAP) in the cell wall peptidoglycans and the amino acid has three stereoisomers (LL-, DD-, and meso-DAP) (Takahashi *et al.,* 1989). Generally, actinomycetes strains contain either LL- or meso-DAP. However, *Kitasatospora spp*. contain both LL- and meso-DAP (Omura *et al*., 1981). Most of the DAP contained in aerial mycelium of this genus is LL-isomer while meso-DAP is found mainly in a substrate mycelium. Major amounts of meso-DAP and a small amounts of both LL- and DD-DAP were also detected in the cell wall of some *Micromonospora spp.* (Hoare and Work, 1957).

The possible application of cell-wall DAP analysis in actinomycetes taxanomy was demonstrated by Cummins and Harris (1956), who also reported that cell wall composition is a stable character, unaffected by cultural factors such as medium and growth conditions. Diaminopemelic acid (DAP) analysis was used successfully to differentiate between non-*Streptomyces* (contains meso-DAP) and *Streptomyces* (contain LL-DAP) strains, including strains with morphology intermediete between the two genera and morphological variants of the same strain (Becker *et al*., 1964). Subsequently, Becker *et al*. (1965) classified various actinomycetes genera into four cell wall types based on their cell-wall DAP and sugar components. Variant strains of the four cell wall groups with different morphology from the parent were found to have no major changes in their cell wall constituents, thus confirming the stable characteristics of cell wall composition is useful in the classification of actinomycetess (Suput *et al*., 1967). However, Hasegawa *et al*. (1983) conducted an improved method from Becker *et al*. (1965) which is more simple, rapid and accurate method for grouping of actinomycetes. In this method, colony of each culture was harvested at a moderate growth phase and used as a whole cell sample hydrolysates. Samples and standard (containing LL- and meso- DAP) were spotted on a same thin cellulose sheet. Separation of DAP isomers from sample hydrolysates by thin-layer chromatography were compared with standard to determine whether LL- or meso- DAP occurred in the sample.

2.3 Secondary metabolites from actinomycetes

Natural product compounds are derived either by primary or secondary metabolism in living organisms. The primary metabolites are common in all biological system while the secondary metabolites are a group of diverse compounds with unclear functions or activities (Berdy, 2005). In general, secondary metabolites can be described as compounds that are synthesized by organisms that are not strictly necessary for the survival of the organism. The concepts of secondary metabolism include products from excess metabolism as a result of nutrient limitation, thus force the production of bioactive secondary metabolites (Sarker *et al*., 2006). Antibiotics are secondary metabolites isolated from microbes and exhibit a wide range of bioactivity (Berdy, 2005). Most antibiotics are excreted when the producing microbes are grown in rich media. The ability of microorganisms to produce diverse and selective bioactive agents through secondary metabolism may lead to the discovery of useful compounds (Araragi, 1979).

Among the bacteria, actinomycetes have proved to be a particularly rich source of antibiotics (Goodfellow and Williams, 1986). Actinomycetes are well known as secondary metabolite producers and also a group of physiologically diverse bacteria. This diversity is seen both in the production of extracellular enzymes and secondary metabolic products that they synthesize and excrete. Many of these products are antibiotics and have a high pharmacological and commercial interest. Most of them showed antibacterial, antifungal, antiviral, anticancer, herbicidal, insecticidal and antiparasitic activities (Kazuro and Satoshi, 2004). Out of 10,000

bioactive compounds produced by actinomycetes, 76% are derived from the genus *Streptomyces* (Berdy, 2005).

The genus *Streptomyces* is the most noteworthy producers of bioactive compound. It is a prolific producer of structurally diverse bioactive metabolites and has yielded some of the most important products in the drug industry including erythromycin, kanamycin, tetracyclines, gentamicin and other classess of antibiotics. They have provided over two-thirds of the naturally occurring antibiotics discovered and continue to be a major source of novel and useful compounds (Berdy, 2005). A large number of *Streptomyces* spp. have been isolated from soil and screened for various bioactivities in the past (Watve *et al.,* 2001). Table 2.1 shows some of the metabolites produced by soil actinomycetes and their activities.

Table 2.1: Actinomycetes isolated from soil and their secondary metabolites

Species	Metabolites	Activity	References
Saccharopolyspora erythrae	Erythromycin	- Antibacterial - Respiratory infectious diseases	El-Enshasy *et al.*, 2008.
Streptomyces kanamyceticus 12-6	Kanamycin	- Antibacterial - Anti-infectives	Murakami *et al.*, 2011.
Streptomyces flavoviridis	Zorbamycin	- Antitumor	Wang *et al.*, 2007.
Streptomyces hygroscopicus	Himastatin	- Antitumor	Leet *et al.*, 1995.
Streptomyces rimosus	Oxytetracycline	- Antibacterial	Hansen *et al.*, 2001.
Streptomyces nodosus	Amphotericin B	- Antifungal - Antileishmania	Caffrey *et al.*, 2001.
Streptomyces venezuelae	Chloramphenicol	- Antibacterial	He *et al.*, 2001.
Streptomyces sp.	Vinylamycin	- Antibacterial	Igarashi *et al.*, 1999.
Streptomyces nanchangensis	Meilingmycin	- Antiparasitic	Sun *et al.*, 2002.
Streptomyces tendae	Nikkomycins	- Antifungal	Decker *et al.*, 1989.
Nocardia sp	Tubelactomicin	- Antibacterial	Igarashi *et al.*, 2000.

