

NANYANG
TECHNOLOGICAL
UNIVERSITY

**Bioprospecting Indigenous Actinomycetes for
Natural Product Discovery**

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SCHOOL OF BIOLOGICAL SCIENCES

2018

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**A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy**

2018

Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor, Assoc. Prof Liang Zhao-Xun for the opportunity to pursue a PhD programme in his laboratory. I am thankful for his patience, support and guidance throughout the past four years allowing me to experience different aspects of research life. His inspirational and motivational talks have brought me through the darkest moments of my research life. I would also like to thank my co-supervisor, Dr Tan Lik Tong (NIE) and thesis advisory committee, Prof James P Tam, Assoc. Prof Liu Chuan Fa and Assoc. Prof Newman Sze for their invaluable advice throughout my PhD. I am thankful for the research platform that NTU and MOE had provided for me to pursue my research.

Next, I would like to thank National Parks Board for their assistance in our sample collection from Sungei Buloh Wetland Reserve. I would like to thank Assoc. Prof Cao Bin (CEE) and team for the providing samples from Pulau Ubin Quarry Lake. I would like to thank Asst. Prof Yang Liang (SCELSE) and his student Ding Yichen for the draft genome assembly of actinomycete strains. I would also like to thank our collaborators Dr Yoganathan K. (BII) and Assoc. Prof Valerie Lin for structure elucidation and estrogen response element assay for 4',5-dihydroxy-7-methoxy-3-methylflavanone. Special thanks to Low Zhen Jie, Ye Hong and Gary Ding for providing the NMR spectra of 4',5-dihydroxy-7-methoxy-3-methylflavanone and echinomycin.

This project would not be made possible without the help of a special team member, Low Zhen Jie, who fought extremely hard to get things to work. I am glad to be able to meet my fiancé during my PhD journey. His strengths complement my weaknesses which allowed me to pull through certain hurdles in experiments and in life. In addition, I am also grateful for the help and support from other lab members, Ye Rui Juan, Hoa Tran, Howard Saw, Xin Lingyi, Cheang Qing Wei, Sheng Shuo, and former FYP/attachment students.

Lastly, I would like to thank my friends and families for their support and encouragement throughout my PhD programme.

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Abbreviations

antiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
<i>C. albicans</i>	<i>Candida albicans</i>
CFU	Colony Forming Unit
DMSO	Dimethylsulfoxide
E2	17 β -estradiol
<i>E. coli</i>	<i>Escherichia coli</i>
EA	Ethyl Acetate
ESI	Electrospray Ionization
HPLC	High Performance Liquid Chromatography
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
ISP	International Streptomyces Project
kb	kilobases
LB	Luria-Bertani medium
LC-HRMS	Liquid Chromatography High-Resolution with Mass Spectrometry
Mbp	Mega base pairs
NaCl	Sodium Chloride
NMR	Nuclear Magnetic Resonance
NRPS	Nonribosomal Peptide-Synthetase
OD	Optical Density
OSMAC	One Strain Many Compound
PCR	Polymerase Chain Reaction
PKS	Polyketide Synthetase
rDNA	ribosomal Deoxyribonucleic Acid
RiPPs	Ribosomally synthesized and post-translationally modified peptides
rpm	Revolutions Per Minute

RPMI	Roswell Park Memorial Institute medium
SBWR	Sungei Buloh Wetland Reserve
v/v	Volume/volume
w/v	Weight/volume
LGT	Lateral Gene Transfer
MRSA	methicillin resistant <i>S. aureus</i>
sp.	Species
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
µg	Microgram
µL	Microliters
mL	Milliliter
mM	Millimolar
nM	Nanomolar
M	Molar
min	Minute

Abstract

Actinomycetes are well-known prolific producers of bioactive secondary metabolites, including some most effective antibiotics in use today. The secondary metabolites isolated from actinomycetes accounts for 45% of natural product derived antimicrobial drugs. After the golden era of antibiotic discovery from 1940s to 1960s, the “Waksman platform” faced diminished returns. The rapid emergence of antimicrobial resistance among many pathogenic bacteria creates an urgent need for more potent antimicrobial compounds to fill the drug pipeline. Many researchers have ventured into underexplored environments to search for novel strains that has potential of producing novel compounds with interesting bioactivities. Little information is currently available for actinomycetes biodiversity in ecologically interesting sites in Singapore. The Sungei Buloh Wetland Reserve (SBWR) and Pulau Ubin Quarry Lake are considered to be unique in biotas because of different environmental conditions such as pressures, nutrient compositions and temperatures. In my Ph.D. research, I aimed to isolate unique actinomycetes strains from the two locations for the discovery of novel biosynthetic pathways and antimicrobial secondary metabolites. To uncover novel actinomycetes, I employed a variety of media recipes and *in situ* cultivation methods in the isolation processes, which yielded a total of 152 actinomycetes strains. The highest number of actinobacteria were found to be affiliated with the genus *Streptomyces*, followed by *Micromonospora*, *Verrucosipora*, *Jishengella*, *Pseudonocardia*, *Isoptericola*, *Mycobacterium* and *Microbacterium*. More than two third of the strains were tested to be bioactive against one or more clinical isolates such as Methicillin-resistant *Staphylococcus aureus* (MRSA). Taking advantage of the falling cost of genome sequencing, we performed genome sequencing and metabolite profiling to further prioritize the strains to identify the strains that have the highest potential to produce novel compounds. Several “high-potential” strains were identified based on the bioactivity assay, genome sequence and metabolite profiling results. We isolated and characterized two bioactive compounds from two such high-potential strains. While the first bioactive compound produced by *Streptomyces* sp. P19 is the known antimicrobial compound echinomycin, the second compound 4',5-dihydroxy-7-methoxy-3-methylflavanone produced by *Streptomyces* sp. SD50 is

a new compound and is a potent agonist of estrogenic receptor. We believe that many more novel biosynthetic pathways and microbial secondary metabolites will be uncovered from the high-potential strains identified by my research work. Hence, the research work described in the dissertation sets the stage for the downstream discovery of novel microbial natural products.

CHAPTER 1: Background

1.1 Actinomycetes as a rich source of bioactive natural product

Actinomycetes are defined as metabolically active group of slow-growing, gram-positive bacteria with high GC contents of ~70%. During its life cycle, actinomycetes produce mycelium, followed by complex morphological differentiation into spores^{1,2}. The formation of spores is commonly observed especially under nutrient limited conditions³. Owing to this unique characteristic, actinomycetes can be found in a wide range of environments such as soils, ocean, intertidal zones, plants, sponges, animals and many more²⁻⁵. Actinomycetes are also known to produce natural pigments as secondary metabolites⁶. Actinomycete colonies may appear to be coloured when cultured on agar plates as depicted in Figure 1.1.

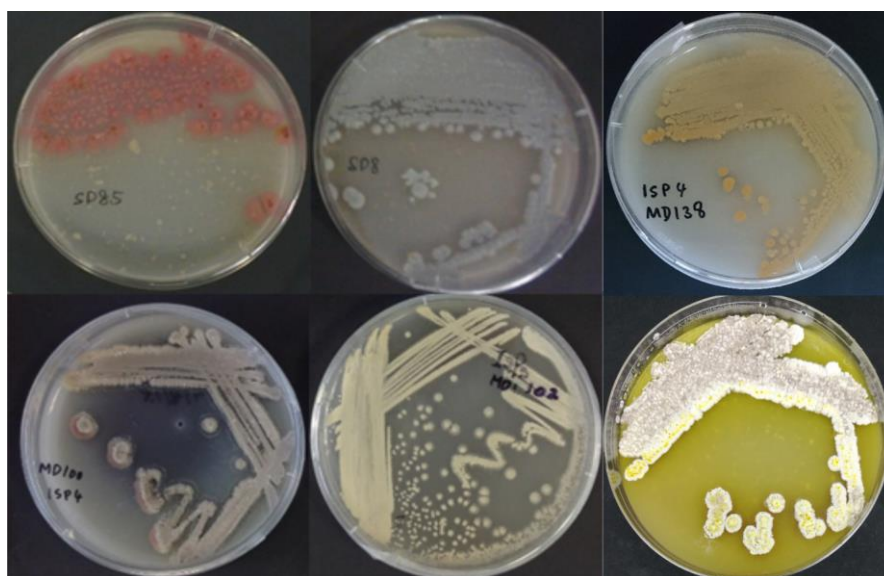


Figure 1.1 Pigmented actinomycete colonies due to the production of natural pigments as secondary metabolites.

Actinomycetes have been a rich source of bioactive secondary metabolites with approximately 12,000 bioactive metabolites^{7,8}. *Streptomyces* genus has been reported to be the largest producer of natural products, accounting for ~39% of all microbial secondary metabolites⁸. *Streptomyces* is also the main source of clinical drugs in the market⁷. Over time, there is an increase in natural products from rare actinomycetes, accounting for ~10% of all microbial secondary metabolites⁸. Rare actinomycetes are known as actinomycetes with lower

frequency of being isolated as compared to *Streptomyces*. Many bioactive compounds from rare actinomycetes such as *Micromonospora*, *Nocardia*, *Salinispora*, *Actinoplanes* and *Streptoverticillium* have also contributed to the repertoire of microbial natural products⁹⁻¹². Secondary metabolites from actinomycetes displayed broad spectrum of activities. They have been widely used as antibacterial agents (daptomycin, fidaxomicin and teicoplanin), antifungal agents (hygromycin B and nystatin), antiparasitic agents (ivermectin), anticancer agents (actinomycin D and mitomycin C) and antidiabetic agents (voglibose)^{4,13,14}. Currently, there are no anti-biofilm drugs available on the market or in the clinical trials yet.

Among prokaryotes, actinomycetes possess some of the largest genomes. Sequencing of actinomycetes whole genomes displayed typical genome size of 8 – 12 Mb with the capacity to produce up to 30 secondary metabolites per strain^{15,16}. The potential of secondary metabolites production from actinomycetes were analysed by looking out for the polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) genes. PKS and NRPS are multi-domain or multi-modular proteins that are involved in the biosynthesis of natural products^{17,18}. PKS and NRPS are uncommon in pathogenic bacteria such as Enterobacteria and Streptococci, where genomes are less than 3 Mb. Genomes with more than 3 Mb have greater coding capacity devoted to biosynthetic gene clusters¹⁶. Actinomycetes with large genomes usually devote 5 – 10% of their coding capacity to secondary metabolism¹⁹.

Due to differences in PKS and NRPS biosynthetic mechanisms, natural products biosynthesized by PKS and NRPS were structurally different as shown in Figure 1.2²⁰. Vancomycin **(1)** and daptomycin **(2)** are classical examples of natural products being biosynthesized by NRPS^{21,22}. Avermectin **(3)** is biosynthesized by Type I PKS and tetracycline **(4)** is biosynthesized by Type II PKS^{23,24}. Some compounds can also be biosynthesized by hybrid PKS and NRPS. Rapamycin **(5)** is a PKS-NRPS hybrid natural product and it comprised of a polyketide backbone incorporated with amino acids²⁵. Bleomycin **(6)** is a NRPS-PKS hybrid natural product that contain a peptidyl chain with ketone groups²⁶.

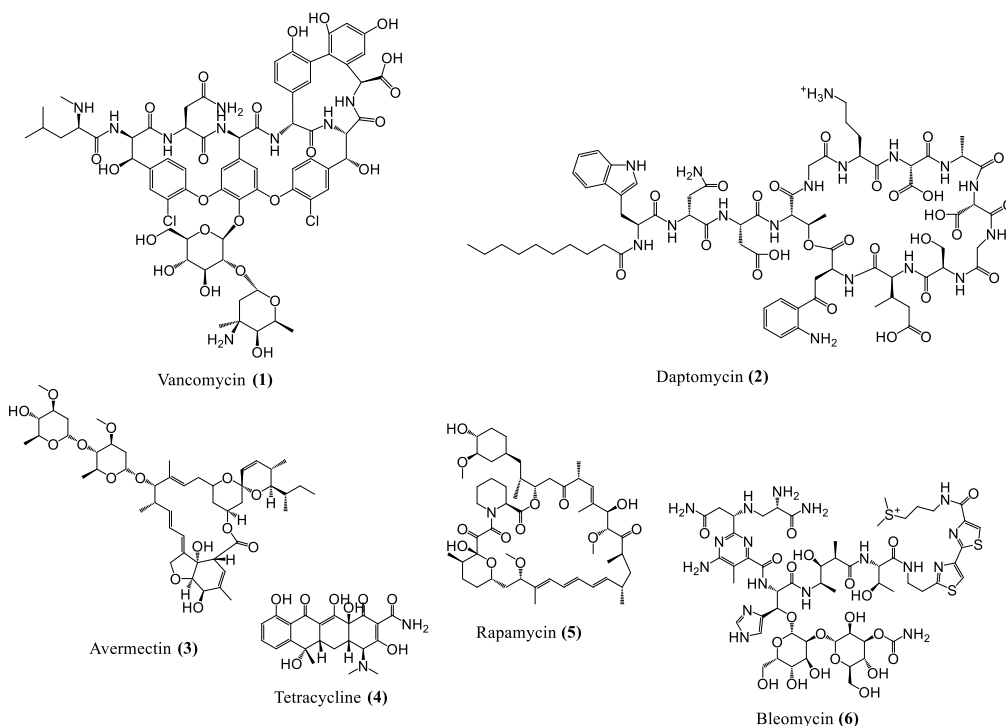


Figure 1.2 Examples of natural products biosynthesized by actinomycetes. Vancomycin (1) and daptomycin (2) are biosynthesized by NRPS. Avermectin (3) and tetracycline (4) are biosynthesized by Type I PKS and Type II PKS. Rapamycin (5) is biosynthesized by PKS-NRPS hybrid gene cluster and bleomycin (6) is biosynthesized by NRPS-PKS hybrid gene cluster.

1.2 Actinomycetes from unique environments

In order to get better odds in finding new or novel microorganisms and compounds, exploring new habitats has been one of the most successful strategies. To gain access to the diversity of actinomycetes from the environment, many groups have been focusing on intensive sampling from many diverse geographical locations and habitats. Large numbers of samples were processed by traditional isolation techniques, resulting in the repeated isolation of predominant species from these locations²⁷. Millions of strains have been isolated since 1940s and the probability of finding a novel actinomycete strain from easily accessible soil samples is currently very low. Current strategies to uncover novel compounds include isolation of rare actinomycetes from underexplored and unique environments associated with rhizospheres, marine sediments, plant endophytes and endosymbionts²⁸. Actinomycetes from these terrestrial niches have gained significant attention for their ability to produce novel bioactive natural products with diverse chemical structure²⁹. Strong environmental

selection, dispersal and evolutionary events are essential in defining the microbial communities^{28,30}.

More than 70% of the earth is covered by ocean. Experts have estimated that the biological diversity in marine ecosystem is higher than terrestrial environment³¹. However, the biological diversity of marine microorganisms has been explored to a limited extent. Actinomycetes can survive on extreme salinity, pH, temperatures and pressures. Reports have demonstrated that novel actinomycetes are present over a wide depth range in ocean, deep sea floor, invertebrates, coral reefs, sponges and plants³¹. Rare actinomycetes *Dietzia maris*, *Kocuria erythromyxa* and *Rhodococcus erythropolis* were isolated from Hokkaido sea floor sediment at the depth of 1225 meters³². *Salinibacterium* under the family of *Microbacteriaceae* was discovered from Amursky Bay of the Gulf of Peter the Great (Japan) at the depth of 5 meters³³. Novel seawater-requiring genus *Salinispora*, under the family of *Micromonosporaceae*, was discovered around the island of Guam^{34,35}.

In recent years, mangrove ecosystem has become a hot spot for biodiversity studies of actinomycetes and source of bioactive compounds³⁶⁻³⁸. Mangrove is unique as it consists of both intertidal and coastal regions. The presence of high salinity, extreme tides and extreme temperatures have created a microbial community that exhibit unique physiological and structural characteristics with the ability to produce novel secondary metabolites that are uniquely different from terrestrial microorganisms³⁹. Many rare actinomycetes have been discovered from different mangrove samples including several novel genera. *Polymorphospora* and *Lysinimicrobium* genera were discovered from rhizosphere mangrove soils in Iriomote Island (Japan)^{40,41}. *Microbacterium*, *Mumia* and *Sinomonas* genera were discovered from mangrove forest in Tanjung Lumpur (Malaysia)⁴²⁻⁴⁴.

Hyper-arid deserts are nutrient-poor, high light intensity and temperature and extremely low in water activity. They harbour numerous of unexplored extremophile species of actinomycetes that could potentially be a source of novel compounds⁴⁵. Novel genus *Jiangella* discovered from a desert in north-west of China, has the capacity to produce seven new compounds given the small genome

size of 5.5 Mbp^{46,47}. Caves are usually nutrient-poor with low light intensity and temperature, yet high in humidity. Despite the extreme environmental conditions, rare actinomycetes have been successfully isolated⁴⁸⁻⁵⁰. Novel strains LM 042T (genus of *Catellatospora*) and N3-2T (genus of *Nocardia*) had been isolated from a gold mine cave in Kongju and a cave on Jeju Island (South Korea)^{49,50}.

1.3 Phenomenon of unculturable bacteria

Sources for drugs derived from cultivable microorganisms are depleting due to highly repetitive isolation and traditional screening strategies^{8,51}. The rediscovery problem of known compounds from cultivable microorganisms pose a major bottleneck for drug discovery. Cultivable actinomycetes are widely considered to be over-mined for its secondary metabolites, thus, the chances of discovery novel compounds are low⁸. Microbial diversity in natural environmental is estimated to be $10^5 - 10^6$, however, only about $10^3 - 10^4$ (~1%) are culturable under laboratory conditions⁵²⁻⁵⁵. This meant that about 99% of the microorganisms remains inaccessible and these are a promising source of novel compounds⁵³⁻⁵⁵. To bridge the gap and tap access into novel species, it brought about a change in cultivation approaches⁵⁵.

The first success story in 2002 was the cultivation of previously uncultured SAR11, a ubiquitous marine clade⁵⁶. The study was carried out via dilution to extinction technique and the use of seawater to promote the growth of marine obligates that requires low levels of nutrients. Since then, there were increasing efforts on novel cultivation approaches such as gel microdroplet, diffusion chamber, microbial trap, iChip, I-Tip and hollow-fiber membrane chamber for the cultivation of uncultivable bacteria^{52,57-63}. These novel cultivation approaches adopted single cell together with high-throughput methodologies for mimicking its native environment, increasing incubation periods and lowering the levels of nutrients available. High-throughput dilution to extinction technique was carried out by diluting the microorganisms from the environment to approximately 1-10 cells per well in nutrient poor condition. These strategies aim to provide the microorganisms with naturally occurring growth factors by incubating them in its native environments.

Scout model hypothesis as shown in Figure 1.3 could be used to explain the phenomenon of microbial uncultivability⁵³. For *in-situ* cultivation, only a few cells would be awakened from dormancy. The scout hypothesis suggested that a stochastic switch might be the deciding factor for the microbial growth. This stochastic awakening happens in Phase (3) whereby dormant cells can wake into activity and scout for available resources. Without the specific nutrients or resources, it would result in the death of 'scout' cells as depicted in Phase (4). Under permissive growth conditions, the production and accumulation of signalling molecules would induce the growth of remaining dormant cells and re-establish the population as depicted in Phase (5) and Phase (6). This stochastic awakening is not due to a genetic change and the re-established population is indeed identical to the active growing population⁵³.

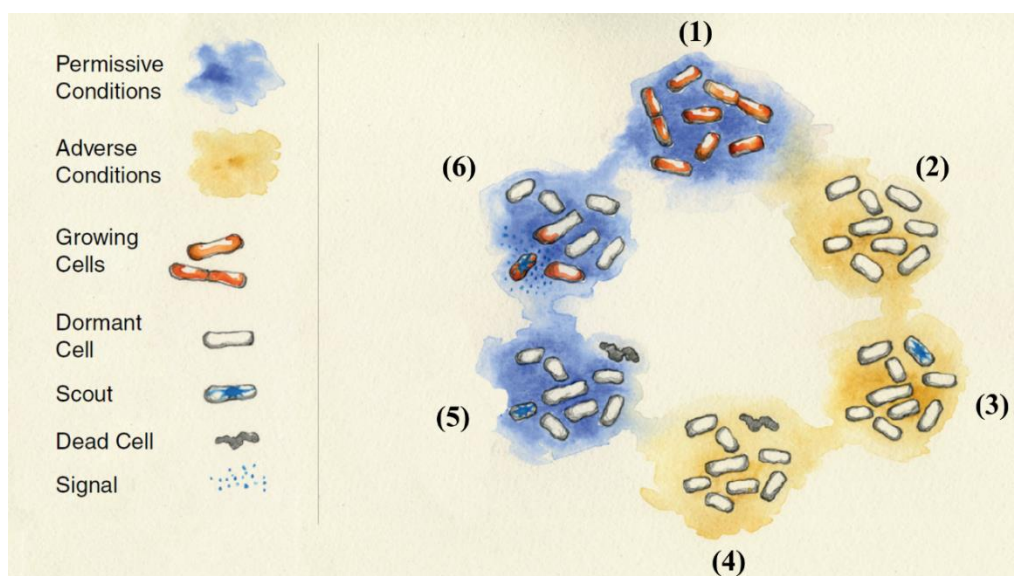


Figure 1.3 Scout model hypothesis on the life cycle of a microorganism. The life cycle starts from Phase (1): growth under permissive conditions, followed by Phase (2): dormancy under adverse conditions and Phase (3): stochastic awakening from dormancy. After Phase (3), cells may choose between death Phase (4) or proliferation Phase (5) and Phase (6), depending on environmental conditions⁵³.

