DISCOVERY OF ANTIBACTERIAL-PRODUCING ACTINOBACTERIA FROM SELECTED MALAYSIAN MANGROVE SEDIMENTS

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by

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LIST OF ABBREVIATIONS

SCA	Starch casein agar
AIA	Actinomycete isolation agar
CVA	Coal-vitamin agar
HVA	Humic acid-vitamin agar
MHT	Moist heat treatment
DHT	Dry heat treatment
CT	Chemical Treatment
CFU	Colony forming unit
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MHB	Muller-Hinton broth
MHA	Muller-Hinton agar
DMSO	Dimethyl sulfoxide
ISP	International *Streptomyces* Project
SSM-T	Synthetically Suter medium with tyrosine
SSM	Synthetically Suter medium without tyrosine
PCR	Polymerase chain reaction
MRSA	Methicillin resistant *Staphylococcus aureus*
TLC	Thin layer chromatography
R_f	Retention factor
UPLC	Ultra-Performance Liquid Chromatography
PAH	Polycyclic aromatic hydrocarbon

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PENEROKAAN AKTINOBAKTERIA PENGHASIL ANTIBIOTIK DARIPADA ENAPAN BAKAU TERPILIH DI MALAYSIA

ABSTRAK

Kemunculan pantas bakteria patogen rintang pelbagai drug telah menyeru penyelidikan intensif dalam meneroka metabolit antibakteria baru dan poten. Aktinobakteria merupakan salah satu penghasil utama metabolit sekunder antibakteria. Antara sumber yang boleh dipercayai bagi pemencilan aktinobakteria adalah tanah hutan dan enapan marin. Bakau adalah persekitaran marin yang kaya dengan biodiversiti, namun kurang diteroka bagi pemencilan aktinobakteria. Sewajarnya, kajian ini dijalankan dengan tujuan utama bagi memencil dan menyaring aktinobakteria penghasil antibiotik daripada enapan bakau. Enapan bakau disampel daripada persekitaran bakau terletak di Balik Pulau, Pulau Pinang, Malaysia. Sebanyak 131 pencilan aktinobakteria telah berjaya diperoleh menggunakan gabungan pra-rawatan selektif dan medium pencilan selektif. Saringan awal pencilan ini terhadap lima spesis bakteria Gram-positif dan Gram-negatif menggunakan kaedah calit silang plat menunjukkan sebanyak 54 % pencilan mampu menghasilkan metabolit antibakteria. Dalam kalangan pencilan aktif ini, 70 % mempunyai ciri antibakteria spektrum luas yang mana ia mampu merencat pertumbuhan bakteria Gram-positif dan Gram-negatif, 24 % mempunyai ciri antibakteria spektrum sempit yang hanya merencat pertumbuhan bakteria Gram-positif dan hanya 6 % merencat pertumbuhan bakteria Gram-negatif. Sebanyak lima pencilan poten dengan ciri antibakteria spektrum luas dipilih bagi kultivasi dalam tiga liter fermentasi kultur tenggelam menggunakan bioreaktor turus buih. Ekstrak mentah kaldu fermentasi diperoleh menggunakan pengestrakan pelarut dengan etil asetat pada nisbah 1:1 dan diuji bagi memperoleh nilai kepekatan perencat minima

(MIC) dan kepekatan bakterisidal minima (MBC) terhadap 17 bakteria ujikaji. Nilai MIC terendah diperoleh adalah 9.77 μg/mL terhadap pencilan klinikal *Pseudomonas aeruginosa*, *Enterococcus faecalis* dan *Listeria monocytogenes* ATCC 19114. Tiada nilai MBC direkodkan walaupun pada kepekatan tertinggi ekstrak etil asetat pada 5000 μg/mL. Penulenan awal bagi ekstrak etil asetat aktinobakteria paling aktif menggunakan kromatografi turus menghasilkan sebanyak 11 pecahan. Hanya Pecahan 1 hingga 4 menunjukkan aktiviti antibakteria terhadap pencilan klinikal *P. aeruginosa*, *E. faecalis*, *Staphylococcus aureus*, *Staphylococcus aureus* rintang metisilin, *Shigella boydii* ATCC 9207, dan *Staphylococcus aureus* ATCC 12600. Pecahan 1 mempamerkan MIC dan MBC terendah dengan nilai 0.15 μg/mL and 2500 μg/mL masing-masing, terhadap *P. aeruginosa* dan *E. faecalis*. Pecahan 1 dipisahkan lagi menggunakan kromatografi lapisan nipis dan menghasilkan tiga tompok. Bioautografi bagi tompok ini menggunakan *S. aureus* ATCC 12600 menunjukkan hanya satu tompok aktif pada nilai R_f 0.88. Analisa kromatografi cecair prestasi ultra mengesan hanya satu puncak utama dalam kromatogram yang mungkin merupakan metabolit yang penting. Satu pencilan aktinobakteria dengan ekstrak etil asetat paling aktif, bernama pencilan PBD-310J dipilih bagi pencirian dan pengenalpastian aktinobakteria. Pencirian melalui ciri morfologi dan fisiologi, dan pengenalpastian molekul 16S rRNA telah memadankan pencilan tersebut sebagai salah satu spesies *Streptomyces* dan berkait rapat dengan *Streptomyces praecox*. Penemuan dalam kajian ini menunjukkan bahawa enapan bakau Malaysia mempunyai simpanan aktinobakteria penghasil antibakteria yang baik, yang mungkin boleh dijadikan calon agen antibakteria pada masa hadapan.

DISCOVERY OF ANTIBACTERIAL-PRODUCING ACTINOBACTERIA FROM SELECTED MALAYSIAN MANGROVE SEDIMENTS

ABSTRACT

The rapid emergence of multi-drug resistant pathogenic bacteria has called for intensive research on the discovery of new and potent antibacterial metabolites. Actinobacteria is one of the major producers of antibacterial secondary metabolites. Among the most reliable sources for the isolation of actinobacteria are forest soil and marine sediments. Mangrove is a rich marine environment, but it is seldom explored for actinobacterial isolation. Accordingly, the present study was carried out with the main aim to isolate and screen for potential antibacterial-producing actinobacteria from mangrove sediments. The mangrove sediments were sampled from the mangrove environment located at Balik Pulau, Pulau Pinang, Malaysia. A total of 131 actinobacterial isolates were successfully isolated by using a combination of selective pre-treatment and isolation media. Preliminary screening of these isolates against five species of Gram-positive and Gram-negative bacteria using cross streak plate method showed that 54 % of the isolates were capable of producing antibacterial metabolites. Among these active isolates, 70 % have broad-spectrum antibacterial activity against both Gram-positive and Gram-negative test bacteria, 24 % have narrow-spectrum antibacterial activity against only Gram-positive bacteria and 6 % against only Gram-negative test bacteria. Five actinobacterial isolates with potent broad-spectrum antibacterial activity were selected for cultivation in three litres submerged culture fermentation using a bubble-column bioreactor. The crude fermentation broth extract was obtained through solvent extraction using ethyl acetate at 1:1 ratio and tested for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against 17 test