Nocardia lurida	Benzanthrins A and B	-	Antibacterial	Theriault *et al.*, 1986
Streptomyces nogalater	Nogalamycin	-	Antitumor	Ylihonko *et al.*, 1996.
Streptomyces rochei	Lankamycin	-	Antibacterial	Suzuki *et al.*, 2010.
Streptomyces platensis	Resormycin	- -	Herbicidal Antifungal	Igarashi *et al.*, 1997.
Streptomyces lavendulae	Ileumycin	-	Antifungal	Kawakami *et al.*, 1978.
Streptomyces lavendulae	Mitomycin C	-	Antitumor	Sheldon *et al.*, 1999.
Micromonospora neihuensis	Neihumicin	-	Cytotoxic	Wu *et al.*, 1988.
Streptomyces niveus	Novobiocin	-	Antibacterial	Kominek, 1972.
Streptomyces sp. HP 530	Saptomycins	-	Antitumor	Abe *et al.*, 1993.
Streptomyces lavendulae	Lavendamycin	-	Antitumor	Balitz *et al.*, 1982.
Streptomyces microflavus	Fattiviracin A1	-	Antiviral	Uyeda *et al.*, 1998.
Actinomadura verrucosospora	Verucopeptin	-	Antitumor	Nishiyama *et al.*, 1993.

2.4 Human African Trypanosomiasis

Human African trypanosomiasis (HAT) or sleeping sickness is caused by the protozoan parasites *Trypanosoma brucei* infection. This disease is endemic in Africa and caused by two subspecies of *Trypanosoma brucei* which are *Trypanosoma brucei rhodosiense* and *Trypanosoma brucei gambiense*. *T. b. rhodosiense* causes more serious health risk in eastern and southern Africa and *T. b. gambiense* causes a major public health problem over a huge areas of western and central African (Fevre *et al.*, 2006). Other subspecies of *T. brucei*, which usually infects domestic and wild animals, are known as African animal trypanosomiasis or nagana disease. This disease does not infect humans and is caused by non-human pathogenic trypanosome species such as *T. b. brucei, T. congolense* and *T. evansi* (Steverding, 2008). However, there are several reports of HAT appearing in human which were caused by non-human pathogenic trypanosome species (Brun *et al.,* 2010). Molecular analysis of blood samples taken from a 10-month old Ghanaian boy recovered from a *T. brucei* infection indicated that the parasites belonged to the *T. b. brucei* species which is normally non-infectious to human (Deborggraeve *et al.*, 2008). In another study, Joshi *et al*. (2005) reported of a human infection by *T. evansi*, the causative agent of animal trypanosomiasis. There was also a report of a woman patient in Cote d'Ivoire who had mixed infection of *T. brucei* and *T. congolense* after the trypanosomes were analysed using DNA identification. The patient was treated successfully with pentamidine and her general health improved within two weeks (Truc *et al*., 1998). Based on the cases that have been reported, Deborggraeve *et al.* (2008) suggested that in a non-endemic region, HAT-suspected cases should be critically diagnosed and managed because misdiagnosis of non-HAT cases increase

the unnecessary administration of toxic HAT drugs and the consequent discomfort and risks.

Trypanosomes are transmitted by the bloodsucking male and female tsetse flies of the genus *Glossina* sp. (Figure 2.3) from one mammalian host to another (Salem *et al*., 2006). As reported by Brun *et al.* (2010), HAT transmission occurs in children and adults during activities such as farming, hunting, fishing or washing cloth. Trypanosoma parasites were first detected in the blood at four to 10 days after infection, showing that migration of parasites from the site of fly bite was very rapid. This will cause anaemia as a key feature of the disease, with a reduction in the numbers and average size of red blood cells and associated decline in numbers of platelets and white blood cells (John *et al*., 2008). The late stage of infection starts when the parasite cross the blood-brain barrier, invading the central nervous system and caused the neurological and endocrine disorder including confusion, sensory disorder and sleep abnormalities which gave the disease its name (Simarro *et al*., 2008). In the absence of a treatment, the disease progresses to the final stage, leading to coma and death.

Figure 2.3: The Tsetse fly

Image source: http://www.nri.org/news/archive/newsarchive2000-01.htm (accessed on Jan 18,2012)