1.4 Drug resistance and the need for new drug discovery

Therapeutic drugs played a vital role in major advances in medicine, surgery and infections⁶⁴. Antibiotics have successfully treated bacterial infections in patients receiving chemotherapy treatments or patients with other chronic illness such as diabetes and rheumatoid arthritis⁶⁵. However, the overuse of antibiotics and

inappropriate prescription drives the evolution of resistance among the pathogens⁶⁵. Drug resistance issue is prevalent in antibiotics research field. The emergence of drug resistance towards clinical antibiotics has created global healthcare crisis⁶⁵. Antibiotic resistant infectious in United States alone has resulted in approximately 23,000 deaths and the estimated cost in US hospital is more than US\$20 billion annually⁶⁶⁻⁶⁸.

Bacterial infections due ESKAPE pathogens has increased with an alarming rate in recent years. The rate at which pathogenic microbes develop resistance against antibiotics is alarming. Table 1.1 highlighted some examples whereby resistance of drugs was observed shortly after the introduction or the discovery of drugs⁶⁹. Due to the long and tedious drug development process, the year of resistance observed for commonly used drugs such as chlortetracycline, streptogramin B and daptomycin were even before the year of introduction. Antibiotic resistance has been attributed by several mechanisms that were able to prevent a drug from reaching its target. Antibiotic resistance can alter the antibiotic action via one of the following mechanisms, (i) modification of antibiotics by inactivation of antibiotics by hydrolysis or by transfer of a chemical group, (ii) target mutation or modification, (iii) restricted access of drug via reduced permeability and increased efflux and (iv) over expression of target⁶⁹⁻⁷¹.

The situation was made worse by increasing number of pharmaceutical companies pulling out from the antibiotic research field. Out of the 18 largest pharmaceutical companies, 15 has withdrawn from research and development of antibiotics⁷². According to Tufts Center for the Study of Drug Development (CSDD), the cost for pharmaceutical drug development exceeds US\$2.5 billion⁷³. The cost of new drug approval has doubled in the past decade. The number of new drug application approvals has declined from 1980 to 2014, as shown in Figure 1.4, due to economic and regulatory barriers^{65,74}. The need for novel drugs with new mode of action against multidrug resistant strains, is extremely crucial for filling the drug pipeline to ensure constant supply of therapeutic agents in the market. Otherwise, the antibiotic crisis that we are facing today may bring us back to pre-antibiotic era, where there was no cure for infectious diseases.

Table 1.1 Period of the discovery, introduction and resistance observed of antibiotics⁶⁹

Class of antibiotics and its example	Discovery	Introduction	Year resistance observed	Target species
Aminoglycosides (streptomycin)	1943	1946	1946	Broad-spectrum activity
Chloramphenicols (chloramphenicol)	1946	1948	1950	Broad-spectrum activity
Macrolides (erythromycin)	1948	1951	1955	Broad-spectrum activity
Tetracyclines (chlortetracycline)	1944	1952	1950	Broad-spectrum activity
Rifamycins (rifampicin)	1957	1958	1962	Gram-positive bacteria
Glycopeptides (vancomycin)	1953	1958	1960	Gram-positive bacteria
Quinolones (ciprofloxacin)	1961	1968	1968	Broad-spectrum activity
Streptogramins (streptogramin B)	1963	1998	1964	Gram-positive bacteria
Oxazolidinones (linezolid)	1955	2000	2001	Gram-positive bacteria
Lipopeptides (daptomycin)	1986	2003	1987	Gram-positive bacteria

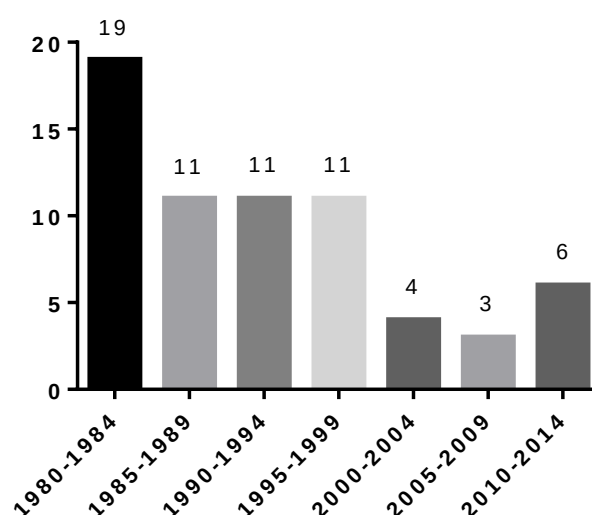


Figure 1.4 Number of antibacterial new drug applications approvals from 1980 – 2014 ⁷⁴.

1.5 Discover bioactive compounds from actinomycetes in the post-genomics era

Traditional screening of crude extracts containing secondary metabolites has been used as the preliminary stage for finding bioactive compounds due to its simplicity and affordability. However, bioactivity guided assays often result in the frequent rediscovery of known compounds such as streptothricin, streptomycin, tetracycline and actinomycin D with frequency magnitude ranging from 10^{-1} to 10^{-3} ⁵¹. The capacity of secondary metabolite production by a microorganism is far greater than it is observed by fermentation¹⁵. The advances in the field of genomics has led to significant reduction in the cost of whole genome sequencing. The availability of various next-generation sequencing platforms have also improved the quality of data obtained for identifying novel compounds and biosynthetic mechanisms through genome mining⁷⁵. This optimism would bring about the renaissance of drug discovery for filling the innovation gap that led to unproductive pipelines¹⁵.

Whole genome sequence information revealed that source of novel secondary metabolites from *Streptomyces* (most commonly found actinomycete) is still not yet exhausted⁷⁶. Whole genome sequence information revealed the potential of several *Streptomyces* can produce more than 20 secondary metabolites encoded by PKS, NRPS, bacteriocins, terpenoids, shikimate-derived metabolites, aminoglycosides and others, but only a small fraction were detected under the tested culture conditions⁷⁷⁻⁸¹. Genome mining efforts have been investigated via homologous expression and heterologous expression⁸². Homologous expression involves the use of chemical stimulants, endogenous transcription, translation or mutation to stimulate the secondary metabolite production. Heterologous expression involves transferring of whole gene clusters into expression strains (heterologous host) for secondary metabolite production of targeted genes. Heterologous expression has added advantage as gene clusters of uncultivable microbes (environmental DNA) could also be expressed via heterologous expression⁸².

It has been reported that the choice of culturing conditions are linked to the numbers and general metabolic profile of secondary metabolites produced by the microorganisms⁸³. One strain many compounds approach, carried out by altering

various nutritional or environmental factors, has shown its effectiveness in discovery of novel bioactive compounds as depicted in Figure 1.5(A)^{84,85}. Environmental cues by the use of chemical elicitors are also ways to activate secondary metabolites from cryptic gene clusters and assists the discovery of novel compounds⁸³. Chemical elicitor *N*-acetylglucosamine (GlcNAc) influences the antibiotic pathway-specific activators via the pleiotropic transcriptional repressor DasR⁸⁶. GlcNAc is a naturally occurring polysaccharide and a major component of cell walls in fungi. Cultivation of several *Streptomyces* strains under nutrient poor conditions with addition of GlcNAc has proven to stimulate the production of secondary metabolites⁸⁷.

Co-culturing can be considered as a tool for increasing the production of existing secondary metabolites and/or inducing silent gene clusters as shown in Figure 1.5(B). Co-culture involves cultivation of two or more microorganisms in the same environment. Microorganisms are omnipresent in nature and the inter-species interaction usually occurs through signalling or defence molecules^{83,88}. Understanding of inter-species communication and the roles in small signalling molecules could create new possibilities to trigger synthesis of novel compounds in microorganisms⁸³. Novel compound rhodostreptomycins A and B were produced by *Rhodococcus fascians* by co-culturing with *Streptomyces padanus*⁸⁹. Increased production of levorin by 25 – 60% was observed after co-culturing of *Actinomyces levoris* together with *Candida tropicalis*⁹⁰. Increased production of istamycins A and B by 50% was observed after co-culturing *Streptomyces tenjimariensis* with an unidentified marine bacteria⁹¹. Though co-culturing may seem a promising approach to trigger silent gene clusters in microorganisms, novel natural products produced by actinomycetes via co-culturing is still very limited⁹².

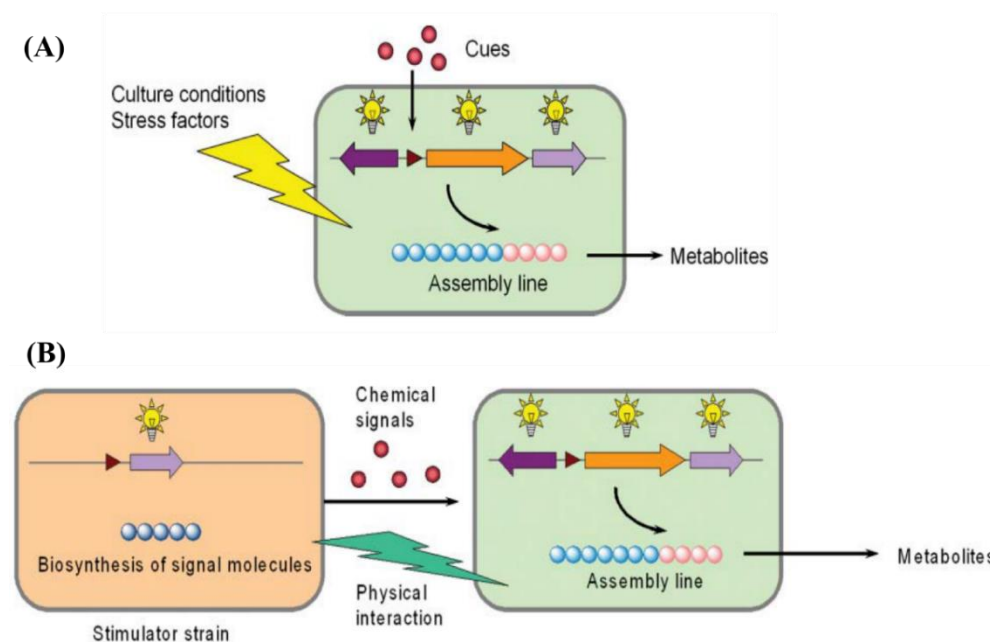


Figure 1.5 (A) Secondary metabolite biosynthesis can be influenced by culture conditions, stress factors and external cues (chemicals). (B) Silent gene clusters can be activated by co-culturing with stimulator strain.⁸³

Heterologous expression involves the transferring of orphan biosynthetic gene clusters into heterologous host. This method has proven to be successful for induction of silent genes and discovery of novel compounds⁹³⁻⁹⁵. However, this method faced a lot of difficulties for expression of large gene clusters over 100 kb^{83,96}. In addition, finding suitable expression hosts may be difficult.

1.6 Overview of thesis

The aim of my Ph.D. research project is to isolate and identify indigenous actinomycetes that have the potential to produce novel bioactive compounds, especially antimicrobial compounds. This thesis is comprised of five chapters. Figure 1.6 depicts the workflow of this project and specific aims for this thesis. Chapter 1 gives a general introduction to actinomycetes and its ability to produce structurally diverse bioactive compounds against infectious diseases. Chapter 2 describes the isolation and characterization of actinomycetes from Sungei Buloh Wetland Reserve and Pulau Ubin Quarry Lake in Singapore. Characterisation of selected strains were carried out to assess the biodiversity of actinomycetes from different locations and isolation strategies. Chapter 3 explores the ability of the isolated actinomycetes to produce bioactive compounds through a series of bioactivity screening. Strains capable of producing bioactive compounds were

shortlisted for genome sequencing. Chapter 4 reveals the genome sequencing information and metabolite profiling of the prioritized strains. Chapter 5 describes the isolation and identification of the bioactive compounds from 2 prioritized strains *Streptomyces* sp. P19 and SD50. References and appendices were presented at the end of the thesis.

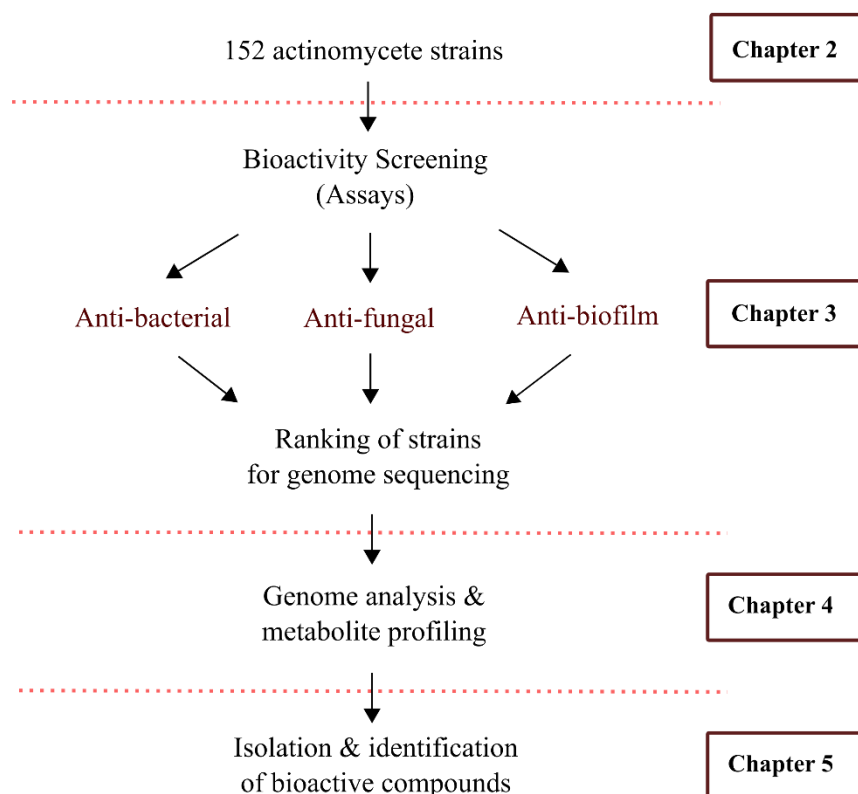


Figure 1.6 Workflow of project and specific aims for this thesis.

CHAPTER 2: Isolation of actinomycetes from mangrove and lake sediment

2.1 Introduction

Actinomycetes have relatively slow growth rates. This also suggest that without proper selective isolation methods and strategies, other fast-growing bacteria and fungi isolated from the same environment would effectively outgrow the actinomycetes, making isolation of actinomycetes impractical under laboratory conditions. Common strategy involves pre-treatment of samples to eliminate other fast-growing bacteria and fungi prior to plating the diluted samples onto nutrient petri dish. Spores of actinomycetes are highly resistant to harsh chemicals, heat and extreme dryness conditions⁵. In addition, actinomycetes can germinate at the condition that disfavours the survival of other bacteria^{2,5}. Owing to these characteristics, pre-treatments could significantly increase the number of actinomycetes being recovered. Pre-treatment methods for sediment or soil samples are classified as chemical, physical and enrichment methods. Chemical methods include 1.5% phenol and calcium carbonate treatments^{97,98}. Physical methods include air drying, wet heat and microwave treatments^{99,100}. Pre-treatment employed for aerial root samples is usually surface sterilization prior to plating¹⁰¹.

Selective isolation media with specific nutrients that favours the growth of actinomycetes can also increase the number of actinomycetes being recovered. International Streptomyces Project (ISP) media were formulated for the effective isolation of actinomycetes, in particularly *Streptomyces* genus¹⁰². To increase the diversity of actinomycetes, many groups have optimized isolation media suitable for the cultivation of rare actinomycetes (non-*Streptomyces*)¹⁰³⁻¹⁰⁵. The complexity and composition of environmental samples are not easily understood. Specialised media containing materials from its native environment was one of the popular options as it increases the chances of preserving nutrients required for growth of specific genus of actinomycetes^{98,106-108}. For example, *Salinispora*, the first obligate marine that requires seawater for cultivation¹⁰⁹. Humic acid component could be extracted from the samples as described previously for the preparation of Humic acid-vitamin (HV) agar¹⁰³. HV agar was also seen to be

superior to other regular media as it permitted the growth of highest number of actinomycete colonies belonging to *Streptomyces*, *Micromonospora*, *Microtetraspora*, *Streptosporangium*, *Dactylosporangium*, *Nocardia* and *Thermomonospora*, while restricting the growth of other bacteria¹⁰³. Soil extract agar has also been reported for its successful isolation of actinomycetes that do not grow on conventional media¹⁰⁸. Preparation of isolation media using materials from its native environment has a lot of added advantages and will be a promising trend for isolating rare actinomycetes.

Apart from altering chemical components in the media, adopting different gelling agent was also seen as a good strategy for recovery of rare actinomycetes¹⁰⁴. Gellan gum, a water-soluble polysaccharide of *Pseudomonas elodea*, has been used for isolation of rare actinomycetes including *Actinobispora*¹⁰⁴. Though the reasons were unclear, it might be possible that actinomycetes utilize the polysaccharide components for its growth.

iChip, an *in-situ* cultivation device, has garnered attention in 2015 after a successful discovery of Texiobactin⁶¹. Texiobactin binds to the molecules that have important roles in cell-wall synthesis, thus, making it impossible for other bacteria to develop resistance. This compound is produced by previously uncultivable *Eleftheria terrae* through *in-situ* cultivation by iChip. *In-situ* cultivation by iChip could increase the growth recovery of microorganisms up to 50% as compared to 1% of cells that grows on regular nutrient agar⁶¹. Incubation of iChip in its natural environment creates an effective incubation strategy that can mimic its native environment closely. The semi-permeable membranes allow molecules of various sizes to diffuse through. Diffusion provides the microorganisms with naturally occurring growth components, nutrients, possible signalling compounds and helps in the removal of metabolic products. It was reported that some environmental microorganisms might acquire the ability to grow in laboratory conditions after repeated cultivation in a diffusion chamber⁶². By exploiting the advantages of novel culturing methods for cultivation, we would be able to uncover more diverse natural products with new modes of action against infectious bacteria.

To gain a better understanding of actinomycete diversity from Singapore natural habitat, culture-dependant studies were carried out using samples collected from several isolations in Sungei Buloh Wetland Reserve and Pulau Ubin Quarry Lake. Various specialised media, made with materials collected from its native environment, were used to increase the chances of isolating rare actinomycetes. Different cultivation methods such as traditional plating method and *in-situ* cultivation method were adopted to investigate the effectiveness of isolating actinomycete strains. Lastly, bacterial identification was carried out to classify strains into various genera and determine whether novel strain could be recovered.

2.2 Materials and methods

All reagents were purchased from Sigma-aldrich, Singapore, unless otherwise stated.

2.2.1 Source and sample collection

A total of 193 strains were isolated from of mangrove and lake sediment in Singapore. Samples were collected from 6 different locations in Singapore, tabulated in

Table 2.1. 1 m depth of mangrove sediments were collected from Sungei Buloh Wetland Reserve (SBWR) using a stainless-steel soil sampler (Keke instruments, China), total length of 1 m and auger diameter 38 mm. The soil sampler was sterilized with 70% ethanol (Aik Moh Paints & Chemicals Pte Ltd, Singapore) before each sampling. 5 cm of aerial root from *Rhizophora* sp. was collected from SBWR by cutting with sterile a pair of stainless-steel scissors. 100 m depth of sediment was collected from Pulau Ubin quarry lake using a gravity-driven soil sampler. Pulau Ubin quarry lake sample was kindly provided by A/Prof Cao Bin's group. All samples collected were kept in sterile 50 mL centrifuge tubes (Greiner, Practical Mediscience Pte Ltd, Singapore) or sterile plastic bags until they were processed. Samples collected were processed within 6 hours.

Table 2.1 Various sampling locations and the description of samples collected in mangrove and lake.