bacteria. The lowest MIC value detected was 9.77 μg/mL against the clinical isolates *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Listeria monocytogenes* ATCC 19114. No MBC value was recorded even at the highest test concentration of 5000 μg/mL of the ethyl acetate extract. Preliminary purification of the most active actinobacterial ethyl acetate extract through column chromatography produced a total of 11 fractions. Only Fractions 1 to 4 showed antibacterial activity against clinical isolates *P. aeruginosa*, *E. faecalis*, *Staphylococcus aureus*, Methicilin resistant *Staphylococcus aureus*, *Shigella boydii* ATCC 9207, and *Staphylococcus aureus* ATCC 12600. Fraction 1 exhibited the lowest MIC and MBC values of 0.15 μg/mL and 2500 μg/mL, respectively against *P. aeruginosa* and *E. faecalis*. Fraction 1 was further separated using thin layer chromatography, which produced three spots. Bioautography of these spots against *S. aureus* ATCC 12600 showed that only one spot with R_f value of 0.88 was active. Ultra-performance liquid chromatography analysis also detected only one major peak in the chromatogram, which might be the possible metabolite of interest. One actinobacterial isolate with the most active ethyl acetate extract, namely isolate PBD-310J was selected for isolate characterization and identification. Characterization through morphological and physiological characteristics, and the 16S rRNA gene sequence showed that the isolate was a species of *Streptomyces* and closely related to *Streptomyces praecox*. Thus, the finding from this study revealed that the Malaysian mangrove sediments showed a good reservoir of antibacterial-producing actinobacteria which could be candidates for future antibacterial agent.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Infectious disease is defined as the illness that is caused by pathogens (bacteria, fungi, parasites or virus) that results from the transmission of the pathogens from an infected person, animal, or environment (Barreto *et al*., 2006). Following the discovery of antimicrobial drugs over the past six decades, the statistics of infectious disease cases had descended steadily and some microbial infectious diseases were eliminated while others became controllable (Trent, 2005; Berdy, 2012). However, the recent emergence and dissemination of multidrug-resistant pathogens had caused the loss in efficacy of the present standard antimicrobial drugs, which jeopardized the infectious disease treatment (Rossolini *et al*., 2014). In 2016, the World Health Organization (WHO) had reported that the infectious diseases were among the top 10 causes of death globally involving lower respiratory infections, where it had caused three millions death worldwide (WHO, 2018). Antibiotic-resistant bacteria such as carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and members of Enterobacteriaceae (e.g. *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* sp., *Serratia* sp., *Proteus* sp., and *Morganella* sp.) have developed a new way to resist antimicrobial drugs and can pass along the resistant genetic materials to other bacteria (WHO, 2017). Carbapenems are β-Lactam class antibiotics, possessed the broadest spectrum of activity and highest potency, which used as a last-line antibiotic agent for treating serious multidrug-resistant infections (Papp-Wallace *et al*., 2011). This critical situation

prompted WHO to issue a warning about these antibiotic-resistant pathogens, which require an immediate action (WHO, 2017). Therefore, discovering a new and potent antimicrobial agent is urgently needed to curb this problem and research in this field is a priority.

Antimicrobial drugs have been discovered from many sources such as from natural products (plants, animals, and microorganisms), as well as through chemical synthesis (Katz & Baltz, 2016). The chemically synthesized antimicrobial drugs such as sulfa drugs and organoarsenicals developed by Domagk and collaborators (1930s) were commercialized for clinical use many years before the commercial use of penicillin by Alexander Fleming in 1940s (Lesch, 2007; Wright *et al*., 2014). These synthetic antimicrobial drugs worked great in killing bacterial pathogens without affecting the host cells and their production was economical due to their simple structures (Wright *et al*., 2014). However, the major limitations of these antimicrobial drugs such as serious side effects, narrow spectrum of activity and the rapid acquirement of resistance by some bacteria pathogens toward these drugs had compromised their public usage (Kaufmann, 2008; Wright *et al*., 2014). On the other hand, antimicrobial drugs also have been developed through the combination of chemical synthesis and the existing antimicrobial drugs from natural products. Derivatives of these semisynthetic antimicrobial drugs could be created with improved safety profiles, greater chemical stability, and increased antimicrobial potencies compared to their original form (Lye, 1998; Wright *et al*., 2014). Nonetheless, the acquirement of resistance by pathogens remains detrimental to the semisynthetic antimicrobial drugs. The expensive production cost and time-consuming development diminished the interest by the pharmaceutical company to develop these drugs (Rai *et al*., 2013). Alternatively, natural product remains as one

of the largest producers of antimicrobial drugs and other therapeutics. The plant-based natural products contained a wide variety of metabolites, which have antimicrobial activity, but were poorly utilized as antimicrobial drugs due to their lack of clinical trials and toxic to human (Liener, 1970; Cowan, 1999). Berdy (2012) also reported that the drugs from higher organisms have less variability and specificity compared to microbial products. Amongst those natural product producers, about 47 % of them were originated from microbial products (Berdy, 2012).

Actinobacteria are known as the major producer of bioactive metabolites from microbial sources. From more than 22,000 of the known microbial bioactive metabolites, 70 % of them originated from actinobacteria (Subramani & Aalbersberg, 2012). The *Streptomyces* genus produced almost 80 % of the metabolites and almost 50 – 55 % of known antimicrobial drugs were introduced from this genus (Berdy, 2012; Subramani & Aalbersberg, 2012). Actinobacteria can be found in a variety of natural and man-made environments. Nature remains the greatest reservoir of these unique metabolites where it was only a tip of the iceberg of actinobacteria and its antibacterial metabolites have been identified (Baltz, 2008). Finding actinobacteria from special niches could increase the chances of finding a novel, potent, or structurally unique antimicrobial metabolite. Hence, in this study, the mangrove environment was selected to isolate the bioactive actinobacteria. Despite being very sensitive to disturbance and is sparsely distributed, mangrove environment is rich in diversity and highly productive (Jennerjahn & Ittekkot, 2002; Thatoi *et al*., 2013). The extreme tides, extreme temperature fluctuations, high salinity, anaerobic soils, and variable sediment types could result in the discovery of unique and hardy actinobacteria and associated metabolites (Azman *et al*., 2015). Although the

discovery of antibacterial-producing actinobacteria from mangrove sediment have increased (Ventola, 2015), studies conducted in Malaysia is limited.

1.2 Aim and specific objectives of research

The main aim of the present study was to isolate and screen for antimicrobial producing actinobacteria, focusing on antibacterial producers from mangrove sediments. The specific objectives were:

1. To isolate actinobacteria from mangrove sediments using different pre-treatments and selective isolation media.
2. To determine the antibacterial activity of the isolated actinobacteria using cross streak method and tetrazolium microdilution assay.
3. To partially purify the antibacterial metabolite present in the active actinobacterial ethyl acetate extract.
4. To characterize and identify the most active antibacterial-producing actinobacterial isolate.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibacterial agents

The discovery of penicillin by Sir Alexander Fleming in 1929 had shed new light on chemotherapy in combating infectious bacterial diseases (Fleming, 1929). The active agent named 'penicillin' produced by the mould, identified as *Penicillium notatum*, was successfully used as a potent antibacterial agent during World War II (Demain & Sanchez, 2009). This finding was a starting point for the discovery of drugs from microbial sources. During the golden era of antibiotics (1940-1970), many new compounds and their derivatives were discovered through screening processes, such as actinomycin, streptomycin, and streptothricin from actinobacteria by Waksman and his colleagues (Barka *et al*., 2016). The chemical substances were termed as 'antibiotics' by the American microbiologist Selman Waksman and his colleagues to describe the metabolites having antagonistic effects towards the growth of other microorganisms (Sengupta *et al*., 2013).

The term 'antimicrobial' and 'antibacterial' are used interchangeably in many articles as they share a similar concept. However, the difference between them is their target organisms. Antibacterial could be anything that can kill or suppress the growth of bacteria while antimicrobial has a broader range of activity, acting against, not only bacteria but also fungi, protozoa, and viruses (Ioan, 2015). Antimicrobial is also used synonymously with the term 'antibiotics' (Ioan, 2015). About 60 % of the currently known bioactive microbial metabolites (about 14,000 compounds) exhibit

antimicrobial activity, which includes antibacterial, antifungal, and antiprotozoal. Despite the tremendous development and success of antibiotics, infectious diseases still remain as one of the major causes of fatality, especially among children and elderly adults (Procópio *et al*., 2012). Often, increasingly more potent and resilient antibiotics are needed to save lives.

2.1.1 Groups of antibacterial compounds

Antibacterial compounds can originate from various sources, such as natural products or even chemical synthesis. Their average molecular weights is around a few hundred Daltons (Da) (Berdy, 2005; Ebejer *et al*., 2016). Actinomycetales species and other rare actinobacteria are known to produce the most complex, yet most versatile structures of antibacterial metabolites. The molecular weights of their antibacterial compounds typically range from 400 to 800 Da for *Streptomyces* sp. and almost double for rare actinobacteria (Berdy, 2005).

Generally, the antibacterial compounds are classified according to their mechanisms of action. Each group has its own and specific interaction, targeting essential cellular functions to inhibit the growth of the bacteria (Kohanski *et al*., 2010). This complex process begins with the interaction between the molecule and specific target, with the eventual structural changes, disrupting the molecular and intracellular system (Procópio *et al*., 2012). Table 2.1 describes the classes of antibacterial compounds with their respective mechanisms of action.

Table 2.1: Classification of antibacterial compounds based on their mechanisms of action.
(Schroeder *et al*., 2002; Kohanski *et al*., 2010)

Mechanism of action	Descriptions	Class of antibacterial compound
Inhibition of DNA replication	The DNA replication is interfered by trapping DNA gyrase (topoisomerase III) and topoisomerase IV (topoIV) at the cleavage stage of DNA and preventing strand re-joining.	Quinolones
Inhibition of RNA synthesis	Binding to DNA-dependent RNA polymerase and preventing the elongation chain of RNA, thereby making the transcription process incomplete.	Rifamycins
Inhibition of cell wall synthesis	Inhibit cell wall synthesis, which results in cell morphological changes, induction of cellular stress response and finally, causing cell lysis.	β-Lactams Glycopeptide
Inhibition of protein synthesis	Blocking 50S and 30S ribosome sub-units from performing translation of mRNA.	Macrolides
Mycolic acid synthesis inhibitor	Inhibit mycolic acid biosynthesis in mycobacterium.	Isoniazid

2.1.2 Antibacterial resistant among pathogenic bacteria

Presently, antibacterial resistance especially multidrug-resistant bacteria (MDR) has become the biggest threat to human health, food security, and also to global evolution as reported by the World Health Organization (WHO, 2016). This life-threatening problem can cause a catastrophe if left unchecked at the early stage. Antibacterial resistance is defined as the resistance of a bacterial pathogen to antibacterial drugs or medicine to, which it was previously sensitive to (Walsh, 2013). This has rendered bacterial infection treatment with commercial antibacterial drugs non-effective and severely limits treatment options (Kaye *et al*., 2017). In February 2017, WHO had come up with a list of priority pathogens that need a new and effective antibiotic treatment due to resistance problem (Table 2.2).

Table 2.2: The priority level of bacterial pathogens that have developed resistance towards currently available antibiotics. (WHO, 2017)

Level of priority	Bacteria pathogens	Resistance
Critical	*Acinetobacter baumannii*	carbapenem-resistant
	Pseudomonas aeruginosa	carbapenem-resistant
	Enterobacteriaceae (e.g. *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter* sp., *Serratia* sp., *Proteus* sp., and *Morganella* sp.)	carbapenem-resistant 3rd generation cephalosporin resistant
High	*Enterococcus faecium*	vancomycin-resistant
	Staphylococcus aureus	methicillin-resistant, vancomycin intermediate and resistant
	Helicobacter pylori	clarithromycin-resistant
	Campylobacter sp.	fluoroquinolone-resistant
	Salmonella sp.	fluoroquinolone-resistant
	Neisseria gonorrhoeae	3rd generation cephalosporin-resistant fluoroquinolone-resistant
Medium	*Streptococcus pneumoniae*	penicillin-non-susceptible
	Haemophilus influenzae	ampicillin-resistant
	Shigella sp.	fluoroquinolone-resistant