Isolates	Description of sample	Coordinates
SE	Sungei Buloh Wetland Reserve (SBWR) aerial root of <i>Rhizophora</i> sp.	1°26'41.6"N 103°43'27.8"E
SW	SBWR 1 m depth dark brown sediment	1°26'58.6"N 103°43'12.8"E
SD	SBWR 1 m depth brown soil	1°26'41.5"N 103°43'19.3"E
MD	SBWR 1 m depth grey sediment;	1°26'38.5"N 103°43'30.4"E;
	SBWR 1 m depth dark brown sediment	1°26'41.2"N 103°43'36.1"E
MI	SBWR 1 m depth grey sediment;	1°26'38.5"N 103°43'30.4"E;
	SBWR 1 m depth dark brown sediment	1°26'41.2"N 103°43'36.1"E
P	Pulau Ubin quarry lake 100 m depth black sediment	1°24'21.3"N 103°57'22.8"E

Temperature of sampling points in SBWR were measured using a digital thermometer (E.T.I. Ltd, UK) during the day of sample collection. Recorded temperatures of sampling points were 28 – 30 °C. Salinity and pH of samples were measured within 24 hours after collection. Salinity of seawater was measured with a hydrometer (Instant Ocean, Fresh & Marine Aquarium, Singapore) according to manufacturer's instructions and the recorded value was approximately 16 ppt. pH of mangrove sediments were prepared in a 2.5:1 water-to-soil ratio and was measured using universal pH indicator (Merck Pte Ltd, Singapore). Recorded pH of mangrove and lake sediment were approximately 7.0.

2.2.2 Pre-treatment methods

Method 1: Surface sterilization ¹⁰¹

5 cm of aerial roots of *Rhizophora* sp. were cut into smaller segments of 1 cm³. External soil was rinsed off with sterile distilled water for at least 3 times. Samples were immersed into 70% ethanol for 30 seconds, followed by 1% sodium hypochlorite solution for 30 seconds. Samples were washed with sterile water and finally dried before plating. Each piece of sample was streaked onto an agar plate using sterile tweezer.

Method 2: Phenol treatment ⁹⁸

1 g of environmental sample was added to 1.5% phenol (w/v) with 10 mL of sterile water. The resulting mixture was vortexed for 5 minutes and allowed to settle for 5 minutes. The supernatant was serially diluted with sterile water from 10⁻¹ to 10⁻⁴. 100 µL from each dilution were inoculated onto various agar plates.

Method 3: Wet heat treatment ¹¹⁰

1 g of environmental sample was added to 10 mL of sterile water. The resulting mixture was heated to 60 °C for 15 minutes using a water bath and then allowed to settle for 5 minutes. The supernatant was serially diluted with sterile water from 10^{-1} to 10^{-4} . 100 μ L from each dilution were inoculated onto various agar plates.

Method 4: Desiccation

5 g of environmental sample was dried for 20 days in a vacuum desiccator loaded with silica gel (Stein Zeiser, Challenger, Singapore). The sample was sieved using a stainless-steel strainer (An Hua, Singapore) for removal of coarse particles. 50 mL of sterile water was added to sample, vortexed for 5 minutes and allowed to settle for 5 minutes. The supernatant was serially diluted with sterile water from 10^{-1} to 10^{-4} . 100 μ L from each dilution were inoculated onto various agar plates.

Method 5: Microwave treatment ¹⁰⁰

Drying of sample was carried out as described in Method 4 before subjecting environmental sample to microwave treatment. The sample was sieved using a strainer (An Hua, Singapore) for removal of coarse particles. 3 g of dried sample was transferred to a 15 mL sterile centrifuge tube (Greiner, Practical Mediscience Pte Ltd, Singapore), moisten with 2 mL of sterile water and incubated at room temperature for 15 minutes. The centrifuge tube was placed in a 600 mL beaker (Schott-Duran, VWR Singapore Pte Ltd, Singapore) containing 400 mL of tap water and microwaved (Cornell, Singapore) at 120 watts for 3 minutes. The resulting mixture was then transferred into 50 mL centrifuge tube under sterile conditions. 45 mL of sterile water was added and vortexed for 15 minutes. The resulting mixture was centrifuged at 5000 g for 10 minutes and supernatant was removed. This washing step was repeated twice. 30 mL of sterile water was added to sample, vortexed for 5 minutes and allowed to settle for 5 minutes. The supernatant was serially diluted with sterile water from 10^{-1} to 10^{-4} . 100 μ L from each dilution were inoculated onto various agar plates.

Method 6: Calcium carbonate treatment ⁹⁷

Drying of sample was carried out as described in Method 4 for 10 days before mixing equal mass of calcium carbonate to the mangrove sediment. The mixture was then incubated for another 10 days at 28 °C in a closed inverted sterile petri dish, maintained by water-saturated discs of filter paper (Whatman, Singapore). 2 g of the mixture was transferred to a 15 mL sterile centrifuge tube and 10 mL of sterile water was added. The resulting mixture was vortexed for 5 minutes and allowed to settle for 5 minutes. The supernatant was serially diluted with sterile water from 10^{-1} to 10^{-4} . 100 μ L from each dilution were inoculated onto various agar plates.

Method 7: Enrichment and rehydration-centrifugation ¹¹¹

Drying of sample was carried out as described in Method 3 for 10 days before adding 0.5 g of calcium carbonate to 5 g of sample. The mixture was then incubated for another 14 days at 28 °C in a closed inverted sterile petri dish, maintained by water-saturated discs of filter paper (Whatman, Singapore). 0.5 g of the mixture was placed in 50 mL centrifuge tube and flooded with 50 mL of 1X phosphate buffer (pH 7.0) containing 10% freshwater from quarry. The mixture was incubated at 30 °C with shaking for 2 hours to liberate actinomycete zoospores. 8 mL of the flooding mixture was transferred to 15 mL centrifuge tube and centrifuged at 1500 g for 20 min. After settling for 30 min, the supernatant was serially diluted with sterile water from 10^{-1} to 10^{-4} . 100 μ L of the supernatant was inoculated on various agar plates.

All plates were incubated at 30 °C for 1 – 2 weeks before the isolates were streaked out into pure colonies.

2.2.3 Isolation media for actinomycetes

All media contained anti-fungal agents cycloheximide (100 μ g/mL), nystatin (50 μ g/mL) and antimicrobial agent nalidixic acid (50 μ g/mL) to eliminate fast growing gram-negative bacteria unless otherwise stated. Sea water for preparation of media were collected at 1°26'60.0"N 103°43'01.0"E and 1°26'38.5"N 103°43'30.4"E.

Medium 1: Humic acid-vitamin agar (HVA) adjusted to pH 7.2 ¹⁰³

Preparation of humic acid was done by suspending 500 g of soil from the sampling locations in 1 L of 0.5% NaOH solution and incubated overnight at room temperature with occasional stirring. Following this, the soil particles were removed by centrifugation at 8000 rpm for 20 minutes. The supernatant was collected and acidified to pH 1.0 with hydrochloric acid. The precipitated humic acid was recovered by centrifugation at 3000 rpm for 20 minutes and washed 3 times with 50 mL of water. Humic acid was resuspended in 100 mL of water and frozen overnight at -20 °C. After thawing, humic acid was recovered using Buchner filtration (Ctech, Singapore) and 150 mm filter paper no. 4 (Whatman, United Scientific Equipment Pte Ltd, Singapore) and dried overnight in a vacuum dessicator loaded with silica gel. Medium consists 18 g bacto agar (BD Biosciences, Singapore), 1 g extracted humic acid, 0.5 g Na₂HPO₄, 1.71 g KCl, 0.05 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.02 g CaCO₃ in 1 L of distilled water. 1x Kao and Michayluk Vitamin solution was added after autoclaving.

Medium 2: Humic acid-vitamin-gellan gum (HVG) adjusted to pH 7.2

Preparation steps were similar to Medium 1 by substituting bacto agar with 7 g gellan gum (MP biomedical, Singapore) and addition of 2 mM CaCl₂ for gelation.

Medium 3: Soil-extract (SE) agar ¹⁰⁸

Preparation of soil extract was done by suspending 500 g of soil from the sampling locations in 1 L of 50 mM NaOH solution and incubated overnight at room temperature with occasional stirring. The soil particles were removed by centrifugation at 8000 rpm for 1 hour. The resulting supernatant was sterile filtered with a 1 L filter unit (Nalgene, Fisher Scientific Pte Ltd, Singapore). Medium consists of 15 g bacto agar in 500 mL of sea water or distilled water and 500 mL soil extract added after autoclaving.

Medium 4: International Streptomyces Project (ISP) 3 adjusted to pH 7.2 ¹⁰²

Preparation of trace salt solution (0.1 g of FeSO₄·7H₂O, 0.1 g of MnCl₂·4H₂O and 0.1 g of ZnSO₄·7H₂O in 100 ml of distilled water) was done separately. 20 g

of oatmeal (Fairprice, Singapore) was boiled with 1 L of distilled water for 20 minutes and filtered using a strainer (An Hua, Singapore). Medium consists of 18 g bacto agar, 1 L filtrate and addition of 1 mL trace salts solution after autoclaving.

Medium 5: ISP 3 gellan gum adjusted to pH 7.2

Preparation steps were similar to Medium 4 by substituting bacto agar with 9 g gellan gum (MP biomedical, Singapore) and addition of 5 mM CaCl_2 for gelation.

Medium 6: ISP 3 sea water adjusted to pH 7.2

Preparation steps were similar to Medium 4 by substituting distilled water with sea water.

Medium 7: ISP 3 instant ocean adjusted to pH 7.2

Preparation steps were similar to Medium 4 by addition of instant ocean synthetic sea salts (Aquarium Systems Inc., Fresh & Marine Aquarium, Singapore) adjusted to salinity of 16 ppt.

Medium 8: Sea water gellan gum

Medium consists of 9 g gellan gum, 5 mM CaCl_2 for gelation and 1 L of sea water.

Medium 9: Nutrient-poor (NP) extract gellan gum ³⁴

Preparation of nutrient-poor extract was done by mixing 900 mL (wet volume) of sand collected from sandy area in SBWR at 1°26'57.9"N 103°43'44.8"E, with 500 mL of sea water with continuous shaking for 2 hours. The supernatant was decanted and stored at 4 °C before use. Medium consists of 4 g gellan gum, 100 mL nutrient-poor extract and 900 ml sea water.

Medium 10: Nutrient-rich (NR) extract gellan gum ³⁴

Preparation of nutrient-rich extract was done by mixing 300 ml (wet volume) of mangrove sediment with 500 mL of sea water with continuous shaking for 2 hours. The supernatant was decanted and stored at 4 °C before use. Medium consists of 4 g gellan gum, 100 mL nutrient-rich extract and 900 mL sea water.

Medium 11: Gauze's synthetic medium No. 1 ¹¹²

Medium consists of 15 g agar, 20 g potato starch (Pagoda, Singapore), 1 g KNO₃, 0.5 g MgSO₄·7H₂O, 0.5 g K₂HPO₄, 0.01 g FeSO₄·7H₂O and 1 L seawater. 50 µg/mL cycloheximide, 20 µg/mL nalixidic acid and 50 µg/ml nystatin were added after autoclaving.

Medium 12: Gauze's medium 2 / SM3 ¹⁰⁵

Medium consists of 15 g agar, 10 g glucose, 5 g peptone, 3 g tryptone, and 1 L of seawater. 50 µg/mL cycloheximide, 10 µg/mL nalixidic acid, 10 µg/mL novobiocin and 50 µg/mL nystatin were added after autoclaving.

Medium 13: SMP ³⁴

Medium consists of 8 g noble agar 0.5 g mannitol, 0.1 g peptone, and 1 L seawater. 5 µg/mL rifampicin was added after autoclaving, together with other antibiotics and antifungal agents stated previously.

Medium 14: Starch casein agar ¹¹³

Medium consists of 15 g agar, 1 g casein powder, 10 g starch, 37 mL seawater and 963 ml distilled water. 50 µg/mL cycloheximide and 25 µg/mL nystatin were added after autoclaving.

Medium 15: Arginine glycerol salt agar ¹¹⁴

Medium consists of 15 g agar, 1 g arginine monohydrochloride, 12.5 g glycerol, 1 g K₂HPO₄, 1 g NaCl, 0.5 g MgSO₄·7H₂O, 0.01 g Fe₂(SO₄)₃·6H₂O, 0.001 g CuSO₄·5H₂O, 0.001 g ZnSO₄·7H₂O, 0.001 g MSO₄·H₂O and 1 L seawater.

Medium 16: SMS agar ⁶¹

Medium consists of 20 g bacto agar, 0.125 g casein, 0.1 g potato starch, 1 g casamino acids and 1 L seawater. SMS agar was used for *in-situ* cultivation; hence, no antibiotics or antifungal agents were added.

2.2.4 Assembly of *in-situ* cultivation disk

Samples collected from 1°26'38.5"N 103°43'30.4"E and 1°26'41.2"N 103°43'36.1"E were pre-treated via desiccation, calcium carbon and microwave treatment as mentioned previously (Section 2.2.2). The *in-situ* cultivation disk was inspired by iChip and was being used in our study⁶². The assembly consist of three identical circular disks with 76 matching holes. The thickness of each disk is 3 mm and the diameter are 80 mm. The central disk was dipped into molten SMS medium containing diluted sediment suspension. Excess agar was removed with a sterile glass slide before placing two 0.03 µm thickness, 47 mm diameter polycarbonate membrane to cover the holes of central plate. Marine glue was used to seal the polycarbonate membrane. The assembly was completed by placing the central plate in between two other plates and screwing them together with 4 stainless-steel screws as shown in Figure 2.1.

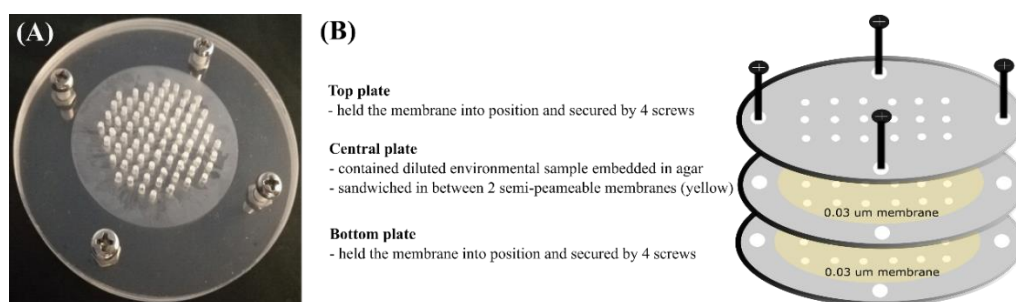


Figure 2.1 Assembled *in-situ* cultivation disk (A) prototype and (B) schematic setup (not drawn to scale) which consists of a central plate which houses diluted environmental sample, semi-permeable membranes and two other plates with 76 matching holes to support the membrane and to allow diffusion of small molecules.

18 *in-situ* cultivation disks were assembled and placed both in the native environment and simulated laboratory incubation conditions for 3 weeks. After which, the *in-situ* cultivation disks were recovered. External surface of disks was washed with sterile water and air-dried. The disks were disassembled by unscrewing and careful opening with a sterile scalpel. The agar from each agar plug were carefully transferred to starch casein agar for incubation in laboratory conditions. All plates were incubated at 30 °C for 1 – 2 weeks before the isolates were streaked out into pure colonies. Some actinomycetes require certain co-factors or signalling molecules from their natural environment in order to be awoken from its dormant state. By allowing single cells to be awoken from its

dormant state, they can then grow into critical cell mass that facilitates their adaptation to lab culture conditions.

2.2.5 Maintenance of strains

All isolates were cultured on 50% ISP3 and ISP4 agar at 28 °C for 3 – 4 weeks¹⁰². Spore stocks were prepared under sterile conditions as described previously¹. Spores and mycelial fragments of pure actinomycete strains were suspended in 30 % (v/v) glycerol stock and stored in cryo-vials at -80 °C for long-term storage.

2.2.6 Isolation of genomic DNA for 16S rDNA PCR amplification

DNA was extracted by colony PCR method with some modifications¹¹⁵. Strains were cultured in 4 mL of CRM medium (10 g/L glucose, 103 g/L sucrose, 10.12 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 15 g/L tryptic soy broth and 5 g/L yeast extract) and were shaken at 180 rpm at 30 °C for 3 – 5 days. Cultures were centrifuged at 8000 rpm and washed with sterile water twice. Cell pellets were homogenised using a glass homogeniser (Hefei biological, China) to break apart the mycelium clumps. Homogenised cells were centrifuged at 5000 rpm for 10 minutes and supernatant were discarded. 2 mL of lysis buffer (400 mM Tris-HCl, 60 mM EDTA, 150 mM NaCl and 1% SDS, pH 8.0) was added to the homogenised cell pellets and vortex for 1 minute. 600 μL of potassium acetate solution (3 M potassium acetate, 11.5% acetic acid, pH 4.8) was added to precipitate the SDS that will inhibit the PCR reactions in the downstream process. The mixture was vortex and centrifuge at 6000 rpm for 5 minutes. The supernatant was transferred to a new tube and centrifugation was repeated twice to remove all the remaining precipitate. The DNA pellet was precipitated with 0.6 volume of isopropyl alcohol and washed twice with 70% ethanol. DNA pellet was left to dry overnight and re-dissolved in 50 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Approximately 100 ng of DNA was used for a PCR reaction. If PCR failed to amplify the 16S rDNA gene, better quality DNA was extracted using CTAB or salting out method as reported previously¹.

2.2.7 16S rDNA PCR amplification

Amplification of 16S rDNA genes from the isolated strains was carried out using universal primers shown in Table 2.2 in a thermal cycler (Bio-rad, Singapore). Sequencing primers used were 27F and 235F.

Table 2.2 ^aDegeneracies according to lane¹¹⁶: R = A:G; W = A:T. ^bBinding site on the 16S rDNA molecule: *Escherichia coli* numbering system¹¹⁷.

Primer	Sequence (5' to 3') ^a	Binding Site ^b		Usage	Source
		5'	3'		
27f	AGAGTTTGATCTGGCTCAG	8	27	PCR & Seq	Lane, DJ. <i>et al.</i> (1991)
1525r	AAGGAGGTG W TCCAR C C	1544	1525	PCR	Lane, DJ. <i>et al.</i> (1991)
235f	CGCGGCCTATCAGCTTGTTG	215	235	PCR & Seq	Bull, AT. <i>et al.</i> (2003)
878r	CCGTACTCCCCAGGCGGGG	897	878	PCR	Bull, AT. <i>et al.</i> (2003)

Q5 High-Fidelity DNA polymerase kits (New England Biolabs, Singapore) and Pfu polymerase (Biotech Rabbit, Germany) were optimised for 16S rDNA PCR reactions. Reactions were prepared in final volume of 25 µL according to respective manufacturer's protocols using 100 ng of genomic DNA. Cycling conditions adopted: initial denaturation at 98 °C for 30 seconds, repeated with 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 57 °C for 15 seconds, extension at 72 °C for 2 minutes, with a final extension at 72 °C for 10 minutes. The amplified products were separated by agarose gel electrophoresis at 100 V for 1 hour with 1 % (w/v) agarose stained with GelRed. PCR products with correct size of 1.5 kb (with 27F and 1525R primers)¹¹⁶ or ~600 bp (with 235F and 878R primers)¹¹⁸ were purified with NucleoSpin Gel and PCR clean up kit (Macherey-Nagel, Biomed Diagnostics Pte Ltd, Singapore), according to manufacturer's protocol. Purified PCR products were stored in 4 °C before sequence was determined by 1st Base, Axil Scientific Pte Ltd (Singapore).

2.2.8 Phylogenetic analysis

The chromatogram file in ABIF was analysed using Unipro UGENE¹¹⁹. The full or partial 16S rDNA sequences of 70 strains were analysed for the percentage DNA similarity via EzBioCloud¹²⁰. The closet match for the isolates were tabulated in Table S.1 (**Appendix**). Phylogeny analysis was constructed by

MEGA 6.0 software using Neighbor-Joining method and the evolutionary distances were computed using the bootstrap method¹²¹. Number of bootstrap replications was set at 1000.

2.3 Results

2.3.1 Summary of strains from mangrove and lake sediment

A total of 193 strains were isolated from mangrove and lake sediment in Singapore. These strains consisted of 155 strains from 5 different locations in Sungei Buloh Wetland Reserve and 38 strains from Pulau Ubin Quarry Lake. Isolates were denoted SE, SW, SD, MD, MI and P to indicate strains isolated from different batch of sampling. Table S.2 (**Appendix**) consolidates the strains isolated from different sampling locations, pre-treatment methods and isolation media. Figure 2.2 taxonomic ordering of strains isolated in this study revealed 12 genera, 10 families, 7 orders and 3 classes. The taxonomy classification for *Actinobacteria* was based on *Bergey's Manual of Systematic Bacteriology*¹²². In this study, genera of isolates were classified based on 16S rDNA identification and morphology. Bacteria found in Phylum (one rank above Class) *Actinobacteria* are usually termed as actinomycetes. *Actinobacteria* or actinomycetes can be divided into two main groups; the *Streptomyces*, representing the dominant species found in natural environmental and the rare actinomycetes (non-*Streptomyces*)¹²². Among the rare actinomycetes, we have isolated 7 different genera; *Micromonospora*, *Verrucosipora*, *Jishengella*, *Pseudonocardia*, *Isoptericola*, *Microbacterium* and *Mycobacterium*. Gammaproteobacteria and alphaproteobacteria belongs to Gram-negative bacteria that were commonly found in the natural environmental^{123,124}.

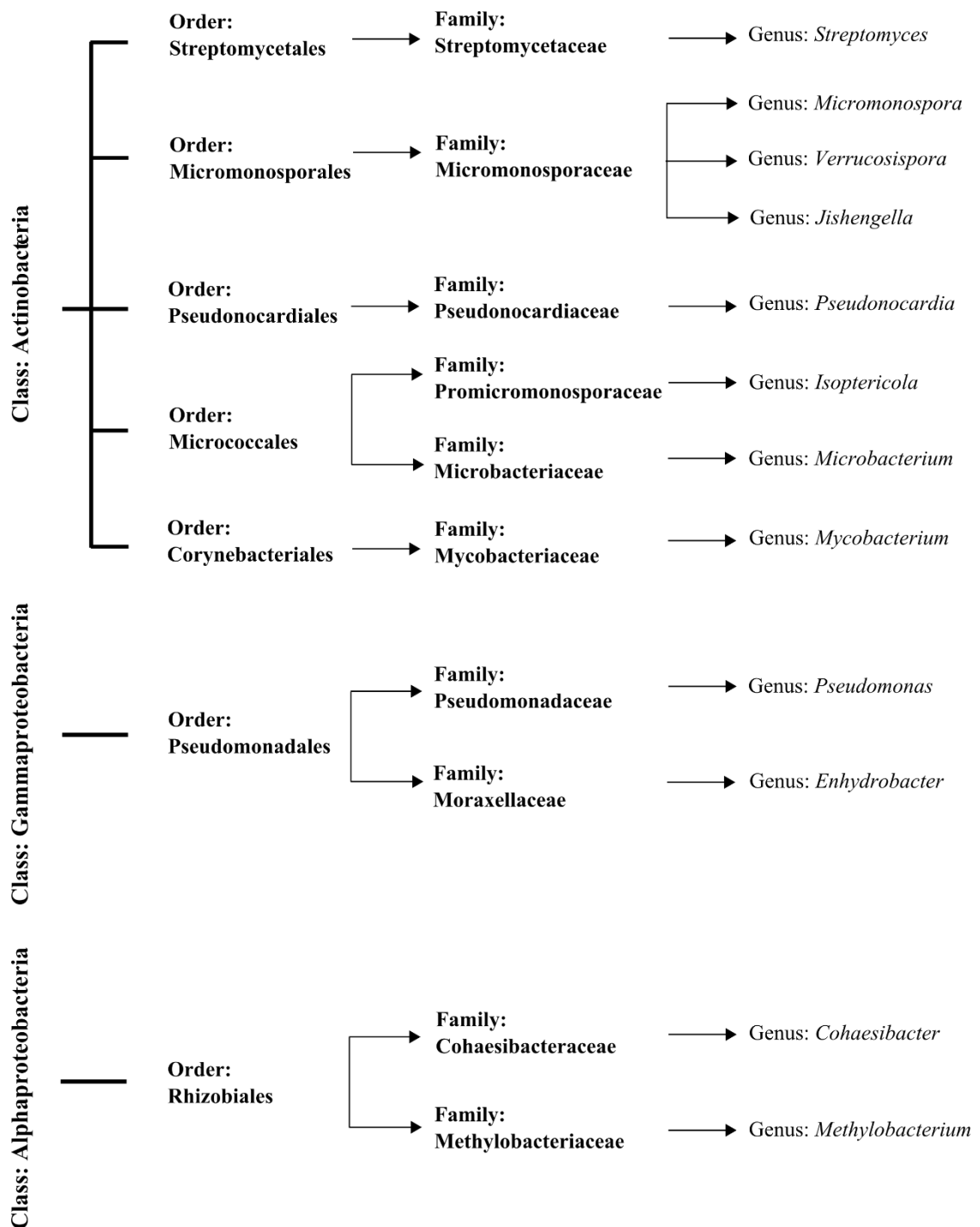


Figure 2.2 Taxonomic ordering of isolated bacteria from mangrove and lake. 12 genera, 10 families, 7 orders and 3 classes identified through 16S rDNA identification.

The strains isolated in this study displayed considerable diversity shown in Figure 2.3. The highest number of actinobacteria were found to be affiliated with the genus *Streptomyces* (59.07%), followed by *Micromonospora* (16.06%), *Verrucosispora* (1.04%), *Jishengella* (0.52%), *Pseudonocardia* (0.52%), *Isoptericola* (0.52%), *Mycobacterium* (0.52%) and *Microbacterium* (0.52%).

The highest number of Gram-negative bacteria were found to be affiliated with the genus *Pseudomonas* (2.07%), followed by *Enhydrobacter* (1.04%), *Cohesibacter* (0.52%) and *Methylobacterium* (0.52%). Among the non-actinomycete species, there were additional 17.10% whereby their genera have not been identified.

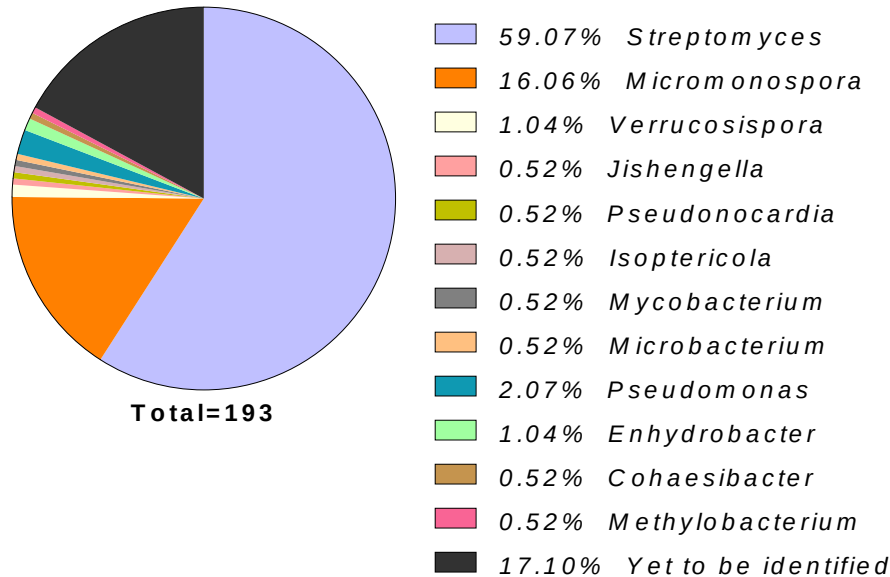


Figure 2.3 Percentage of different genera isolated from Mangrove and Lake sediment.

The number of strains and genera isolated from mangrove and lake sediment were shown in Figure 2.4. The number of *Streptomyces* isolated from both mangrove (83 isolates) and lake (31 isolates) were significantly higher than the rest of the genera. This was due to rare actinomycetes being considered to have lower isolation rates as compared to *Streptomyces* via conventional isolation strategies¹¹. This was followed by *Micromonospora* from mangrove (29 isolates) and lake (2 isolates). *Verrucosipora* (2 isolates), *Jishengella* (1 isolate) and *Isoptericola* (1 isolate) were isolated from mangrove samples, while *Pseudonocardia* (1 isolate), *Mycobacterium* (1 isolate) and *Microbacterium* (1 isolate) were isolated from lake sample. The remaining ones which were classified as unknown were non-actinomycetes which 16S rDNA identification has not been done. These results showed that the biodiversity of actinomycetes differs from one location to another.

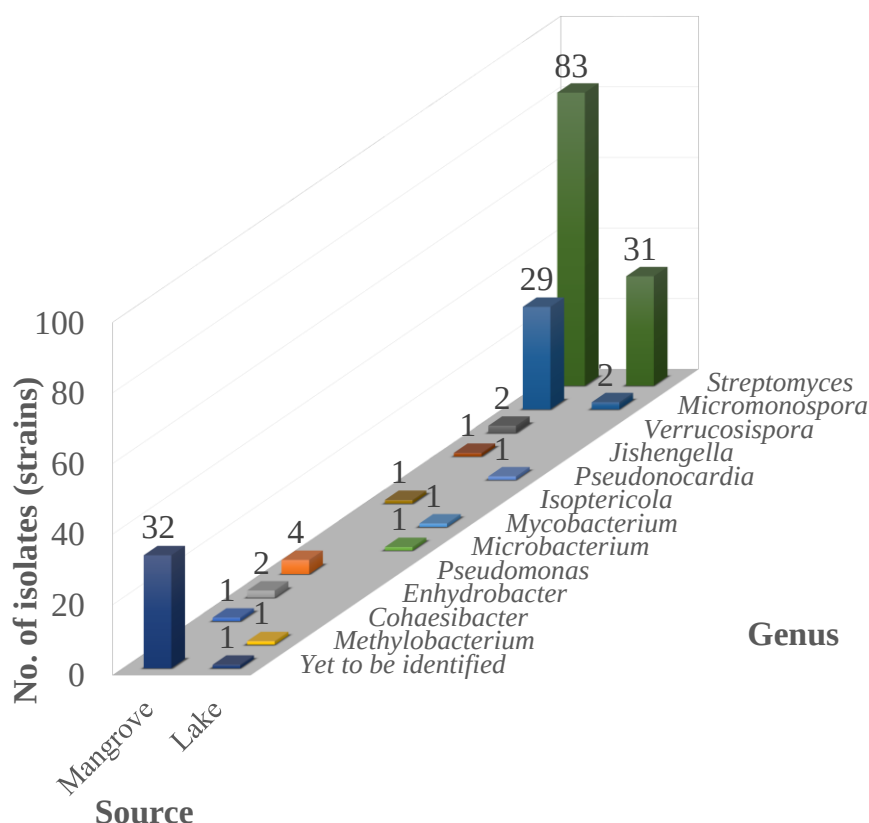
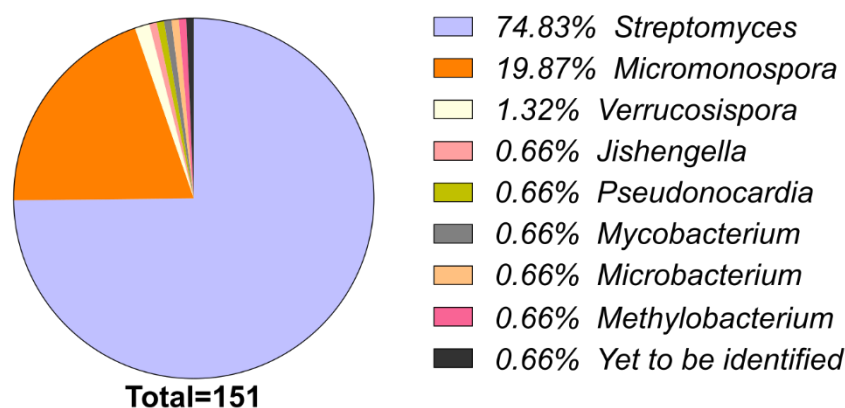


Figure 2.4 Number of isolates (strains) and the genera isolated from mangrove and lake sediment.

2.3.2 Comparison between direct plating and *in-situ* cultivation method

The actinomycete isolation rate was much higher in direct plating method (98.68%) as compared to *in-situ* cultivation (7.14%) as shown in Figure 2.5. *Streptomyces* and *Micromonospora* were isolated from both methods. These two genera are found in high abundance in our study. Interestingly, the diversity of rare actinomycetes differs from each cultivation methods. *Verrucospora* (1.32%), *Jishengella* (0.66%), *Pseudonocardia* (0.66%), *Mycobacterium* (0.66%) and *Microbacterium* (0.66%) were isolated only by direct plating method from various selected medium. *Isoptericola* (2.38%) was isolated only *in-situ* cultivation. *In-situ* cultivation also increased the rate of isolation for non-actinomycete colonies (92.86%) as compared to direct plating (1.32%). These results were not surprising as *in-situ* cultivation has previously been used for isolation of unculturable non-actinomycete strains^{59,61}. In addition, antibiotics such as nalixidic acid has been added to agar plates via direct plating method. Nalixidic acid significantly decreased the number of fast growing bacteria and increased the number of actinomycete colonies formed on the agar plates.

(A) Direct plating



(B) In-situ cultivation

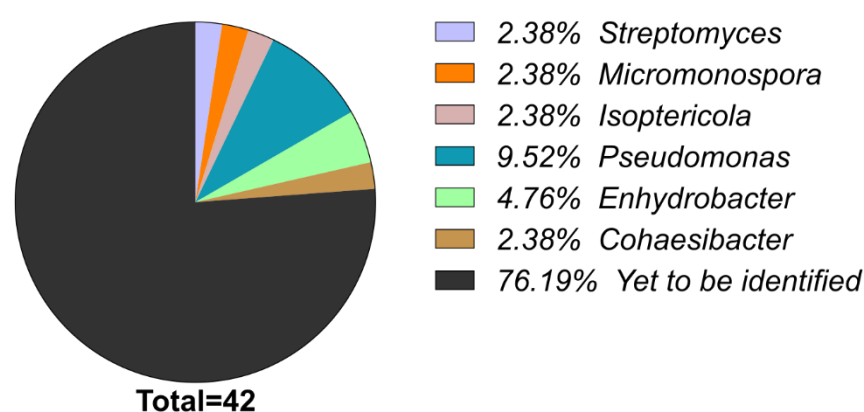


Figure 2.5 Comparison of actinomycete isolation rates between conventional (A) direct plating method and (B) *in-situ* cultivation method.

2.3.3 Strain identification by 16S rDNA sequencing

Of these strains, 70 were selected for partial or full 16S rDNA gene sequencing (ranging from 510 to 1530 nucleotides). These strains were selected based on their bioactivities or interesting morphology. Figure 2.6 depicts a circular phylogenetic analysis of the 70 strains. Of these strains, 43 belong to *Streptomyces*, 12 belong to *Micromonospora*, 2 belong to *Verrucosispora*, 1 belongs to *Jishengella*, 1 belongs to *Mycobacterium*, 1 belongs to *Pseudonocardia*, 1 belongs to *Isoptericola*, 1 belongs to *Microbacterium*, 1 belongs to *Cohaesibacter*, 2 belong to *Enhydrobacter*, 1 belongs to *Methylobacterium* and 4 belong to *Pseudomonas*.

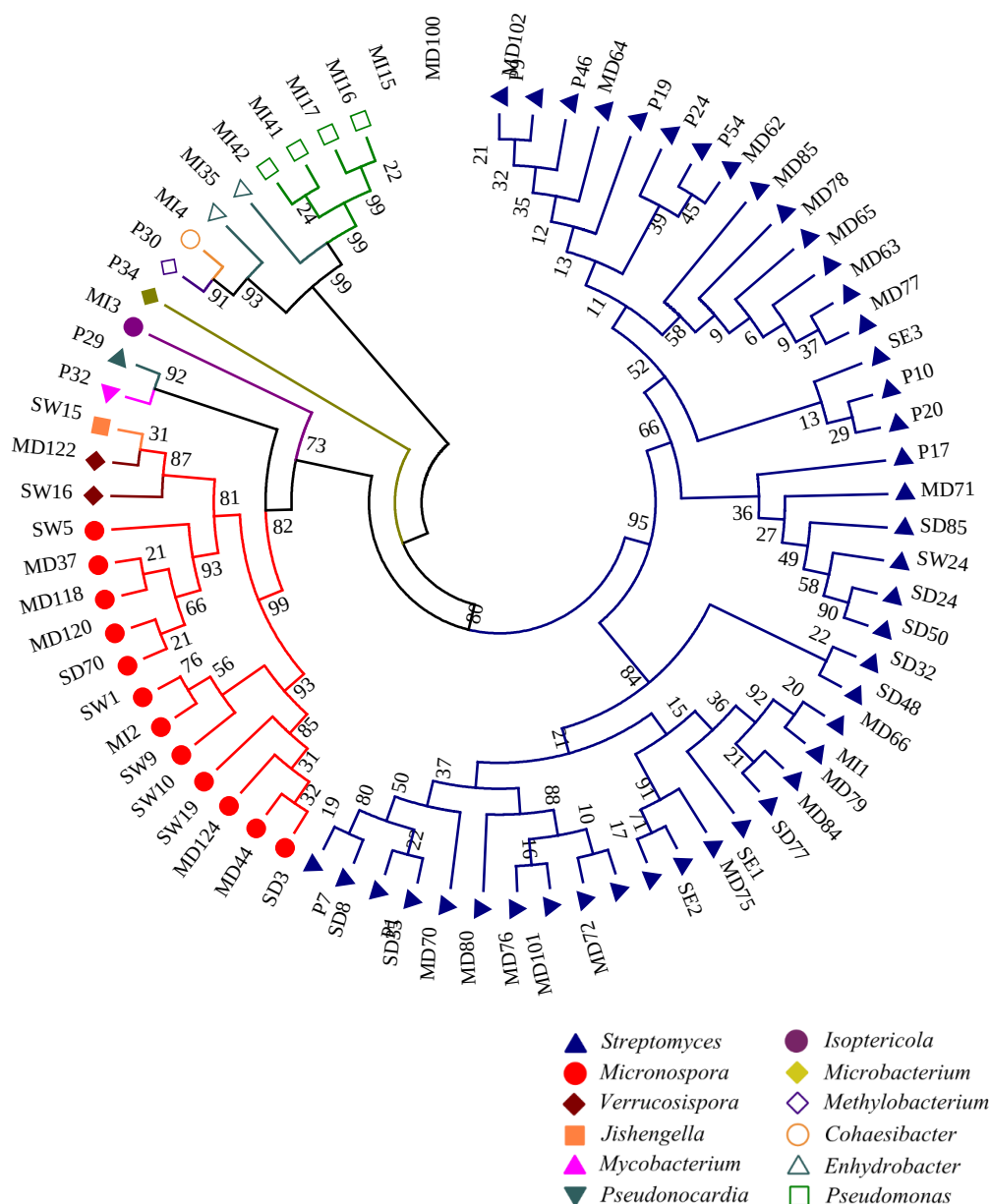


Figure 2.6 Circular phylogenetic analysis of the characterised 70 strains was plotted based on Neighbor-Joining method¹²⁵. Maximum composite likelihood method was used to compute the distances.

16S rDNA identification is not only used for identifying strains and genera, percentage similarities against a known strain can also be accessed for the novelty in strain isolated. It has been reported that a strain can be consider novel if the percentage similarity is less than 98.5% of any strain found in the database¹²⁶. Out of the strains isolated so far, none of the strains met the criteria of less than 98.5% similarity. From our strain collection, the strains with lowest percentage similarities (less than 99.2%) were tabulated in Table 2.3. The strain with lowest percentage similarity was SD32 with 99.10% similarity to *Streptomyces collinus*

Tu365, followed by SW16 with 99.16% similarity to *Verrucosispora sonchi* NEAU-QY3 and SD35 with 99.17% similarity to *Streptomyces cylabdanicus* K04-0144.

Table 2.3 Strains SW16, SD32 and SD35 with the lowest percentage similarities (less than 99.2%) to a known strain from NCBI database

Isolates	Closest match based on 16S rDNA gene sequencing	Similarity (%)	Coverage (%)
SW16	<i>Verrucosispora sonchi</i> NEAU-QY3	99.16	100.00
SD32	<i>Streptomyces collinus</i> Tu365	99.10	100.00
SD35	<i>Streptomyces cylabdanicus</i> K04-0144	99.17	100.00

Phylogenetic tree of selected strain with full 16S rDNA sequences were plotted against their close relative in Figure 2.7. 16S rDNA is a useful tool for identification of strains especially for genera that belongs to the family of Micromonosporaceae. The phylogenetic distances of Micromonosporaceae were closely related as shown in Figure 2.7. Without the use of 16S rDNA identification, it is unlikely to differentiate between the genera base on morphology. From Figure 2.8, the morphology of *Micromonospora* (SW1), *Verrucosispora* (SW16) and *Jishengella* (SW15) showed high similarities when cultured on ISP4 agar plates.

Among the Streptomycetaceae family, isolate SD24 and SD50 are different strains based on 16S identification and the difference was only 2 nucleotides out of 1501. They are very closely related as seen in the phylogenetic distances in Figure 2.7. Both SD24 and SD50 are highly similar to *Streptomyces bingchengensis*. From Figure 2.9, the morphology of SD24 and SD50 showed high similarities when cultured on MS agar (20 g of mannitol, 20 g of soy, 20 g of agar in 1 L of water).