There are a few causes for their resistance among these deadly pathogens. Most of the resistance cases are acquired from the clinical environment (Walsh, 2013). The aging and invasive surgical procedures have increased the risk of contracting a severe infection (Walsh, 2013). As reported by Ventola (2015), factors such as the overuse of antibiotics, inappropriate prescription by physicians, extensive use in the agricultural sector, and poor regulatory barriers, are among the major contributors to this problem. In a non-clinical or environmental setting, resistance can be acquired through the sharing of antibacterial-resistant plasmid from the resistant bacteria, especially from soil. Studies by Oliver *et al*. (2001), Nordmann and Poirel (2005), and Zheng *et al*. (2011) have identified a few environmental bacteria such as *Kluyvera* sp., *Shewanella* sp., and *Erythrobacterlitoratis* sp. carrying antimicrobial resistance gene $bla_{\text{CTX-M}}$, *qnr*A, and bla_{NDM}, respectively. However, the origin of plasmid-mediated resistance gene are still unknown (Walsh, 2013). In

the natural relevance standpoint, it can be hypothesized that this might happen as a part of the ecological change and evolution of the bacteria (Walsh, 2013).

To address this crisis, several approaches have been proposed by researchers. They include high-throughput screening, selection, enrichment process of new antimicrobial metabolites from non-traditional or less traditional sources, mining the microbial genomes to explore the gene specific for antimicrobial biosynthesis, studying of the microbial DNA from the environment by using metagenomics analysis, and transforming the old and forgotten natural compounds for new metabolic pathways (Baltz, 2008). Malaysia is a country harbouring a great biodiversity in its ecosystem. Thus, finding a potent antibacterial producer from the local environment can be possible with the aid of selective isolation and screening.

2.1.3 Sources of antibacterial metabolites

Antibiotics and any other natural products can be found in many kinds of organisms including prokaryotes and eukaryotes (Berdy, 2005). They are the products of secondary metabolism, which might not appear beneficial for the host endurance under laboratory conditions, but might be the key of their survival in their native environment (Katz & Baltz, 2016). Over the past 75 years, the majority of natural products, especially antimicrobial metabolites, have been extracted from bacteria, mainly by the *Actinomycetaceae* family (Berdy, 2012). Among ~28,500 antimicrobial metabolites from microbial sources, almost half of them were originated from actinobacteria (Table 2.3). Besides microorganisms, higher plants and animals also contribute to the number. In higher plant, active compounds such as alkaloids, flavonoids, phenolic derivates, as well as other natural products were found to exhibit antimicrobial activity (Sánchez & Demain, 2015). Even though the

estimated number of compounds could reach up to the thousands, only few hundreds are being used in clinical practices (Spížek *et al*., 2016). As many as 877 drugs have been commercialized between 1988 to 2008 and almost 60 % of them were derived from natural sources (Lefevre *et al*., 2008). Synthetic compounds or chemically-synthesized antibiotics also contributed to the number of drugs available. However, only 0.001 % of them were developed into consumable drugs compared to 0.2 – 0.3 % of the microbial metabolites (Spížek *et al*., 2010).

Table 2.3: Estimated number of antibiotic producers from microorganisms and higher organisms.
Adapted from Berdy (2015)

Antibiotic producers	Number of antibiotics produced
Microorganisms	
Eubacteriales	3500
Cyanobacteria	400
Myxobacteria	450
Actinobacteria	14,500
Streptomyces sp.	11,000
Other Actinobacteria	3400
Other bacteria including proteobacteria	800
Fungi	10,500
Microscopic fungi (*Penicillium*, *Aspergillus*)	9000
Basidiomycetes	2900
Other fungi (including slime moulds, yeast, etc)	110
Total microorganisms	~28,500
Higher organisms	
Terrestrial animals	1000-5000
Marine products (animals and plants)	6000-8000
Higher plants	15,000-16,000
Lower plants (mosses, lichens, and algae)	1500-2000
Total	~23,500-31,000
Total estimated number of natural antibiotics	~59,000

The actual number of microorganisms may be much higher than reported (Spížek *et al*., 2016)

2.2 The actinobacteria

The phylum Actinobacteria is famously known primarily for their complex morphological criteria with the ability to form branching hyphae at some stages of their growth (Goodfellow & Williams, 1983). This phylum is recognized as the largest taxonomic unit among the major lineages within the domain of Bacteria (Whitman *et al*., 2012). Their genomic DNA comprises a high guanine (G) and cytosine (C) content, ranging from 51 % in some *Corynebacteria* to more than 70 % in some *Streptomyces* species (Ventura *et al*., 2007). With their filamentous growth ability and distinct pellet formation in liquid cultures, actinobacteria were once referred to as ray fungi ("Strahlenpilze") or mould bacteria and were mistakenly classified as a group of fungi in the past (Benson & Schultz, 1990; Stackebrandt & Schumann, 2006; Margulis & Chapman, 2009). However, following the discovery of streptomycin from *Streptomyces griseus*, the rapid increase in research focusing on this bacteria provided more evidence to later classify them as Gram-positive filamentous bacteria (Kokare, 2008). It is believed that their 'fungal' features evolved from the daughter cells that did not separate after binary fission, thereby forming a mass of visible filaments called mycelium (Margulis & Chapman, 2009).

Actinobacteria are one of the successful, wide spread group of bacteria that can be found in a diversity of natural and man-made environment. Most of them are strict saprophytes while others can form a parasitic and mutualistic associations with lower and higher plants and animals (Barka *et al*., 2016). The presence of various types and numbers of actinobacteria in soil are totally influenced by the geographical conditions. Normally, in comparison to the population of soil microbes, the actinobacterial population are relatively low and contains a predominance of *Streptomyces* species, which is tolerant to low pH environment (Davies & Williams,

1970). However, rare genera of actinobacteria can be found in alkaline pH arid soils but may contain few *Streptomycetes* populations. Alkaliphilic actinobacteria are a valuable source for new products of industrial interest, including enzymes and antimicrobial agents (Mitsuiki *et al*., 2004).

2.2.1 Taxonomy and classification of actinobacteria

Traditionally, the identification of bacteria was very laborious and time-consuming, except for some pathogenic species (Muramatsu, 2008). However, the advancement in the molecular identification systems, such as the rapid genome sequencing, has successfully transformed the landscape of taxonomic branching of microorganisms, especially the actinobacteria. As a consequence, the phylogenetic relationships of actinobacteria have to be reconstructed on the basis of 16S rRNA gene sequence analysis to meet the requirement of the latest technology (Zhi *et al*., 2009). In recent classification by Whitman *et al*. (2012), this phylum has undergone some modifications by eliminating the subclass and suborder levels, and has been elevated to the rank of class and order, respectively, as shown in Figure 2.1 (Gao & Gupta, 2012). Hence, this phylum now consists of six classes and 22 orders with a majority of orders belonging to the class Actinobacteria (Gao & Gupta, 2012).