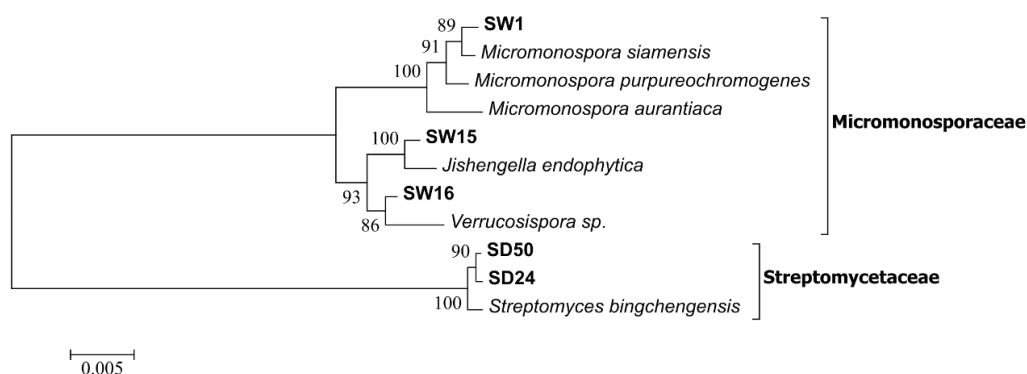


Figure 2.7 Phylogenetic tree of selected strains was plotted based on Neighbor-Joining method¹²⁵. The distances were computed using maximum composite likelihood displayed in the units of number of base per substitutions site. Numbers of substitution is 0.005.

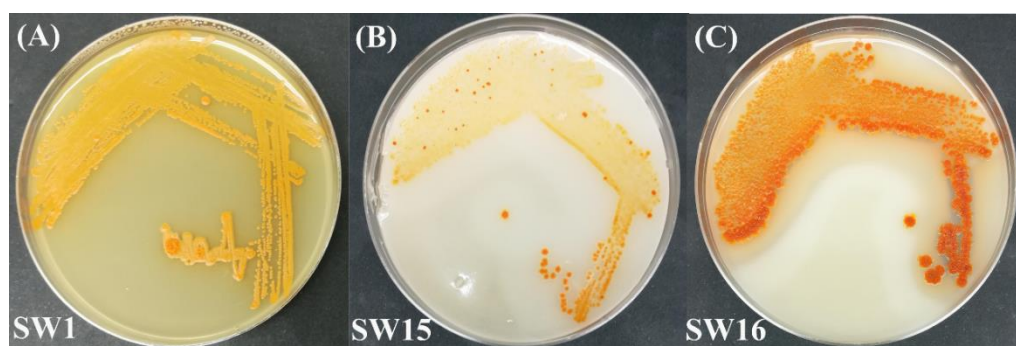


Figure 2.8 Morphology of (A) *Micromonospora* (isolate SW1), (B) *Jishengella* (isolate SW15) and (C) *Verrucosipora* (isolate SW16) cultured on ISP4 for two weeks.

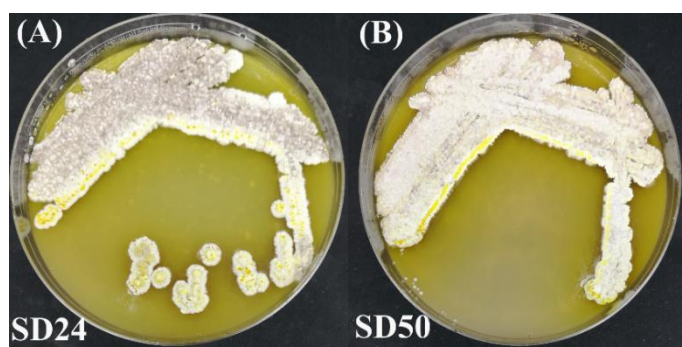


Figure 2.9 Morphology of *Streptomyces bingchengensis* (A) SD24 and (B) SD50 cultured on MS agar for a week.

2.4 Discussion

Intensive screening efforts have been carried out worldwide to access the actinomycete biodiversity. Many have ventured into unique environments such as deep sea, mangroves, deserts and caves^{31,34,36,46,49,50}. Mangrove has become a hotspot for discovery of secondary metabolites due to its special ecosystem between land and sea, superiority of easy sampling and the rich biodiversity³⁸. There were increasing successful discovery of novel genera from mangrove environment⁴⁰⁻⁴⁴. The largest mangrove in Singapore main island is Sungei Buloh Wetland Reserve (SBWR) with area of 130 hectares¹²⁷. However, little research has been carried out to assess the actinomycete biodiversity from SBWR^{128,129}. Pulau Ubin quarry lake, situated at the offshore island of Singapore, has never been studied for actinomycete biodiversity. Both SBWR and Pulau Ubin quarry lake were very different in biotas because of different environmental conditions such as pressures, nutrient compositions and temperatures. Since these environments were underexplored, we hope to do our part and contribute to this research by uncovering unique strains that are exclusive to these locations.

Among the actinomycetes, *Streptomyces* was seen to be the most abundant whereby its growth rates exceed other genera¹⁹. This was in agreement with our observations from mangrove and lake sediments. The isolation rates for *Streptomyces* were much higher than other genera of actinomycetes. Conventional isolation methods also faced serious drawback of repeated isolation of same abundant *Streptomyces* strains²⁷. In view of this bottleneck, selective isolation media had been improvised to favour the growth of rare actinomycetes^{103,108,114}. Specialised media containing sea water, soil extract and humic acid that were extracted using the sediments at the sampling location were incorporated into our isolation media to increase the rates of isolation of different genera. This was done to mimic the nutrient requirements of the native environment since the compositions were largely unknown. It was observed that media containing materials from that native environment tend to favour the growth of rare actinomycetes. For example, Gauze's medium 2 (SM3) which was prepared using seawater collected from SBWR, favoured the growth of mostly *Micromonospora* isolates (including isolate MD118 which would be covered in

the following chapters). *Jishengella* (SW15) was isolated from seawater ISP3, another medium that was prepared using seawater. *Verrucosipora* (SW16) was isolated from nutrient poor gellan gum. Nutrient poor gellan gum media made use of two different strategies. Materials from native environment, sand extract and seawater, were used to prepare the media. In addition, the use of different gelling agent has shown to favour the growth of certain rare actinomycetes¹⁰⁴.

Many industrial and research laboratories have processed a large number of samples across wide range of unique habitats and geographically diverse areas. As a result of the conventional screening methods, rediscovery of similar strains and known compounds were prevalent¹³⁰. The chance of finding novel compounds from conventional screening methods can be as low as 10^{-7} ¹³⁰. Only about 1% of the microbial diversity were culturable under laboratory conditions⁵³. This implies that there might be a potential source of novel compounds with more diverse chemistry waiting to be uncovered from the previously uncultured actinomycetes. Novel culturing methods have made its way since the first successful isolation of previously uncultured SAR11, a ubiquitous marine clade, in 2002⁵⁶. These novel culturing methods could better mimic the natural environment by incubation the cells at its native environment. Inspired by the ideology behind *in-situ* cultivation with a diffusion chamber, we designed circular *in-situ* cultivating disks for this purpose. I investigated the ability to culture actinomycetes via *in-situ* cultivation by loading diluted pre-treated samples into the wells of circular *in-situ* cultivating disks. From the findings, only a small percentage (7.14%) of the strains isolated from *in-situ* were actinomycetes. This result was not surprising as this *in-situ* cultivation set up has been successful for the isolation of Gram-negative bacteria^{59,61,62}.

Actinomycetes have relatively slow growth rates as compared to other bacteria. *In-situ* cultivation requires sufficiently dilute environmental samples being trapped into molten agar and loaded into the central plate of the *in-situ* cultivation disks. In my experiment, the environmental samples were pre-treated prior to dilution so as to increase the number of actinomycetes. This was the mandatory step in typical isolation of actinomycetes and was seen to be effective for direct plating method. However, some other species could also survive under harsh environmental conditions and extreme dryness. For example, *Pseudomonas* were

resistant to chemical treatments¹³¹. *Enhydrobacter* can survive desiccation for up to 2 weeks¹³². *Bacillus* and *Clostridium* were known to form endospores which are quite resistant to the pre-treatment methods applied¹³³. If these species could survive the pre-treatment methods, the number of cells for non-actinomycete after prolong incubation would effectively outgrow the actinomycetes making isolation of actinomycetes impractical. Direct plating method have higher success rate for isolation of actinomycetes due to selective media and nutrient that favours growth of actinomycetes and the addition of antifungal and antibiotics retards the growth of other bacteria and fungi.

There were many *in situ* cultivation methods that were not selective for actinomycetes. In view of these difficulties, Kim Lewis and group has devised a microbial trap for *in situ* cultivation that specifically targets actinomycetes⁵⁸. There were several differences between a microbial trap and a diffusion chamber (iChip concept). A diffusion chamber contains agar matrix that were pre-inoculated with microbes and sandwiched between two 0.03 μm membrane. Whereas, a microbial trap contains agar matrix without pre-inoculated with cells and sandwiched between a 0.2 μm membrane (only at the side that was in contact with the soil or aquatic sediment) and a 0.03 μm membrane⁵³. The membrane pore size of 0.2 μm allows actinomycetes with thinner hyphae to get trapped and effectively excludes fungi with much thicker hyphae⁵⁸. A microbial trap is a promising approach for gaining access to unique filamentous actinomycetes such as *Catellatospora*, *Catenulispora*, *Dactylosporangium*, *Lentzea* and *Streptacidiphilus* that were not recovered by traditional direct plating method⁵⁸.

From this study, 8 different genera of actinomycetes; *Streptomyces*, *Micromonospora*, *Verrucosispora*, *Jishengella*, *Pseudonocardia*, *Isoptericola*, *Microbacterium* and *Mycobacterium* were isolated from SBWR and Pulau Ubin quarry lake. In the process, 4 other genera of non-actinomycetes; *Pseudomonas*, *Enhydrobacter*, *Cohaesibacter* and *Methylobacterium* were also isolated. Though there are many strains yet to be identified, none of the strains characterised so far were considered as very unique based on the criteria of less than 98.5% similarity in 16S rDNA identity¹²⁶. Nevertheless, our findings shed some light of the actinomycete biodiversity from SBWR and Pulau Ubin quarry lake in Singapore. In addition, some of these strains contain novel biosynthetic

gene clusters and I will discuss in Chapter 4. *Streptomyces* and *Micromonospora* were present in abundance from both locations. Different locations and cultivation methods resulted in different variety of strains and genera being recovered from the environment.

2.5 Conclusion

Despite the dramatic slow-down in natural product antibiotic discovery in the last two decades, it is believed that actinomycetes are still a valuable source of natural products yet to be discovered. The biodiversity of Singapore's environmental samples is largely underexplored; and there is still a high potential of finding strains that are unique to this geographical location. In this study, isolation of actinomycetes were carried out using mangrove (SBWR) and lake (Pulau Ubin Quarry Lake) environmental samples as these geographical locations are unique and well preserved. A total of 193 strains were isolated and the genera includes *Streptomyces*, *Micromonospora*, *Verrucosipora*, *Jishengella*, *Pseudonocardia*, *Isoptricola*, *Microbacterium*, *Mycobacterium*, *Cohaesibacter*, *Enhydrobacter*, *Pseudomonas* and *Methylobacterium*. Different isolation strategy and cultivation has been employed to increase the phylogenetic diversity of the strains. Bacterial classification was carried out by 16s rDNA to classify strains into various genera and determine the novelty of strains. Though none of the strains met the criteria of less than 98.5% similarities among the strains we have characterized, there were still a lot more strains yet to be characterized. A total of 152 actinomycete strains isolated from this study laid down the foundation for the next stage of research work to uncover new biosynthetic pathways and compounds. The potential of finding novel bioactive compounds from these strains would be discussed in the next two chapters.

CHAPTER 3: Strain prioritization by bioactivity screening

3.1 Introduction

The Waksman platform had been widely adopted by pharmaceutical companies and research laboratories during the golden era. This involved isolation of soil-derived microorganisms from various sources. By exploring the diversity and taxonomy of microorganisms, it increased the chances of uncovering novel repertoire of secondary metabolites¹³⁴. This traditional screening method has resulted in the discovery of many classes of antibiotics including β -lactams, sulfadruugs, macrolides, tetracyclines and quinolones over the period between 1940s to 1960s⁶⁹. However, the platform became less efficient shortly after when same compounds were repeatedly discovered against the background of known compounds. With the diminished returns and high risk, many big pharmaceutical companies such as Merck and Eli Lilly have withdrawn from the field^{51,68}.

The aim of bioactivity screening is usually to trigger a specific biological response that can be measured *in vitro* or *in vivo*¹³⁵. For *in vitro* experiments, it can be examined by probing the biological responses towards inhibition of cell growth or ability to inhibit biofilm formation. Failure of traditional screening platforms and diminishing returns led to the need to revamp screening methodologies¹³⁶. The application of high-throughput screening (HTS) has resulted in increased sensitivity for detection and development of different bioactivity assays¹³⁷. These changes were crucial as bioactive compounds are usually produced in small quantities. In addition, development of whole-cell assays such as cross streak assay and overlay assay enabled the observations of biological response of a cell to perturbation of the test organisms¹³⁶. HTS platforms have been developed for antibacterial, antifungal and anti-biofilm assays in search for bioactive compounds with different targets¹³⁷.

One strain many compound (OSMAC) approach refers to the potential of a single bacterial strain being able to produce chemically diverse compounds. Whole genome sequencing of bacteria strains that produce secondary metabolites revealed the potential to generate more than 20 secondary metabolites per

strain^{75,82}. One effective strategy of increasing the chances of finding novel bioactive compound was to focus on a small number of bacteria strains and to investigate in detail. OSMAC approach has been successful in eliciting novel compounds that were only expressed under certain conditions^{84,138}. It is not possible for a strain to produce all possible secondary metabolites found in its entire genome under a set of environmental conditions as it would be metabolically and energetically costly¹³⁹. Biosynthesis of secondary metabolites is very dependent on internal and external cues. The production of specific secondary metabolite only occurs when there is a competitive advantage under different conditions¹³⁹. In addition, OSMAC approach is a pleiotropic approach whereby it does not allow the development of common rules for all strains¹⁴⁰. OSMAC approach involves changing parameters such as nutrients, salinity, incubation time, temperature, pH and more for the activation of different secondary metabolites from a single strain¹⁴⁰. This would be a promising trend if screening through bacterial extracts is the focus of the preliminary process.

In this study, bioactivity screening was used as the initial phase for streamlining the workflow. OSMAC approach of altering nutrients and culturing conditions were adopted. Antibacterial, antifungal and anti-biofilm assays were conducted for screening of cell extracts produced by the actinomycete strains. As different bioactivity assays have its own advantages and limitations, screening with different types of assays would provide a more comprehensive and conclusive data for analysis. Bioactivity guided approach often results in rediscovery of known compounds with strong bioactivities. Thus, bioactivity screening approach was only used to prioritize the strains for genome sequencing. Bioinformatics analysis, covered in my next chapter (**Chapter 4**), would then be used for assessing the potential of finding novel compounds before compound isolation.

3.2 Materials and methods

3.2.1 Cross streak assay

Antibacterial assay by cross streak assay was carried out as mentioned previously with slight modifications¹⁴¹. Actinomycetes isolated from mangrove were

streaked on one corner of the petri plates on 25 % ISP 2 (1 g/L glucose, 1 g/L yeast extract, 2.5 g/L malt extract and 6 g/L of bacto agar). Plates were incubated for 7 to 21 days at 28 °C for secondary metabolite production before introducing 3 test organisms. The test organisms include 2 gram-positive bacteria *Bacillus subtilis* 168, *Staphylococcus aureus* ATCC 14775 and a gram-negative bacteria *Pseudomonas aeruginosa* PA01. A pure colony of test strains was transferred into fresh Luria-Bertani (LB) (10 g/L of tryptone, 5 g/L of yeast extract and 10 g/L of NaCl) and incubated overnight to early exponential phase (OD600 of approximately 1.0). 10 µL inoculating loops (NUNC, Fisher Scientific Pte Ltd, Singapore) were dipped into the bacterial suspension and streaked perpendicular to the isolates on the agar medium. The plates were then incubated at 28 °C for 16 hours. The microbial inhibitions observed were indications of antimicrobial metabolites produced by the isolates. Experiments were repeated at least three times and the results were reproducible. Data from one experiment was presented.

3.2.2 Overlay assay

Antibacterial assay by overlay assay was carried out as mentioned previously¹⁴². Actinomycetes isolated from mangrove were tested against 6 clinical isolates. These clinical isolates are Methicillin-resistant *Staphylococcus aureus* (MRSA) strain DR42412 from sputum, MRSA strain DB68004 from blood, *Pseudomonas aeruginosa* strain DU144476/07 (*P. aeruginosa*), *Candida albicans* strain DF0002672R (*C. albicans*), *Escherichia coli* strain Y9 (*E. coli*) and *Klebsiella pneumoniae* strain Y10 (*K. pneumoniae*). Actinomycete strains were cultured in CRM media (103 g of sucrose, 10.12 g of MgCl₂·6H₂O, 15 g of tryptic soy broth, 5 g of yeast extract and 10 g of glucose in 1 L of water) to exponential phase prior to the experiment. 2 µL of actinomycete liquid culture was inoculated on minimal media agar plate (0.5 g of L-asparagine, 0.655 g of K₂HPO₄·3H₂O, 0.2 g of MgSO₄·7H₂O, 0.01 g of FeSO₄·7H₂O, 20 g of instant ocean salts, 10 g of agar and 10 g of mannitol in 1 L of distilled water, pH 7) and incubated at 30 °C for a week. One agar plate can accommodate 8 actinomycete strains which were positioned 1.5 cm apart from one another.

The test organisms were inoculated in 5 ml of Luria-Bertani (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L of water) and incubated at 37 °C

overnight with shaking. 1% of overnight culture was inoculated to 5 ml of fresh LB and incubated for a few hours at 37 °C until the OD₆₀₀ of the culture reaches 0.5. For the overlay layer, the freshly grown culture was diluted to a final OD₆₀₀ of 0.0025 in 12 mL of 0.7% LB agar, and poured over the actinomycete-containing plate. The plates were incubated for 24 h at 30 °C. Zones of inhibition around the actinomycete colonies were observed and measurements were recorded in millimeters (mm).

3.2.3 Fermentation of strains for bioactivity assays

The actinomycetes were inoculated in 2 mL of ISP2 (4 g glucose, 4 g yeast extract, 10 g malt extract in 1 litre distilled water) and Pharmamedia (5 g glucose, 5 g cornsteep powder, 10 g oatmeal, 10 g cottonseed flour, 5 g K₂HPO₄, 5 g MgSO₄·7H₂O, and 1 mL trace metal solution in 1 litre distilled water) at 30 °C 170 revolutions per minute (rpm) for several days to high density. They were then subcultured into 5 mL fresh medium and incubated for 1 week at 30 °C 170 rpm. The biomass was then separated from the supernatant via centrifugation at 8,500 rpm for 10 minutes. The biomass and supernatant were mixed with equivalent amount of acetone and ethyl acetate respectively. After centrifugation, the organic portions were aliquoted into 48-well ABgene storage plate (ThermoFisher Scientific). The solvents were then evaporated using Genevac EZ-2 elite personal evaporator (Genevac Ltd, United Kingdom) at low boiling point setting before switching to aqueous setting to remove traces of water. The dried extracts were dissolved in 20 µL of dimethyl sulfoxide (DMSO) and mixed with 180 µL of distilled water to achieve 10% DMSO concentration.

3.2.4 Microtiter plate based antibacterial assay

Crude extracts or pure compounds produced by actinomycete strains were tested against clinical isolates MRSA, *E. coli* and *P. aeruginosa*. Crude extracts or pure compounds were reconstituted with 10% DMSO prior to the assay. The test organisms were inoculated in 5 mL of LB media and incubated at 37 °C overnight with shaking. 1% of overnight culture was inoculated to 5 mL of fresh LB and incubated for a few hours at 37 °C until the OD₆₀₀ of the culture reaches 0.5. The assay was carried out in 96 well microtiter plate (Thermo Scientific, USA). 90

μL of the diluted test organism culture and 10 μL of the crude extract or pure compound were added into each well of a microtiter plate. Final testing concentration of DMSO in each well would be 1% and final testing OD₆₀₀ of test organisms would be 0.0025. 10 μL of rifampicin (stock concentration 2 mg/ml) was used as the positive control against MRSA, while 10 μL of tetracycline (stock concentration 2 mg/mL) was used as the positive control against *E. coli* and *P. aeruginosa*. 1% DMSO was used as negative control for the assay. The 0 hour absorbance reading at 600 nm was obtained using Tecan Infinite M200 Pro microplate reader (Tecan, Switzerland). After 0 hour readings were taken, the microplates were covered with aluminium foil to improve aeration among the wells. The microtiter plates were then incubated at 37 °C for 16 hours. After 16 hours, the absorbance reading at 600 nm was obtained. 0 hour readings were subtracted from the 20 hour readings before calculating the percentage inhibition. Each extract was tested in triplicate.

The percentage inhibition was calculated based on the following formula:

$$\text{Percentage Inhibition} = \left[1 - \left(\frac{\text{OD}_{\text{test}} - \text{OD}_{\text{positive}}}{\text{OD}_{\text{negative}} - \text{OD}_{\text{positive}}} \right) \right] \times 100$$

Where:

OD_{test} = OD of testing well with crude extract or pure compound

OD_{positive} = OD of positive control with 200 μg/mL of rifampicin or tetracycline

OD_{negative} = OD of negative control with 1% DMSO

Some crude extracts do not have good solubility in 10% DMSO (solvent for reconstituting the compound). Therefore, when the extracts were added into the 96 well plates together with the cells, it precipitates. This caused the initial OD measurement of the cells to be slightly higher than negative controls. After 16 hours incubation at 37 °C, the extracts that exhibit bioactivity managed to dissolve better at elevated temperature, causing the percentage inhibition to be greater than 100%.

3.2.5 Microtiter plate based antifungal assay

Crude extracts or pure compound produced by actinomycete strains were tested against clinical isolate *C. albicans* DF2672R in 96 well microtiter plates. *C.*

albicans was inoculated in 5 mL of yeast extract peptone dextrose broth (YPD broth: 20 g peptone, 10 g yeast extract, 20 g glucose in 1 L distilled water) and incubated at 30 °C overnight with shaking. 1% of overnight culture was inoculated to fresh YPD broth and incubated at 30 °C to log phase. The cells were centrifuged and washed twice with YPD before being resuspended in fresh YPD. The assay was carried out in a microtiter plate (Thermo Scientific, USA). 90 µL of the diluted test organism culture and 10 µL of the crude extract or pure compound were added into each well of a microtiter plate. Final testing concentration of DMSO in each well would be 1% and final testing OD₆₀₀ of *C. albicans* would be 0.1. 10 µL of nystatin (stock concentration 2 mg/ml) was used as the positive control. 1% DMSO was used as negative control for the assay. The 0 hour absorbance reading at 600 nm was obtained using Tecan Infinite M200 Pro microplate reader (Tecan, Switzerland). After 0 hour readings were taken, the microplates were covered with aluminium foil to improve aeration among the wells. The microtiter plates were then incubated at 30 °C for 20 hours with shaking. After 20 hours, the absorbance reading at 600 nm was obtained. 0 hour readings were subtracted from the 20 hour readings before calculating the percentage inhibition using the formula in Section 3.2.4. Each extract was tested in triplicate.

3.2.6 Anti-biofilm assay

Crude extracts produced by actinomycete isolates were tested against clinical isolate *P. aeruginosa* PA01 due to its strong biofilm forming ability^{143,144}. Experiment was conducted in microtiter plates by static biofilm assay as mentioned previously with some modifications^{145,146}. Isolates were inoculated in 96 well plates containing 200 µL of production medium 3 (20 g/L of oatmeal, 2.5 g/L of glycerol, 1 mL of trace solution and 10 g/L agarose) per well for production of secondary metabolites¹⁴⁷. Isolates were incubated at 28 °C for 3 weeks. Due to long incubation period, to prevent the agar from drying up, the plates were kept in tuber ware containing a reservoir of distilled water. After 3 weeks of incubation, the medium was dried for a week in the incubator without supplying moisture. Following that, the secondary metabolites of isolates were extracted with 150 µL of dimethyl sulfoxide (DMSO) per well and placed on rotary shaker for 2 hours before use. 2% DMSO extracts were used for the assay.

Pseudomonas aeruginosa PA01 was inoculated in LB medium and incubated overnight to early stationary phase (OD₆₀₀ of ~1.0). 1% of the overnight culture was sub-cultured into fresh LB medium and incubated at 37 °C for 3 hours (OD₆₀₀ <0.5). The culture was diluted to OD₆₀₀ 0.05 using M63 medium (13.6 g/L of KH₂PO₄, 0.5 mg/L of FeSO₄, 0.2 g/L of MgSO₄·7H₂O, 5 g/L of casamino acids and 0.2% of glucose) and used for the assay.

Quantitative analysis using 96 well plates: The quantitative analysis for biofilm assay was carried out using 96 well plates (NUNC, Fisher Scientific Pte Ltd, Singapore). 200 µL of the diluted bacterial suspension were added to each well. For positive control, 1 µL of DNase I Solution (1 unit/µL), RNase-free (Thermo Scientific) was mixed with 99 µL of the diluted culture. DNASE I was used as a positive control because it is known to inhibit biofilm formation by destroying extracellular DNA, which is one of the essential components of biofilm extracellular matrix¹⁴⁸. Negative control wells contained diluted bacterial suspension with 2% pure DMSO.

Qualitative analysis using test tubes: The qualitative analysis for biofilm assay was carried out using test tubes (diameter: 1 cm; length: 6 cm). 400 µL of the diluted bacterial suspension were added to each test tube.

To ensure consistency and reliability of results at least 3 replicates per samples were carried out for this experiment. After 24 hours incubation, the content of each well was emptied and washed 3 times with distilled water. The plates were washed gently to remove all non-adherent bacteria without disturbing the biofilm attached to the sides of the wells. The remaining biofilm was fixed with 200 µL of methanol per well for 15 minutes. Then, the plates were emptied and left to dry at room temperature. The plates were stained with 0.1% crystal violet solution for 15 minutes. Then, the plates were washed gently to remove excess stain that may interfere with the results and left to dry overnight. 300 µL of 30% acetic acid were added to each well to solubilize the crystal violet stain for 15 minutes. The contents were mixed well by pipetting and 200 µL of the contents were transferred to a new 96 well plate for quantification. This dilution was necessary to prevent the quantification of crystal violet stain from exceeding the detection limit of microplate reader. The OD of each well was measured at 600 nm with a

TECAN Infinite M200 Pro. Experiments were performed in triplicates and the data obtained from the experiments were presented as mean values with standard deviations plotted as error bars. The anti-biofilm activity was calculated as percentage inhibition using the formula in Section 3.2.4.

3.3 Results

152 actinomycete strains isolated from SBWR and Pulau Ubin Quarry Lake, as discussed in Chapter 2, were screened using various bioactivity assays. In this Chapter, I would be focusing on the bioactivity assays performed to assess the potential of the 152 actinomycete strains in producing bioactive compounds. This step is crucial as strains that exhibit interesting bioactivities were prioritized for genome sequencing and metabolite profiling. Various bioactivity assays were carried out using extracts generated from 152 actinomycete strains against test organisms which consist of Methicillin-resistance *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*) and *Candida albicans* (*C. albicans*).

110 strains (72.37%) were tested to be bioactive against at least one of the test organisms. The bioactivity assays results were tabulated in Table S.3 - Table S.9 (**Appendix**). Figure 3.1 shows the number of actinomycete strains tested to be bioactive against the various test organisms in this study. There were higher proportion of strains showing bioactivity against Gram-positive bacteria, *S. aureus* (84 strains) and *B. subtilis* (46 strains), as compared to Gram-negative bacteria, *E. coli* (54 strains), *K. pneumoniae* (5 strains) and *P. aeruginosa* (8 strains). There was also a small proportion of the strains that exhibit bioactivity against fungus, *C. albicans* (11 strains).

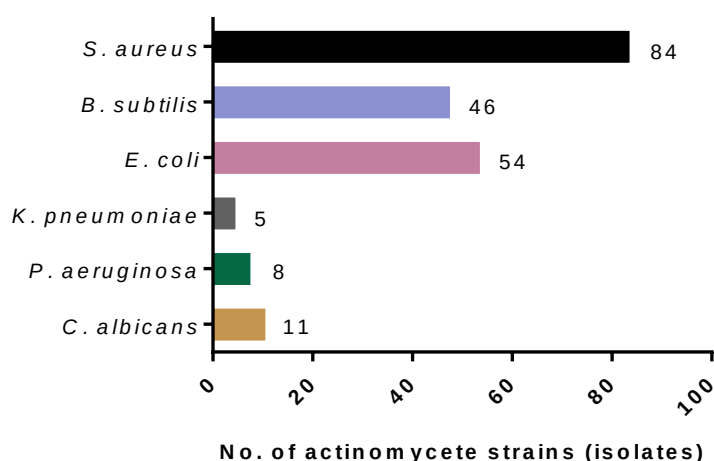


Figure 3.1 Number of actinomycete strains tested to be bioactive against the various test organisms under tested conditions.

3.3.1 Antibacterial activities from *Streptomyces* sp. SW24, SD24 and SD50

SW24, SD24 and SD50 exhibited strong activities against *S. aureus* and *B. subtilis* in cross streak antibacterial assay shown in Figure 3.2. Limited growth can be observed from the test organisms *S. aureus* and *B. subtilis* due to the presence of bioactive compounds produced by the actinomycete strains. SW24 showed the strongest activity against *S. aureus* (62 mm) and *B. subtilis* (72 mm), followed by SD24 and SD50 with zones of inhibition between 48 mm to 55 mm.

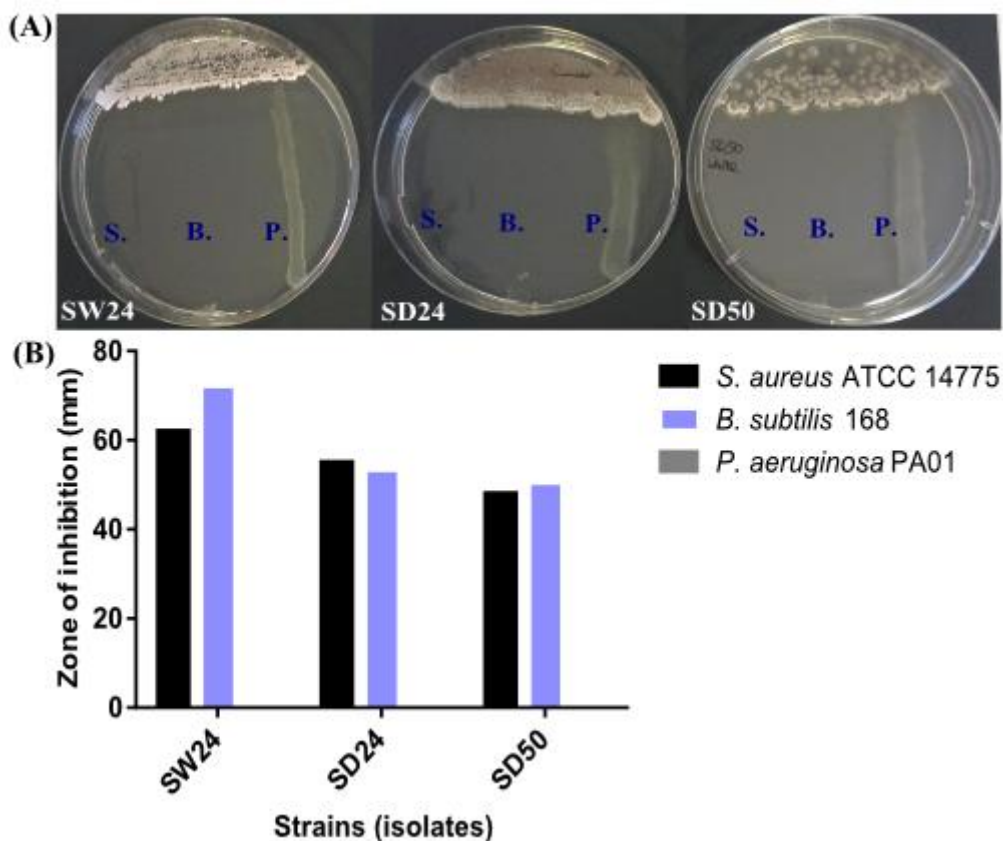


Figure 3.2 (A) Cross streak antibacterial assay for selected strains (*Streptomyces* sp. SW24, SD24 and SD50) that exhibit strong bioactivity against *S. aureus* ATCC 14775 and *B. subtilis* 168. (B) Selected strains with their zone of inhibition (mm) >50 mm against *S. aureus* ATCC 14775, *B. subtilis* 168 and *P. aeruginosa* PA01.

3.3.2 Antibacterial activities from *Streptomyces* sp. P19

P19 extracts from culture broth and biomass exhibit strong bioactivities (>98% inhibition) against clinical isolates MRSA, *E. coli* and *P. aeruginosa* as shown in Figure 3.3. The bioactive compound was produced only when cultured in Pharmamedia and the bioactive compound was present in both culture broth and biomass. From our collection of strains, only 8 of them showed activity against *P. aeruginosa*, an opportunistic pathogen. Out of the 8 strains, only P19 crude extracts showed strongest activity against *P. aeruginosa* with $117 \pm 6.7\%$ (EA extract) and $98.9 \pm 1.1\%$ (Acetone extract) inhibition.

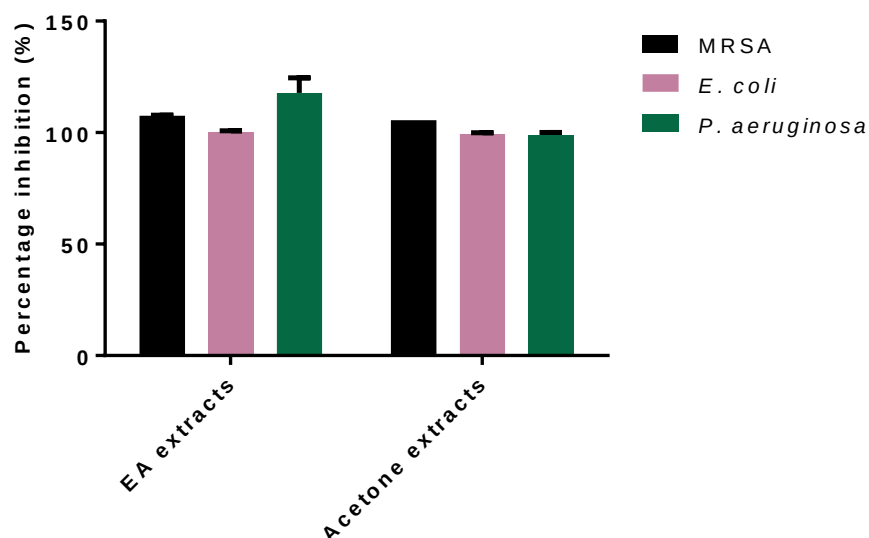


Figure 3.3 *Streptomyces* sp. P19 was cultured in Pharmamedia for 10 days before extracting culture broth with ethyl acetate (EA) and biomass with acetone. Microtiter based antibacterial assay for P19 extracts exhibit strong activity (>98% inhibition) against clinical isolates MRSA, *E. coli* and *P. aeruginosa*.

3.3.3 Antibacterial and antifungal activities from *Streptomyces* sp. P9

From our collection of strains, P9 is the only strain that produces compounds that target both bacterial and fungal strains as shown in Figure 3.4. The significantly strong bioactivities were observed only from overlay antibacterial and antifungal assays. P9 exhibited strong activities against Gram-positive bacteria MRSA and fungus *C. albicans* with 17 mm and 16 mm zones of inhibition respectively. P9 exhibited moderate activities against Gram-negative bacteria *K. pneumoniae* and *E. coli* with 9 mm and 7 mm zones of inhibition respectively.

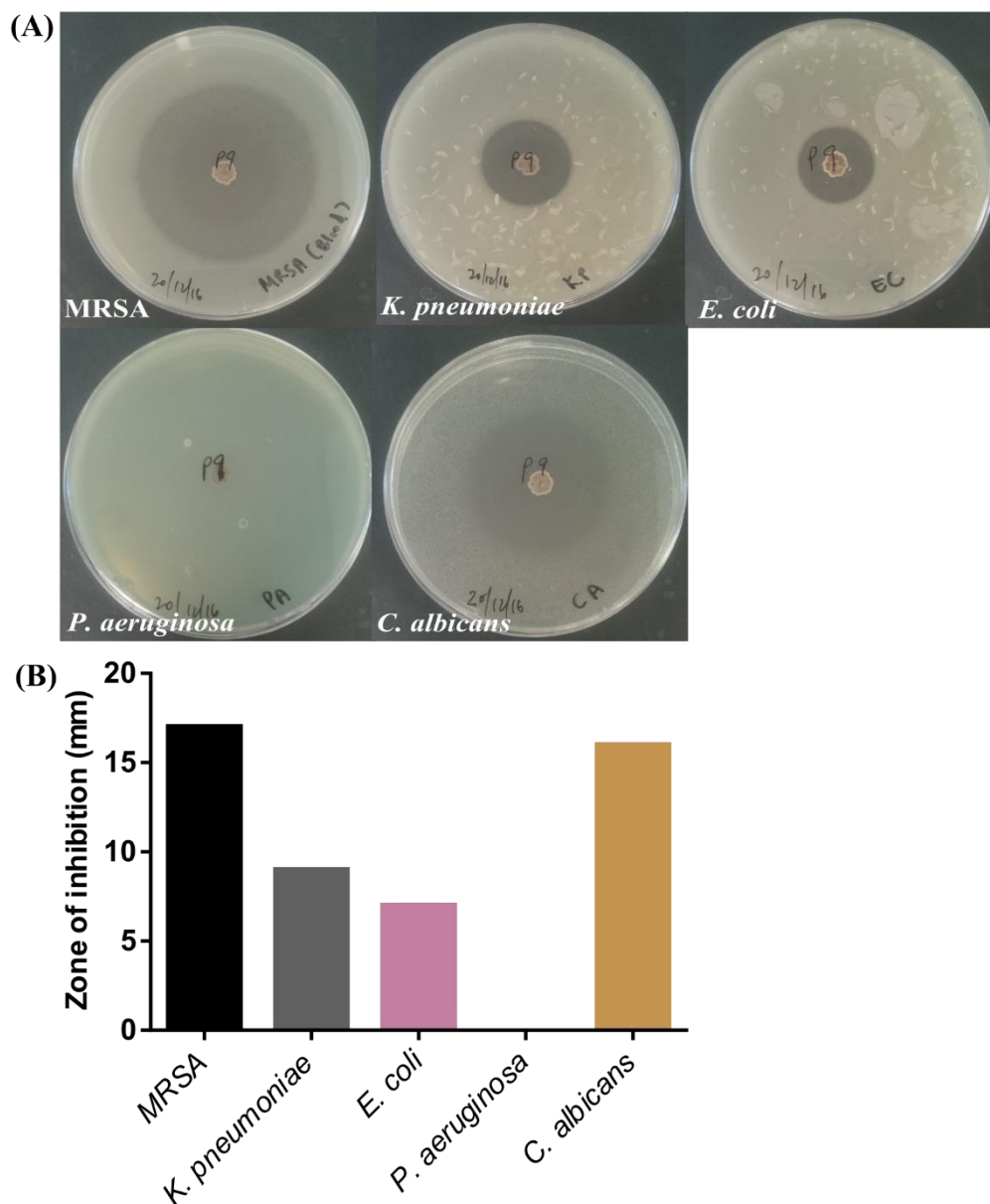


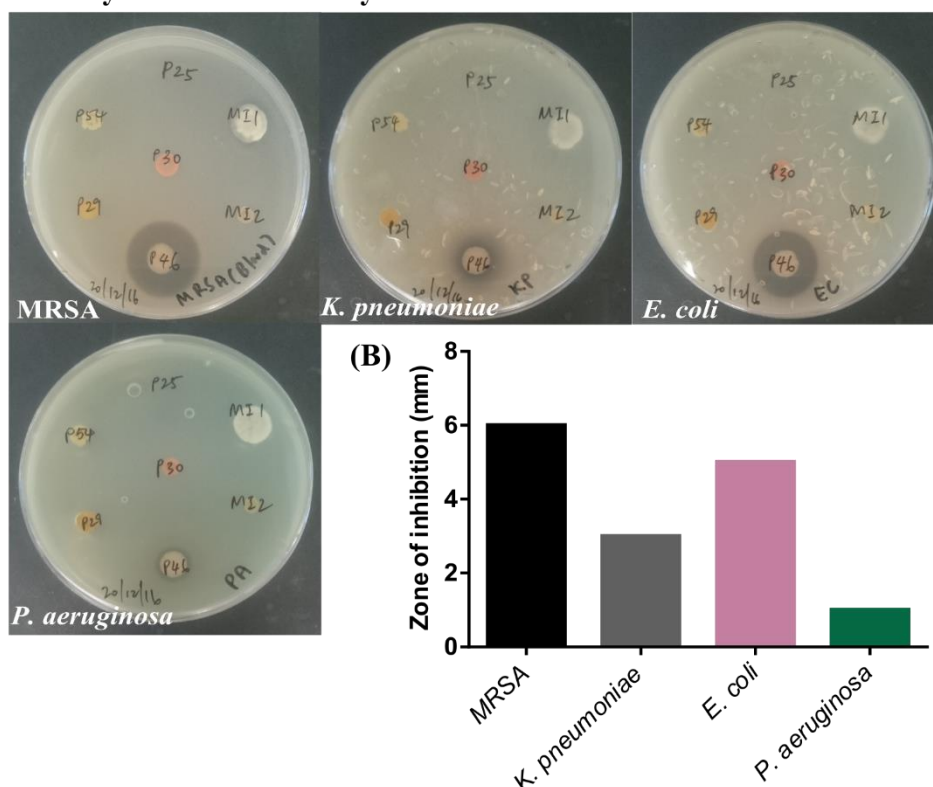
Figure 3.4 (A) Overlay antibacterial and antifungal assay plate images for *Streptomyces* sp. P9. (B) P9 exhibited strong activity against Gram-positive bacteria MRSA (17 mm) and fungus *C. albicans* (16 mm). It also exhibited moderate activities against Gram-negative bacteria *K. pneumoniae* (9 mm) and *E. coli* (7 mm).