The conventional approaches for classification of actinobacteria involve their morphological, physiological and biochemical characteristics. Early taxonomic works took advantage of the unique morphological characteristics and true branching of actinobacteria for their classification (Haley, 1954). The International *Streptomyces* Project (ISP) by Shirling and Gottlieb (1966) is one of the identification and classification system based on the morphological appearance and pigmentation. Through this system, the growth performance, aerial mass colour, the

presence of the reverse side pigment, and melanin formation of isolates were studied and compared (Radhakrishnan *et al*., 2013). These cultural characteristics can also be used for recognizing an individual strain (Kumar *et al*., 2005).

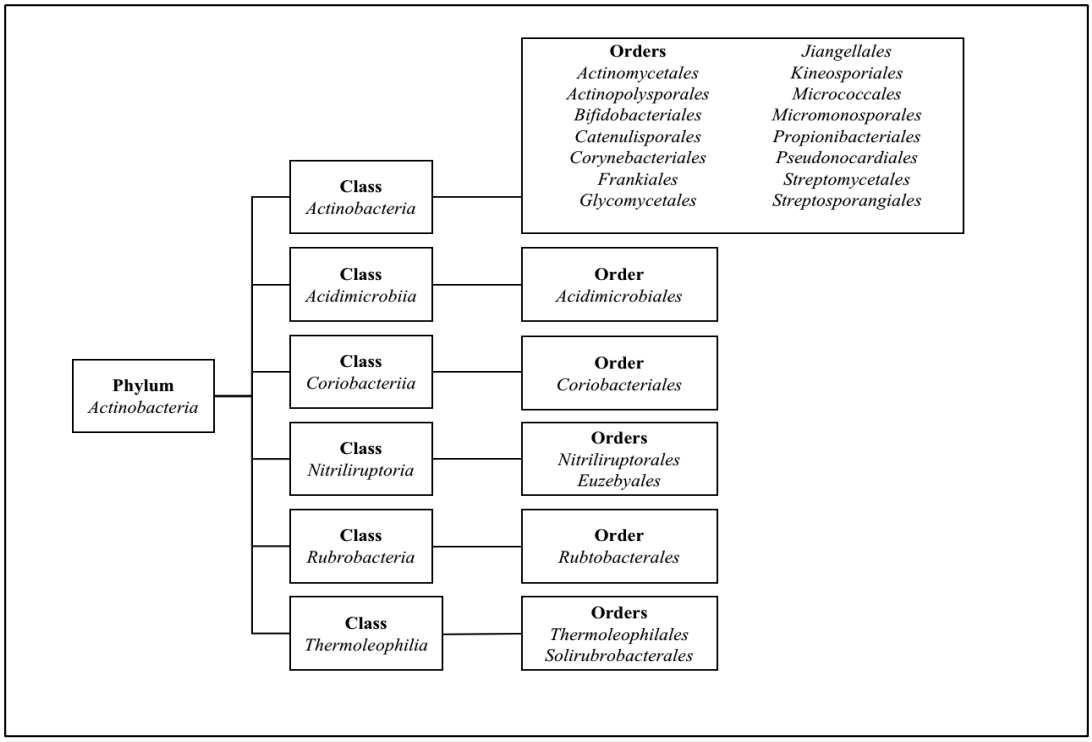

Figure 2.1: Proposed taxonomic branching for actinobacteria in the Bergey's Manual of Systematic Bacteriology.
Adapted from Gao and Gupta (2012)

Streptomyces is a famous genus from the order of Streptomycetales as it is the richest known source of antibacterial metabolites. This genus received particular attention due to their abundance and important soil bacteria for recycling an organic matter, exhibits wide phylogenetic spread, and their important bioactive metabolites producers (Barka *et al*., 2016). *Streptomyces* are chemoorganotrophic, filamentous bacteria, non-motile and non-acid-fast stain (Ikeda *et al*., 2003). The colonies are slow growing and have a relatively smooth surface at the beginning, but later they develop into a thick and weft aerial mycelium that appear powdery, velvety, floccose

or granular (Ambarwati *et al*., 2012). Spore chain morphology is critical in identifying certain actinobacteria for their respective taxonomic classes. In *Streptomyces*, the spores are formed by the fragmentation of the hyphae growing out from the aerial mycelium, borne in a structure of straight to flexuous (rectus-flexibilis), verticillate, open or closed spirals (spira), and open loops (relinaculam-apertum) as shown Figure 2.2 (Pridham *et al*., 1958; Chater, 1993; Barka *et al*., 2016).

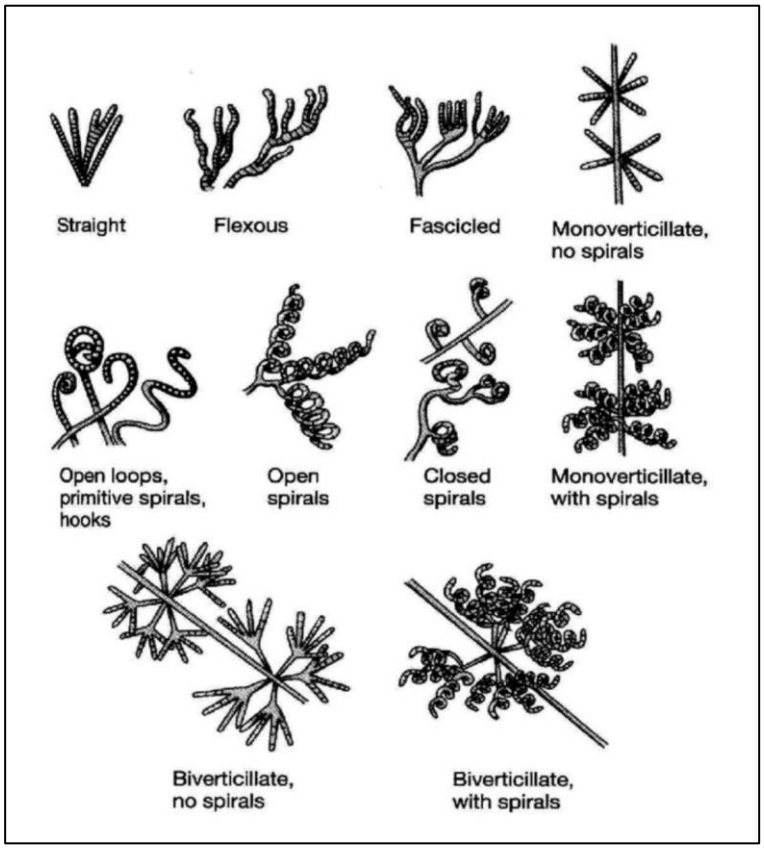

Figure 2.2: Structure of spore-bearing hyphae formed by *Streptomyces*.
Adapted from Madigan and Brock (2011) and Anandan *et al*. (2016).