3.3.4 Antibacterial activities from *Streptomyces* sp. P46

P46 exhibited bioactivities in overlay and microtiter plate based antibacterial assays as shown in Figure 3.5. P46 exhibited moderate activities against MRSA, *K. pneumoniae*, *E. coli* and *P. aeruginosa* in overlay assay (Figure 3.5(A)) with zone of inhibitions 6 mm, 3 mm, 5 mm and 1 mm. P46 was one of the strains that displayed bioactivities against many test organisms, including *K. pneumoniae* and *P. aeruginosa*, in microtiter plate based antibacterial assay (Figure 3.5(B)).

Surprisingly, P46 ethyl acetate extracts obtained from GYM fermentation medium exhibited strong activities against MRSA ($73.8 \pm 0.2\%$ inhibition) and *P. aeruginosa* ($73.8 \pm 0.8\%$ inhibition). Acetone extracts on the other hand only displayed slight activities against different test organisms with percentage inhibition ranging from $19.9 \pm 16.8\%$ to $36.7 \pm 5.9\%$.

(A) Overlay antibacterial assay



(C) Microtiter plate based antibacterial assay

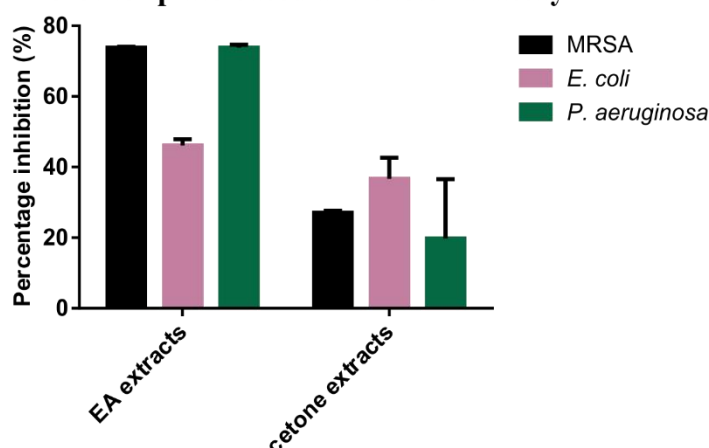


Figure 3.5 (A) Overlay antibacterial assay for *Streptomyces* sp. P46. (B) P46 shows moderate activities against MRSA (6 mm), *K. pneumoniae* (3 mm), *E. coli* (5 mm) and *P. aeruginosa* (1 mm). (C) P46 was cultured in GYM fermentation media for 7 days before extracting culture broth with ethyl acetate (EA) and biomass with acetone. Microtiter plate based antibacterial assay shows that the

extracts from culture broth exhibited strong activities against MRSA ($73.9 \pm 0.2\%$ inhibition) and *E. coli* ($73.8 \pm 0.8\%$ inhibition).

3.3.5 Antibacterial activities from *Micromonospora* sp. MD118

MD118 exhibited strong bioactivity against Gram-positive bacteria MRSA with zone of inhibition of 38 mm and moderate activities against Gram-negative bacteria *K. pneumoniae*, *E. coli* and *P. aeruginosa* with zones of inhibition of 10 mm, 16 mm and 4 mm respectively as seen Figure 3.6. MD118 was one of the strains tested to be bioactive against *P. aeruginosa* from overlay antibacterial assay. Out of the 2 strains (MD118 and P46) that displayed bioactivities against clinical isolate *P. aeruginosa*, MD118 displayed stronger activity with larger zone of inhibition. Interestingly, these bioactivities were only observed when cultured on minimal medium agar plates in overlay assay.

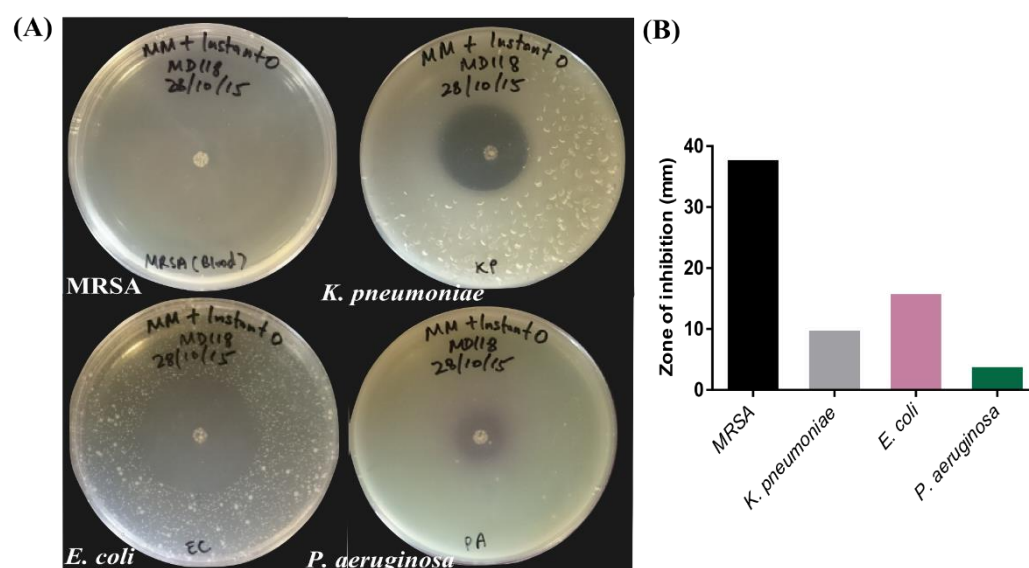


Figure 3.6 (A) Overlay antibacterial assay plate images for *Micromonospora* sp. MD118. (B) MD118 exhibited strong activities against Gram-positive bacteria MRSA (38 mm) and moderate activities against Gram-negative bacteria *K. pneumoniae* (10 mm), *E. coli* (16 mm) and *P. aeruginosa* (4 mm).

3.3.6 Antibacterial activities from *Streptomyces* sp. MD100

MD100 was the only strain that exhibited strong activities against Gram-positive bacteria MRSA with zone of inhibition 25 mm and Gram-negative bacteria *E. coli* with zone of inhibition 23 mm from overlay antibacterial assay (Figure 3.7).

However, it does not display any activities against other Gram-negative bacteria *K. pneumoniae* and *P. aeruginosa*.

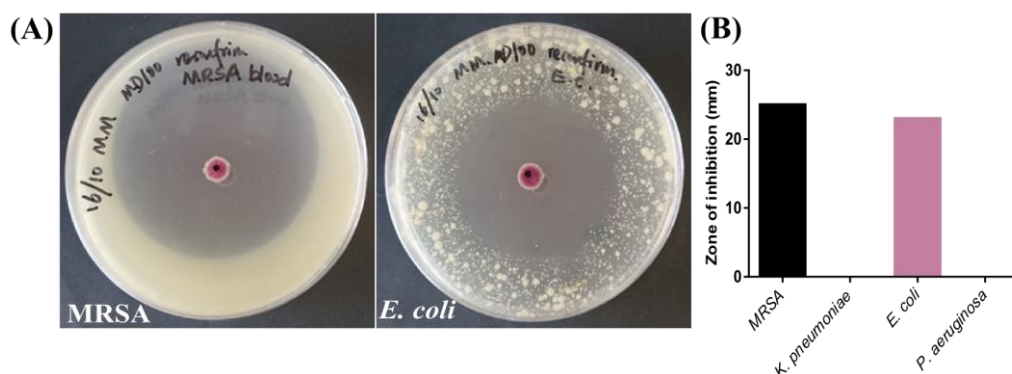


Figure 3.7 (A) Overlay antibacterial assay plate images for *Streptomyces* sp. MD100. (B) MD100 exhibited strong activities against both Gram-positive bacteria MRSA (25 mm) and Gram-negative bacteria *E. coli* (23 mm).

3.3.7 Antifungal activities from *Streptomyces* sp. MD102 and P7

Out of our strain collection, MD102 and P7 have the strongest activities against fungal strain, *C. albicans* as shown in Figure 3.8. Zone of inhibition for MD102 and P7 were 21 mm and 20 mm respectively.

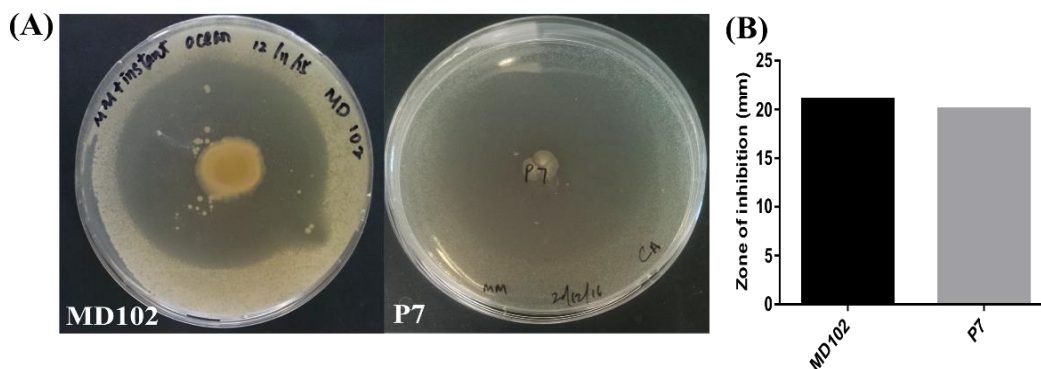


Figure 3.8 (A) Overlay antifungal assay plate images for *Streptomyces* sp. MD102 and P7. (B) Both MD102 and P7 exhibited strong activity against *C. albicans* with zone of inhibitions 21 mm and 20 mm respectively.

3.3.8 Anti-biofilm activities from *Streptomyces* sp. SD9, SD35 and SD48

Isolates were screened using static biofilm assay to observe the ability to inhibit biofilm formation in *P. aeruginosa* PA01. Interestingly, crude extracts from SD9, SD35 and SD48 were able to inhibit biofilm formation from an opportunistic pathogen *P. aeruginosa* PA01 by at least 80%. Figure 3.9(A) showed the visible reduction of biofilm density as compared to 2% DMSO when cultured in test

tubes. Figure 3.9(B) showed the percentage inhibition of biofilm by SD9 ($88.5 \pm 12.5\%$), SD35 ($80.8 \pm 13.5\%$) and SD48 ($81.9 \pm 16.8\%$).

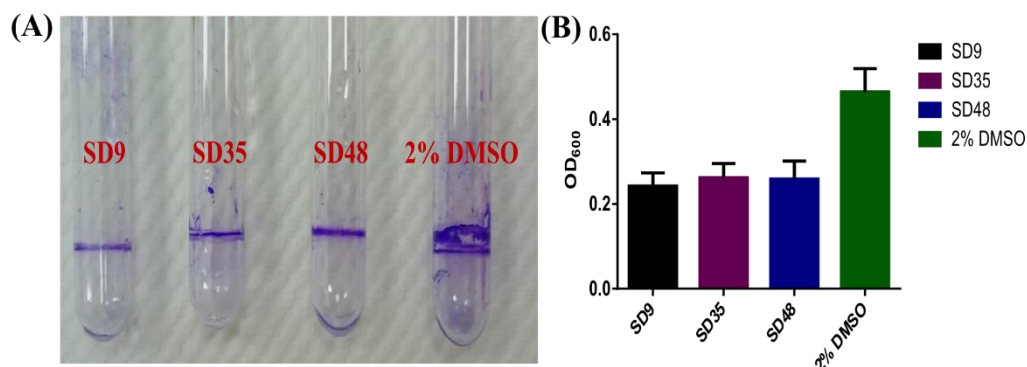


Figure 3.9 (A) Qualitative analysis of anti-biofilm assay for *Streptomyces* sp. SD9, SD35 and SD48 performed in test tubes to show the visible reduction of biofilm density as compared to the negative control (2% DMSO). (B) Quantitative analysis of anti-biofilm assay for SD9, SD35 and SD48 performed in 96 well plate showing the reduction of OD₆₀₀ values as compared to the negative control (2% DMSO).

3.4 Discussion

Screening of actinomycetes can be carried out by screening for enzymatic activities or antimicrobial activities¹⁴⁹. Strain prioritization by screening against a panel of test organisms is one of the most common strategy. Bioactivity screening is a fast and cost-efficient way of screening hundreds of strains. Thus, bioactivity assays were used in this study for ranking of strains. Successful screening strategies involve increasing the quantity of screening and quality of screening¹⁵⁰. Academic research laboratories are considered to be small scale operations as compared to the previous efforts by big pharmaceutical companies with high throughput screening methods and standard operational procedures. The challenge faced by academic research laboratories would be to increase the quality of screening so as to avoid rediscovery of known compounds from the huge background of already existing compounds. Even though the challenges faced by small academic research laboratories can be tremendous, many academic laboratories were able to reap success from the advances in technology and experimental techniques. For example, liquid chromatography coupled with mass spectrometry (LCMS) could be used as a dereplication tool from the initial stages¹⁵¹. With the improved sensitivity of biochemical assays and instruments, assays can be conducted in smaller volumes. For example, 1 mL assays

conducted during 1980s can be replaced with less than 400 μ L for 96 well microplate or even 1 – 2 μ L for 384 well plate¹⁵². The invention of high-throughput screening via microtiter plate greatly reduced the amount of labor required. In addition, the results more reproducible, reliable and less expensive. As one of the academic research laboratories which has strong interest in discovery of bioactive compounds, we would like to contribute our efforts into finding potential drug leads by tapping on the tools available.

The ESKAPE pathogens accounts for the highest number of nosocomial infections worldwide¹⁵³. For our study, we have included 3 clinical isolates, Methicillin-resistant *Staphylococcus aureus* (MRSA), *K. pneumoniae* and *P. aeruginosa* from the ESKAPE pathogens, as our test organisms for bioactivity assays. In addition, we have included clinical isolates of *E. coli* and *C. albicans* which are also major concerns in healthcare settings. The cell wall components and generation time of bacteria and fungi are very different. Thus, antibacterial and antifungal agents are structurally different and selectively targets the most relevant organisms¹⁵⁴. For example, antibacterial agents inhibit steps required for the formation of peptidoglycan (the essential component of bacterial cell wall), inhibit nucleic acid synthesis or protein synthesis. Whereas, most antifungal agents target either the formation or the function of ergosterol (the essential component of the fungal cell membrane). Despite the differences, the mechanisms of resistance of bacterial and fungi were found to be quite similar for the ability to modify its drug target, alteration in cell membrane biosynthesis, overexpression of drug target and active efflux¹⁵⁴.

The need for potential drug candidate with the ability to target Gram-negative bacteria remains a pressing issue as the industrial pipeline of new drugs remains quite limited¹⁵⁵. One characteristic feature of most Gram-negative bacteria is the ability to form biofilm, causing a significant amount of hospital acquired infections¹⁵⁶. These organisms include *P. aeruginosa*, *K. pneumonia*, *E. coli* and many more. Anti-biofilm assay has been carried on *P. aeruginosa* PA01 as it is the model organisms for biofilm studies^{143,144}. It was known that some compounds exhibit anti-biofilm properties though these compounds were not potent enough to inhibit the growth of *P. aeruginosa*^{157,158}.

We have included a few types of antibacterial and antifungal assays as each has their pros and cons. For example, whole-cell assay such as cross streak assay and overlay assay were cost effective primary screening methods. Not only that, it enabled the observations of biological response of a cell to perturbation of the test organisms by skipping the secondary metabolites extraction procedures. Microtiter plate based antibacterial and antifungal assays requires the extraction of secondary metabolites prior to the testing. But it allows throughput analysis and comparison between a single strain cultured under different fermentation media. OSMAC approach of altering nutrients in fermentation media and culturing conditions were employed to vary the secondary metabolites being produced by a single strain. Different nutrients and culturing conditions often led to production of different set of secondary metabolites¹⁴⁰. By doing so, it also helped in increasing the chances of finding a hit from the bioactivity assays and the conditions required to produce high quantities of the bioactive compound.

From the results, strains that exhibit bioactivity against Gram-negative bacteria were rare especially for the case of *P. aeruginosa* and *K. pneumoniae*. In addition, strains that exhibit bioactivity against fungus *C. albicans* were also limited. There is a possibility that these metabolites possess interesting features or chemistry that may interact with more resistant test organisms. In addition, there were also strains that exhibit broad spectrum activity that targets both Gram-positive bacteria and Gram-negative bacteria. MD118 and P19 were examples that exhibit broad spectrum activity and would be discussed further in Chapter 4.

3.5 Conclusion

Bioactivity assays have been conducted for 152 actinomycete strains. Bioactivity assays were used as a tool for strain prioritization. 72.37% of the strains exhibited bioactivities against one or more test organisms, with a higher proportion of strains tested to be bioactive against Gram-positive bacteria. Antibacterial assays were conducted against MRSA, *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*. Antifungal assays were conducted against *C. albicans*. Anti-biofilm assay was conducted against *P. aeruginosa* PA01. Out of the strains, 13 displayed interesting bioactivities under the tested conditions. Only one of them belongs to the genus of *Micromonospora* and the rest were *Streptomyces*. *Streptomyces* sp. SW24, SD24 and SD50 exhibited strong activities against *S. aureus* and *B. subtilis* in cross streak antibacterial assay. Crude extracts of *Streptomyces* sp. P19 showed strong activities against clinical isolates of MRSA, *E. coli* and *P. aeruginosa*. *Streptomyces* sp. P9 is the only strain that was tested to be bioactive against bacterial and fungi. *Streptomyces* sp. P46 exhibited moderate bioactivity against tested bacterial strains including Gram-negative bacteria. *Micromonospora* sp. MD118 was the only *Micromonospora* strain that exhibited strong bioactivity against Gram-positive and Gram-negative bacteria with the largest zone of inhibitions against MRSA (38 mm), *K. pneumoniae* (10 mm) and *P. aeruginosa* (4 mm) in overlay assay. *Streptomyces* sp. MD100 exhibited the strong activity against MRSA (25 mm) and *E. coli* (23 mm) in overlay assay. *Streptomyces* sp. MD102 and P7 displayed strong activity against *C. albicans* with zone of inhibitions 21 mm and 20 mm respectively in overlay assay. These two strains only displayed selective activity against fungi *C. albicans*. *Streptomyces* sp. SD9, SD35 and SD48 exhibited strong anti-biofilm activity with more than 80% inhibition of biofilm formation. Each of the strains highlighted in the results were capable of producing bioactive compounds under the tested conditions. These strains were shortlisted for genome sequencing and the findings would be discussed in detail in the following chapter.

CHAPTER 4: Strain prioritization by genome sequencing and metabolite profiling

4.1 Introduction

The biosynthetic potential of actinomycetes encoded in the genome are usually not being expressed as compounds under laboratory conditions. This brings the need for genetic approaches to tap on this hidden potential⁷⁵. In early 2000s, only Sanger sequencing technique was available and it was costly and labour intensive to provide sufficient coverage for whole-genome shotgun. The advances in Next Generation DNA Sequencing (NGS), high-throughput and non-Sanger-based, in recent years has led to the significant reduction in cost of whole genome sequencing and also the improvements in the quality of data obtained⁷⁵. Draft genome sequence that consists of several hundred short contigs in the past, has been replaced by complete and accurate DNA sequence in a single contig⁷⁵. High quality whole genome sequences coupled with computing algorithms and databases for annotations of secondary metabolite gene clusters like antiSMASH, have provided researchers with valuable insights for identification of novel chemistry and enzymology involved^{159,160}. Genome mining of actinomycetes has successfully contributed to the discovery of novel natural products and biosynthetic pathways that would otherwise remained undetected^{161,162}. Hence, genome mining from actinomycetes in the post-genomics era is seen as the promising approach for discovery of novel natural products¹⁵⁹.