In terms of spore chain length and number, *Streptomyces* are able to produce very long spore chains containing up to 100 spores (Pridham *et al*., 1958). On the other hand, they also produce a volatile metabolite called geosmin, which

responsible for the earthy or soil-like smell (Jüttner & Watson, 2007). *Streptomyces* also produce wide variety of pigments, which responsible for the colour of vegetative and aerial mycelia (Flardh & Buttner, 2009). Biochemical tests also contributed a significant part in identification and characterization of *Streptomyces*. They are catalase positive, able to degrade casein, gelatine, starch, and L-tyrosine (Smaoui *et al*., 2011).

2.2.2 Actinobacteria as antibacterial producers

Actinobacteria are gaining attention as the gold mine of bioactive natural product producers as they have the ability to produce antifungals, antivirals, anti-hypertensives, antitumor, immunosuppressive agents, and mainly antibiotics (Procópio *et al*., 2012). Even though the advancement in the field of chemical synthesis and engineered biosynthesis has greatly improved the production of synthetic drugs, nature remains the richest source for isolation of unique and versatile antibacterial compounds, which can be utilized directly or indirectly as reference compounds (Baltz, 2006; Pelaez, 2006). Until now, actinobacteria remain as the major producers of antibiotics from the bacteria kingdom, making them very crucial and useful in antibiotic discovery (Berdy, 2005).

The first antibiotic from actinobacteria was discovered back in 1940 with the discovery of actinomycin A and B produced by *Streptomyces antibioticus*. This was followed by streptothricin antibiotics from *Streptomyces lavendulae* (Waksman & Woodruff, 1940, 1942). In a span of almost eight decades of actinobacterial discovery, *Streptomyces* have contributed almost 80 % of all antibiotics from actinobacteria origin (Iliĉ *et al*., 2007). Table 2.4 presents a few antimicrobial metabolites produced by actinobacteria.

Table 2.4: Selected antibacterial producers among actinobacteria genera and their bioactive agents.
Adapted from Barka *et al*. (2016)

Orders	Species	Bioactive agent	Reference
Micromonosporales	*Micromonospora purpurea*	Gentamicin	Weinstein *et al*. (1963)
	Verrucosispora sp.	Abyssomycin	Bister *et al*. (2004)
Actinomycetales	*Marinispora* sp.	Marinomycin	Kwon *et al*. (2006)
Corynebacteriales	*Nocardia lurida*	Ristocetin	Williams *et al*. (1980)
Pseudonocardiales	*Amycolatopsis orientalis*	Vancomycin	Brigham and Pittenger (1956)
Streptomycetales	*Streptomyces venezuelae*	Chloramphenicol	Shapiro and Vining (1983)
	Streptomyces griseus	Cycloheximide	Kominek (1975)
	Streptomyces kanamyceticus	Kanamycin	Umezawa *et al*. (1957)
	Streptomyces niveus	Novobiocin	Kominek (1972)
	Streptomyces lavendulae	Streptothricin	Waksman and Woodruff (1942)
	Streptomyces aureofaciens	Tetracycline	Darken *et al*. (1960)
	Streptomyces mediterranei	Rifamycin	Margalith and Beretta (1960)

2.3 Extreme environment as a source of actinobacterial isolation

Actinobacteria were traditionally isolated from terrestrial sources and marine sediments. For example, *Streptomyces*, *Micromonospora*, *Rhodococcus*, and *Salinispora* species can be found from soil and aquatic environment. They also can be found as plant symbionts (e.g. *Frankia* sp.), animal or plant pathogens (e.g. *Mycobacterium*, *Corynebacterium*, and *Nocardia* species), and gastrointestinal commensals (e.g. *Bifodobacterium* sp.) (Barka *et al*., 2016). The first report in the isolation of mycelium-forming actinobacteria was published by Weyland (1969) from marine sediment several decades ago (Baskaran *et al*., 2011).

In recent studies, many researchers have focused their research to isolate actinobacteria from non-traditional sources. The extreme environment, such as deep-sea hydrothermal vents, hot springs, high salinity water reservoirs, and deserts, were explored for actinobacterial isolation (Kokare *et al*., 2004; Spížek *et al*., 2010; Ballav *et al*., 2015; Tiwari *et al*., 2015; Al-Dhabi *et al*., 2016). These unusual and extreme

environment may have an impact on the adaption mechanism of microorganisms, resulting in the production of unusual compounds (Meklat *et al*., 2011). For example, the discovery of angucycline antibiotic with its nine derivatives including three new compounds by Hu *et al*. (2016) from a deep-sea marine sediment isolated *Streptomyces* with antimicrobial and antitumor properties.

2.3.1 Ecology of actinobacteria

Most of the actinobacteria, especially the *Streptomyces*, are saprophytic in nature. They are soil-dwelling microorganisms that have the most number of morphological changes compared to other bacteria and spend a majority of their life cycles as semi-dormant spores, especially under nutrient-limited condition (Mayfield *et al*., 1972). They can be found in wide range of ecological environments, such as in soils and rocks (Arasu *et al*., 2009; Saravana *et al*., 2014), marine and freshwater (Gebreyohannes *et al*., 2013; Govindarajan *et al*., 2014), and even in desert and Antarctic soils (Balagurunathan *et al*., 2011; Marta *et al*., 2014).

Actinobacteria are commonly abundant in alkaline soils and soils with rich organic matter compared to in other habitats. They can be found not only on the soil surface but also below the ground down to two metres in depth. However, their population density can be influenced by the ecological habitat and climate condition, with typical population densities ranging from 10^6 to 10^9 per gram of soil (Goodfellow & Williams, 1983; Williams & Vickers, 1988). Most actinobacteria are mesophilic, having optimal growing temperatures in the range of 25 to 30 °C. However, other factors such as soil moisture, pH, and salinity also influence their growth (Edwards, 1993). Actinobacteria grow best at neutral pH but they can adapt well in the pH range of 6 to 9 (Kim *et al*., 2003).

Actinobacteria have been found to have interactions with other organisms. They have developed a symbiosis interaction with others. For example, the interaction with invertebrates such as termites, ants, gall midges and beetles, benefits the host or food source while at the same time, helps the mobility of microorganisms, as well as providing food and shelters (Seipke *et al*., 2012). However, the interaction may not necessarily be only as symbionts as they can be in the form of antagonism, commensalism or even mutualism, and from facultative to obligate (Leung & Poulin, 2008).

In addition to invertebrates, actinobacteria have been observed to interact with vertebrates, namely as gut microbiota. They can be found in various locations in the gastrointestinal tract, such as throat, distal oesophagus, gastric fluid, stomach mucosa, as well as in faeces (Barka *et al*., 2016). They also play distinct roles as a plant-associated microbial community. Since they are abundantly present in the soil, it is not surprising that actinobacteria have a crucial interaction with plants in the recycling of nutrient, symbionts, endophytes and even as pathogens (Bulgarelli *et al*., 2013; Barka *et al*., 2016). Many endophytic actinobacteria have been isolated from various plants for their metabolites. Besides protecting the plants from infections, endophytes can improve the growth of their hosts by producing hormones (auxins) that can promote root growth and development (Coombs *et al*., 2004; Overvoorde *et al*., 2010).

On the contrary, some actinobacteria also causes a serious human and animal infections. *Mycobacterium tuberculosis* is the most pathogenic actinobacteria, which caused a deadly infectious disease called tuberculosis or TB (Smith, 2003). The genus *Nocardia* (e.g. *Nocardia asteroides*) is one of the aerobic actinobacteria genera, which causes a wide spectrum of clinical diseases in human and also

animals, such as dogs, cattle, horses, and swine (McNeil & Brown, 1994). Other pathogenic aerobic actinobacteria is *Rhodococcus* sp., which can cause infection in immunocompromised individuals. Primary pulmonary *Rhodococcus equi* infection (pneumonia and lung abscesses) is one of the frequently reported bacterial infections caused by *Rhodococcus* sp. (McNeil & Brown, 1994). On the other hand, *Streptomyces* sp. rarely caused an infection in human. Human infections, such as mycetoma (chronic skin and subcutaneous tissue infection) linked to this genus are rare (Kapadia *et al*., 2007). Most of the invasive infection caused by *Streptomyces* sp. are commonly associated with bacteraemia and lung infection, such as pneumonitis, abscess, and pneumonia (Dunne *et al*., 1998).

2.3.2 Actinobacteria from mangrove environment

Mangrove is a vegetated ecosystem comprising of unique plants species (ferns, shrubs, palms, and trees) found in the intertidal zone of coastal and estuarine environments above the mean sea level (Duke, 2013). Mangroves occupy almost 150,000 km^2 globally, spread across tropical and subtropical coastlines, and highly rich in diversity with at least 73 mangrove species and hybrids recorded (Holguin *et al*., 2001; Krauss & Friess, 2011). In Malaysia, only 1.4 % (4691 km^2) of its land is covered with mangrove (Hamilton & Casey, 2016). Peninsular Malaysia constitute 17 % of the total mangrove area while majority are found in Eastern Malaysia in Sabah (58.6 %) and Sarawak (24.4 %) (Kanniah *et al*., 2015). The majority of mangroves are river-dominated type found on large deltaic plains while the rest are tide-dominated, drowned valley settings, and also carbonate settings (Chong, 2006). Malaysia is also the home among the most diverse mangrove ecosystem in the world, namely the Merbok Estuary (Spalding, 2010). Despite being diverse and

socioeconomically important (Chong, 2006), mangroves are under constant threat from human activities (Heumann, 2011). It was estimated about one-third of the mangrove forest was converted into urban areas, aquaculture, and deforestation within the past 50 years (Holguin *et al*., 2001; Heumann, 2011; Sahriman *et al*., 2017). In Malaysia, about 30 % of mangrove loss was recorded in 1990 and this trend is expected to continue with 1 % mangrove loss annually (Gong & Ong, 1990; Sahriman *et al*., 2017).

The mangrove ecosystem is inhabited by resident and associated mangrove species demonstrating tolerance to extreme changes in their environment (Kathiresan & Bingham, 2001; Tomlinson, 2016). The sediment or substrate consists of sand and mud (mixture of silt and clay), and rich in organic matter (Hossain & Nuruddin, 2016). A strong hydrogen sulphide (H_2S) smell also can be found in muddy sediment, indicating the completely anaerobic property of water logged-soil (Nickerson & Thibodeau, 1985; Tomlinson, 2016).

Microorganisms are important biotic components in influencing the productivity of mangroves (Holguin *et al*., 2001). They help in the decomposition of organic matter, mineralization of organic compound, and also supply nutrients to plants (McGuire *et al*., 2012). Metagenomic study of the microbial population by Basak *et al*. (2015) in Sundarbans mangrove, India, showed that Actinobacteria was one of the dominant phyla along with Proteobacteria, Chloroflexi, Firmicutes, Bacteroidetes, Acidobacteria, and Nitrospirae. A study by Andreote *et al*. (2012) and Thompson *et al*. (2013) on microbial populations in mangrove area along San Paulo coast, Rio de Janeiro, and Bahia, showed that the percentage of actinobacteria were 5.4 - 12.2 %, 8.4 %, and 7.5 %, respectively. Recent studies by Ruan *et al*. (2015), Ser *et al*. (2015), and Zainal *et al*. (2016) also showed that the mangrove

environment was a promising source for isolation of novel species of actinobacteria, as these authors have successfully isolated novel *Streptomyces ferrugineus*, *Streptomyces gilvigriseus*, and *Streptomyces humi* from mangrove sediments.

2.3.3 Previous discoveries of antibacterial-producing actinobacteria from mangrove sediments

Recently, the mangrove ecosystem has become popular for the discovery of novel bioactive metabolites (Azman *et al*., 2015). The discovery of a uniquely structured compound from this ecosystem lead directly to the development of novel antibacterial agents that are effective against multidrug resistant pathogens (Lam, 2006). In 2014, almost 73 novel compounds proven as a potential new antibiotics, antiviral, antitumor, and antioxidant have been isolated from mangrove Actinobacteria (Xu *et al*., 2014). For instance, the discovery of new Azalomycin F analogs from mangrove *Streptomyces* species showed a potent antimicrobial activity and anticancer activity (MIC 1.56 – 25.0 μg/mL) (Yuan *et al*., 2013). Isolation of *Streptomyces olivaceus* (MSU3) from rhizosphere soil of mangrove plant, *Rhizophora mucronat*a also showed a potent antibacterial activity against bacterial pathogens (MIC 0.625 μg/mL against *Streptococcus* sp.) (Sanjivkumar *et al*., 2016).

However, studies on isolation of actinobacteria from Malaysian mangrove for their antibacterial activity are still scarce. There were few studies reported for the isolation of actinobacteria from Malaysian mangrove sediments and their antimicrobial activity by Lee *et al*. (2014), Zainal Abidin *et al*. (2016), Ariffin *et al*. (2017), and Azman *et al*. (2017). Study by Lee *et al*. (2014) was the first report for isolation of actinobacteria from mangrove sediments from the east coast of Malaysia. Nevertheless, most of the antimicrobial assays conducted in their studies were mostly

preliminary study without the quantitative determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

2.4 Antimicrobial assay

There are many *in vitro* antimicrobial susceptibility assays that have been introduced for the detection and evaluation of antimicrobial activity, both qualitatively and quantitatively. Even though these methods share the same final goals, the principles behind them are not necessarily the same. The final results are often influenced by the method, the microorganisms used, and the solubility of the compound tested (Tripoli *et al*., 2007; Valgas *et al*., 2007). Ideally, the assays should be simple to conduct, inexpensive, reproducible, rapid, and maximize sample throughput (Hostettmann *et al*., 1997).

Standard methods have been proposed by various parties in order to standardize the methodology for antimicrobial assays. For example, methods for dilution in the antimicrobial susceptibility test by Clinical and Laboratory Standards Institute (CLSI), and antimicrobial susceptibility testing, European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method by the European Society of Clinical Microbiology and Infectious Diseases, have been introduced for global reference (Cockerill *et al*., 2012; EUCAST, 2015). Modifications and improvements of the methodology have also been made to enhance their reliability (Balouiri *et al*., 2016). For example, the use of tetrazolium salts as an indicator of bacteria growth in the colorimetric method helps in faster and safer bioassay test, giving more reliable results (Eloff, 1998; Grare *et al*., 2008).

The agar disk-diffusion method is the earliest antimicrobial susceptibility test developed by Heatley (1944). This method is still widely used in many clinical

microbiology laboratories for routine antimicrobial susceptibility assay (Balouiri *et al*., 2016). In addition to the agar disk-diffusion, other diffusion methods, such as the agar plug diffusion method, the agar well diffusion method, the cross streak method and the antimicrobial gradient method (E-test) are other available susceptibility tests (Hausdorfer *et al*., 1998; Magaldi *et al*., 2004; Lertcanawanichakul & Sawangnop, 2008; Elleuch *et al*., 2010). The agar plug diffusion is frequently used to emphasize the antagonism activity between microorganism while the E-test is a combination of the dilution and the diffusion method for the determination of minimum inhibitory concentration value (MIC). On the other hand, the cross streak method is the most suitable method for rapid screening of antimicrobial producers and antagonism bioassay (Lertcanawanichakul & Sawangnop, 2008).

The dilution method is considered as the only standard and reliable *in vitro* method for MIC determination to obtain a quantitative value of the antagonistic activity. This method provides a way to estimate the concentration of the antimicrobial agent needed against the tested pathogens (Balouiri *et al*., 2016). The most common dilution method used is the broth macro- or microdilution method and the agar dilution method (Cockerill *et al*., 2012). The antimicrobial assay coupled with thin layer chromatography (TLC) is also becoming a great tool for the separation and the detection of antimicrobial compounds. This method was pioneered by Goodall and Levi (1947) using a combination of paper chromatography and contact bioautography to detect different penicillin compounds. Later, this method was improved by Fischer and Lautner (1961) using thin layer chromatography (TLC). As this method combines both biological and chemical detection methods, it is widely used for screening of organic extracts for

antimicrobial and antifungal activity (Mehrabani *et al*., 2013; Messaoudi *et al*., 2015).

2.5 Actinobacterial fermentation

In term of metabolite production strategies, many researchers are still restricted to the shake flask level in the laboratory scale. This method is quite laborious, offering a limited prospect of parameter controls (Marwick *et al*., 1999). It is also unsuitable when cultivating organisms with low product yields. Unlike any other unicellular microorganisms, filamentous microorganisms tend to prefer the growth on solid media compared to liquid media due to their growth physiology (Prosser & Tough, 1991; Minas *et al*., 2000). However, most metabolite production still takes place in submerged cultures, especially the production of antibiotics from *Streptomyces* (Fernandez & Sanchez, 2001).

Actinobacterial morphology can vary depending on the cultivation medium (Dobson *et al*., 2008). On solid media, colonization takes place by the formation of apical and branched growth whereas in liquid media, they tend to grow in the form of dispersed mycelia, pellets or clumps (entangled filaments) (Minas *et al*., 2000; Pamboukian *et al*., 2002). This physiology may vary depending on the species, size of spore inoculum and also the cultivation condition in the liquid culture (Dobson *et al*., 2008). The stirred tank bioreactor and the bubble column bioreactor are two commonly utilized bioreactors in a variety of bioprocesses (Felix & Gomez, 2009). These types of bioreactors have a very different system, mechanical setup and favour a different physiology of microorganisms. Cultivation of actinobacteria in submerged culture fermentation is not an easy process since their unique morphology may influence the fermentation broth rheological properties and cause problems in

mass transfer and mixing (Olsvik *et al*., 1993; Pamboukian *et al*., 2002). The bubble column and airlift bioreactor systems are more suitable fermentation systems to cultivate filamentous bacteria due to the low-shear environment they provide compared to that of the stirred tank bioreactor (Felix & Gomez, 2009).

When running a fermentation, it is important to consider the whole bioprocess system. Downstream processing is one of the crucial parts in bioprocessing, contributing significantly to the total cost of production. Hence, the bioreactor needs to be set up to complement an easy integration of downstream product separation and purification strategies (Marwick *et al*., 1999). A few approaches have been used by researchers for an easy downstream process, such as the use of the biopolymer matrix polycaprolactone/polyethylene glycol based membrane (PCL/PEG) for cell growth support and separation, and ceramic microfiltration membrane for cell recycle (Lu *et al*., 2012; Scaffaro *et al*., 2016). In this study, a fabric mesh is used as a matrix for cell attachment and biomass separation for convenience and availability.