The first NGS technologies, referred to as second-generation sequencing (SGS), depends on cycles of the termination of DNA polymerisation and detection of the incorporated nucleotides per cycle. SGS technology consists of 454 pyrosequencing released in 2005, followed by Illumina and SOLid released in 2006 and Ion Torrent released in 2010^{163,164}. In year 2011, the realisation of whole genome was made possible by third-generation sequencing technology; Single Molecule Real Time (SMRT) referred to as PacBio¹⁶⁵. A fourth-generation sequencing technology, nanopore-based sequencers, is currently making its way into the market. It has the potential to sequence the entire human genome at the cost of less than \$1000 with quick and reliable data¹⁶⁶. However,

the application of nanopore-based sequencers to actinomycetes is still limited due to the high GC content⁷⁵.

From the bioactivity results, unique morphology or 16S identification as discussed in previous chapters, 36 strains have been shortlisted for genome sequencing. Out of the 36 genomes, 9 strains (*Streptomyces* sp. SD24, SD50, SW24, P7, SD9, P46, P9, P19 and MD100) appeared to have large numbers of biosynthetic gene clusters (≥ 30 BGCs) that are capable of producing more than one bioactive compounds per strain. MD118 was the only strain with full genome sequence by third-generation sequencing (TGS) technology, PacBio. According to antiSMASH analysis, MD118 contains novel BGCs with low homology against known BGCs.

Metabolite profiling of several strains were carried out to assess the relations between genetic potential and actual secondary metabolite expression. From strains we have studied, *Streptomyces* sp. P19 and SD50 displayed high potential for producing a wide variety of secondary metabolites when cultured under specific conditions. The peaks responsible for its bioactivity as discussed in Chapter 3 were also identified.

4.2 Materials and methods

4.2.1 Isolation of genomic DNA

Strains were cultured in 20 ml of CRM medium (10 g/L glucose, 103 g/L sucrose, 10.12 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 15 g/L tryptic soy broth and 5 g/L yeast extract) in 100 ml flask and incubated at 30 °C for 2 – 3 days (early stationary phase). Mycelium of strains were prepared as described previously¹⁵⁹. The culture was divided into 5 ml aliquots in 15 ml centrifuge tubes (Greiner, Singapore) and the mycelium were harvested by centrifugation at 4000 rpm for 5 min. The supernatant was discarded, and mycelium was washed with 5 ml of 20% glycerol. Aliquots that were not processed immediately were stored in 20% glycerol at -20°C. Washed mycelium was harvested by centrifugation at 4000 rpm for 5 min. High quality of genomic DNA were extracted using salting out method as described in Practical *Streptomyces* Genetics¹. Mycelium was resuspended in 5 ml of SET

buffer and homogenized using a dounce homogeniser (Bellco, Sigma Aldrich Singapore). 2 mg/ml lysozyme was added and incubated at 37 °C for 1.5 hours. 0.5 mg/ml proteinase K solution and 1% SDS were added and incubated at 55 °C for another 2 hours. 1.25 M of NaCl was added to the mixture and cooled to 37 °C before chloroform extraction. 5 ml of chloroform was added and mixed gently by inversion until the mixture was homogenous. The mixture was centrifuge at 6000 rpm for 5 min. Aqueous layer (top) was transferred to a new tube and chloroform extraction step was repeated until clear layer was obtained. 100 µg/ml of RNase A was added to the aqueous layer and incubated at 37 °C for 1.5 hours. 0.6 vol isopropanol was added and mixed by inversion to precipitate the genomic DNA. Genomic DNA was spooled out with a Pastuer pipette and washed with 70% ethanol. Genomic DNA was dried overnight in 2 ml centrifuge tube at room temperature. Tris buffer was added and shaken in cold room for a few days for dissolving genomic DNA. Quality of genomic DNA were analysed by nanodrop readings, qubit readings and visualization of agarose gel. 260/280 ratio should be within 1.8 – 2.0 and 260/230 ratio should be within 2.0 – 2.2 from nanodrop readings. The concentration of genomic DNA obtained from nanodrop and qubit readings should be within the range of ± 50 µg/µl. The integrity of genomic DNA (at least 300 ng) has to be determined on low percentage (0.6%) agarose gel at 100V for 2.5 hours with 1 kb DNA extension ladder. Genomic DNA should not show signs of degradation (smearing DNA bands).

4.2.2 Genome sequencing and assembly

The genome sequences of MD118 and MD100 were determined using Pacific Biosciences SMRT (PacBio) sequencing platform (University of Washington). Assembly of data for MD118 yielded 1 contig and a total of 6,761,541 bp. Assembly of data for MD100 yielded 4 contigs and a total of 8,943,018 bp. The genome sequences of SD24, SD50, SW24, P7, SD9, P46, P9 and P19 were determined using Illumina MiSeq sequencing platform. Assembly of Illumina MiSeq sequencing data were performed by Ding Yichen (Graduate Student from Asst Prof Yang Liang's group). Assembly of data for SD24 yielded 357 contigs and a total of 12,179,798 bp. Assembly of data for SD50 yielded 304 contigs and a total of 11,537,587 bp. Assembly of data for SW24 yielded 324 contigs and a

total of 9,314,730 bp. Assembly of data for P7 yielded 261 contigs and a total of 7,994,283 bp. Assembly of data for SD9 yielded 198 contigs and a total of 11,306,095 bp. Assembly of data for P46 yielded 73 contigs and a total of 9,735,389 bp. Assembly of data for P9 yielded 169 contigs and a total of 8,114,780 bp. Assembly of data for P19 yielded 155 contigs and a total of 7,353,541 bp.

4.2.3 Genome visualization of *Micromonospora* sp. MD118 with DNAPlotter

Coding sequences (CDS) annotations of MD118 was created by Rapid Annotation using Subsystem Technology (RAST) server¹⁶⁷. Secondary metabolite gene clusters were identified by antibiotics & Secondary Metabolite Analysis Shell (antiSMASH). antiSMASH aligned known biosynthetic gene clusters to its closest relative from databases¹⁶⁰. Genome information was then downloaded in Genbank format from RAST server. Gene clusters information obtained from antiSMASH was included using DNAPlotter¹⁶⁸.

4.2.4 Analytical HPLC of crude extracts

The following procedure was performed for P19 and SD50 crude extracts with Grace VisionHTTM C18-HL (4.6 mm x 250 mm, 5 μ m) using Agilent 1200 HPLC system equipped with DAD for UV detection. Mobile phase used: Buffer A consists of water + 0.1% formic acid and Buffer B consists of acetonitrile + 0.1% formic acid. HPLC gradient elution program was set with 10% B at 0 min, 20% B at 5 min, 70% B at 35 min, 90% B at 50 min, 100% B at 60 min. 20 μ L sample volume was injected. Analytes were monitored at λ = 220 nm, 260 nm, 284 nm, 314 nm, 330 nm, 360 nm, 400 nm and 420 nm.

4.2.5 Fermentation of *Streptomyces* sp. P19

P19 was inoculated into 5 x 100 mL flasks containing 50 mL of Pharmamedia (5 g glucose, 5 g cornsteep powder, 10 g oatmeal, 10 g cottonseed flour, 5 g K₂HPO₄, 5 g MgSO₄·7H₂O, and 1 mL trace metal solution in 1 L distilled water) and cultured for 4 days at 30 °C with shaking. 1% of the starter culture was used to inoculate into 4 x 2 L flasks containing 400 mL of Pharmamedia, 4 x 5 L flasks

containing 500 mL of Pharmamedia and 2 x 500 mL flasks containing 200 ml of Pharmamedia and 20 x 100 ml flasks containing 50 mL of Pharmamedia. The cultures were incubated at 30 °C with shaking for 10 days. The fermentation culture (5 L) was centrifuged for 6 min at 8500 rpm and the culture broth (supernatant) was collected. The culture broth was extracted with equal volume of ethyl acetate in a separatory funnel. The ethyl acetate solution (organic layer) was then concentrated under reduced pressure to a brown oil. The biomass was extracted with 500 mL of acetone.

4.2.6 Fermentation of *Streptomyces* sp. SD50

A tiny circle (1-2 mm diameter) of sporulated mycelia was picked from a well sporulated SD50 culture grown on ISP2 and spread evenly on a fresh ISP2 agar plates. 120 ISP2 agar plates were kept at 30 °C for 14 days. After incubation, SD50 ISP2 agar plates were extracted by mixing 70 mL of acetone per plate and blending using a handheld homogenizer. The cell debris and agar were pelleted by centrifuging at 8,000 rpm for 4 minutes. The acetone solution was then concentrated under reduced pressure to a dark brown oil.

4.3 Results

4.3.1 *Micromonospora* sp. MD118 is a high potential strain that contains many novel biosynthetic gene clusters (BGCs)

Pacific Biosciences SMRT (PacBio) sequencing revealed a genome size of 6,761,541 bp with GC content of 73.0% for MD118 as depicted in Figure 4.1. antiSMASH prediction revealed 16 secondary metabolite gene clusters, tabulated in Table S.10. The highest number of biosynthetic gene clusters (BGC) were found to be hybrid BGC (6), followed by terpene BGC (5), polyketide synthase (PKS) BGC (2), non-ribosomal peptide synthase (NRPS) BGC (2) and phosphonate BGC (1). From the antiSMASH prediction, only one BGC is 100% similar to a known cluster, sioxanthin. Sioxanthin is a carotenoid compound which gives characteristic orange pigment in *Micromonosporaceae* family. Sioxanthin BGC were commonly found in *Micromonospora*, *Salinispora*, *Verrucosipora* and some species belonging to *Actinoplanes*. Another BGC that has high percentage similarity is alkyl-O-dihydrogeranyl-

methoxyhydroquinones with 71% similarity. Alkyl-O-dihydrogeranyl-methoxyhydroquinones is biosynthesized by type III PKS. Apart from these two BGCs which have high similarities with known gene clusters, other predicted BGCs had low percentage similarities (<50%) to known cluster. This also meant that MD118 has high potential of producing novel compounds whereby the gene clusters have not been characterized yet.

According to 16S rDNA identification, MD118 has 100% similarities to *Micromonospora aurantiaca* ATCC 27029 and *Micromonospora* sp. L5. Their genome sizes and GC content were relatively similar as well. MD118 has slightly smaller genome 6,761,541 bp as compared to *M. aurantiaca* ATCC 27029 and *M. sp. L5* with 7,025,559 bp and 6,962,533 bp respectively. MD118 has slightly higher GC content of 73.0% GC as compared to *M. aurantiaca* ATCC 27029 and *M. sp. L5* with 72.8% GC. *M. aurantiaca* ATCC 27029 has one additional BGC as compared to MD118 and *M. sp. L5*.

Based on the antiSMASH predictions, MD118 contains the highest number of unique BGCs that were not found in *M. aurantiaca* ATCC 27029 and *M. sp. L5*. These results were tabulated in Table 4.1. 9 BGCs (cluster 2: alkyl-O-dihydrogeranyl-methoxyhydroquinones; cluster 5: sioxanthin; cluster 6: phosphonoglycans; cluster 9: bleomycin; cluster 10: nocathiacin; cluster 11: xantholipin; cluster 12: lobosamide; cluster 13: lobosamide and cluster 15: lymphostin) from MD118 were found to be similar to *M. aurantiaca* ATCC 27029 and *M. sp. L5*. 6 BGCs (cluster 3: gentamicin; cluster 4: dynemicin; cluster 7: macbecin; cluster 8: dynemicin; cluster 14: teicoplanin and cluster 16: apramycin) were found to be unique to MD118. 2 BGCs (cluster 3: landepoxcin and cluster 12: cosmomycin D) were found to be unique to *M. aurantiaca* ATCC 27029. 2 BGCs (cluster 3: herboxidiene and cluster 8: cinerubin B) were found to be unique to *M. sp. L5*. In addition, rifamycin, tiancimycin and azicemicin were found in both *M. aurantiaca* and *M. sp. L5* but were absent in MD118. Even though MD118 shares some similarities with *M. aurantiaca* and *M. sp. L5*, there are novelty in BGCs that are worth pursuing.

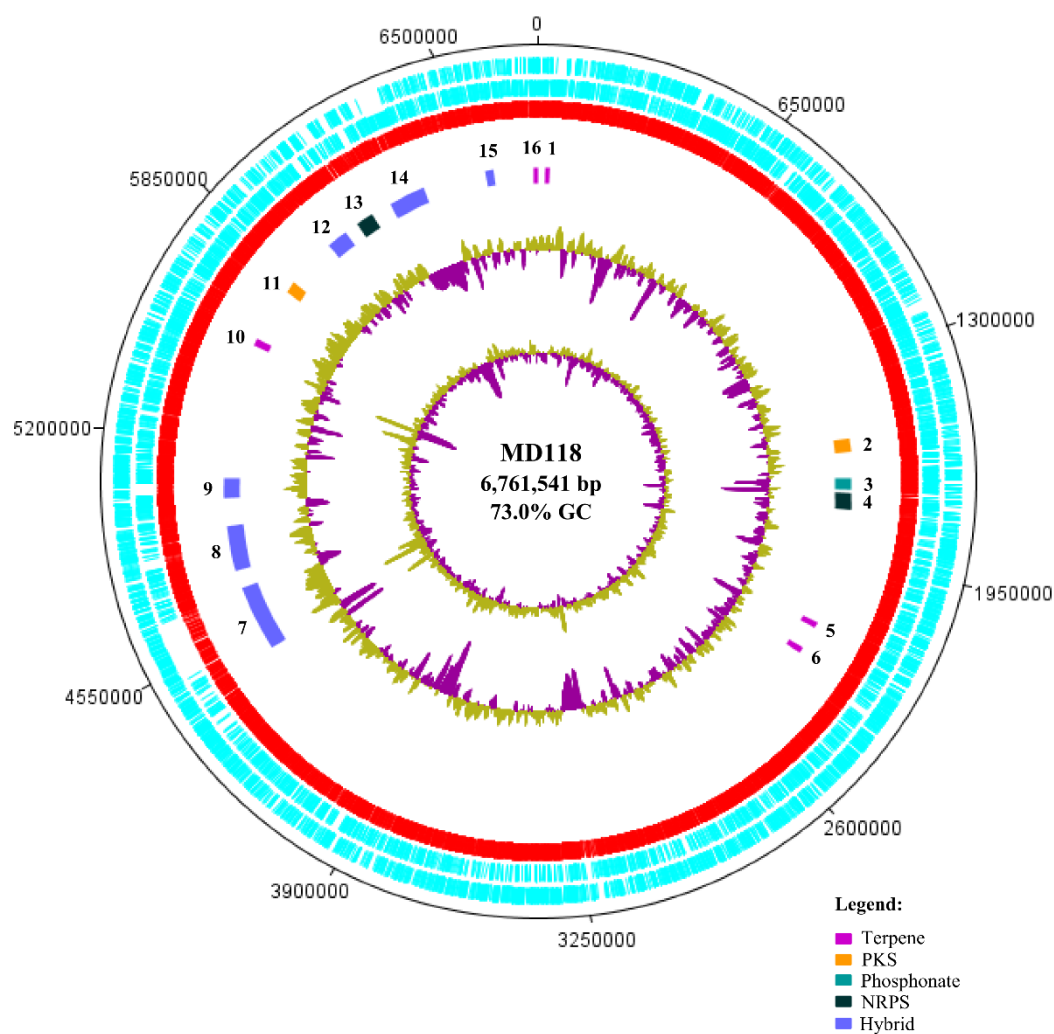


Figure 4.1 *Micromonospora* sp. MD118 circular genome with gene clusters annotations. The representation of tracks (starting from outer track): Forward Coding DNA Sequence; Reverse Coding DNA Sequence; Forward and Reverse Coding DNA Sequence; Secondary metabolite gene clusters annotations with reference to antiSMASH predictions; %GC plot; GC skew [(GC)/(G+C)].

Table 4.1 antiSMASH-predicted BGCs for *Micromonospora* sp. MD118 closest strains *Micromonospora aurantiaca* ATCC 27029 and *Micromonospora* sp. L5. BGCs unique to a particular strain is bold.

Cluster No.	Type descriptor / Most similar known cluster (% similarity)		
	MD118	<i>Micromonospora aurantiaca</i> ATCC 27029	<i>Micromonospora</i> sp. L5
1	Terpene / -	Terpene / Sioxanthin (100%)	Terpene / Sioxanthin (100%)
2	Type 3 PKS / Alkyl-O-dihydrogeranyl-methoxyhydroquinones (71%)	Terpene / Phosphonoglycans (3%)	Terpene / Phosphonglycans (3%)
3	Phosphonate / Gentamicin (7%)	NRPS / Landepoxcin (11%)	NRPS / Herboxidiene (2%)
4	NRPS / Dynemicin (13%)	Type 1 PKS-NRPS / Rifamycin (35%)	Type 3 PKS / Alkyl-O-dihydrogeranyl-methoxyhydroquinones (71%)
5	Terpene / Sioxanthin (100%)	Type 1 PKS / Tiancimycin (19%)	Terpene / -
6	Terpene / Phosphonoglycans (3%)	NRPS-Type 1 PKS-Other KS-Siderophore / Azicemicin (13%)	Bacteriocin-Terpene / Lymphostin (33%)
7	Type 1 PKS-NRPS / Macbecin (43%)	Type 1 PKS-NRPS / Bleomycin (12%)	Siderophore / -
8	NRPS-Siderophore-Type 1 PKS-Other KS / Dynemicin (23%)	Terpene / Nocathiacin (4%)	Type 2 PKS-Oligosaccharide / Cinerubin B (62%)
9	Type 1 PKS-NRPS / Bleomycin (12%)	Type 2 PKS / Xantholipin (16%)	Trans AT PKS-Other KS-NRPS / Lenamycin (15%)
10	Terpene / Nocathiacin (4%)	Oligosaccharide-Terpene-NRPS / Lobosamide (13%)	Oligosaccharide-Terpene-NRPS / Lobosamide (13%)
11	Type 2 PKS / Xantholipin (16%)	Trans AT PKS-NRPS-Other KS / Leinamycin (15%)	Type 2 PKS / Xantholipin (14%)
12	Oligosaccharide-Terpene-NRPS / Lobosamide (13%)	Type 2 PKS-Oligosaccharide / Cosmomycin D (55%)	Terpene / Nocathiacin (4%)
13	NRPS / Lobosamide (6%)	Lantipeptide / -	Type 1 PKS-NRPS / Bleomycin (12%)
14	Type 3 PKS-Other KS-NRPS / Teicoplanin (26%)	Bacteriocin-Terpene / Lymphostin (33%)	NRPS-Type 1 PKS-Other KS-Siderophore / Azicemicin (13%)
15	Bacteriocin-Terpene / Lymphostin (38%)	Terpene / -	Type 1 PKS / Tiancimycin (19%)
16	Terpene / Apramycin (6%)	Type 3 PKS / Alkyl-O-dihydrogeranyl-methoxyhydroquinones (71%)	Type 1 PKS-NRPS / Rifamycin (35%)
17	-	Lantipeptide / -	-

4.3.2 Other high-potential strains containing large number of gene clusters

According to antiSMASH predictions, the strains that we sequenced varies in the number of biosynthetic gene clusters. The number of gene clusters predicted ranged from 16 to more than 60 BGCs. Figure 4.2 shows 9 high-potential strains containing large number of BGCs. antiSMASH-predicted BGCs for *Streptomyces* sp. SD24, SD50, SW24, P7, SD9, P46, P9, P19 and MD100 were tabulated in Table S.11 - Table S.19 (**Appendix**). Strains containing large genomes, SD24 and SD50 with more than 10 Mbp, contains the most number of

BGCs. As both SD24 and SD50 are closely related strains, about 60% of the BGCs predicted were similar.

From Figure 4.2, there are significant proportion of BGCs attributed from PKS and NRPS gene clusters. PKS and NRPS are known to be the most important classes bioactive compounds produced by actinomycetes¹⁶⁹. Terpene gene clusters were also prevalent among the 9 strains. Terpenes and terpenoids are the most chemically diverse pool of antibacterial and anticancer compounds found in soil and marine actinomycetes^{170,171}. Ribosomally synthesized and post-translationally modified peptides (RiPPs) were also found in the 8 strains except for *Streptomyces* sp. SW24. Both lantipeptide and lasso peptide are classified under RiPPs. Lantipeptides usually have characteristic thioether cross-links formed between Ser/Thr and Cys residues. Lanthionine (from Ser) and Methyl-lanthionine (From Thr) are the post-translational modifications that results in thioether structures¹⁷². Lasso peptides usually forms specific interlocked topology and is stabilized by steric interactions¹⁷³. They consist of a macrolactam ring with 7 to 9 residues and a linear C terminal peptide tail¹⁷³. Lasso peptides also displayed unique bioactivities that targets bacterial and cancer cell lines¹⁷³.

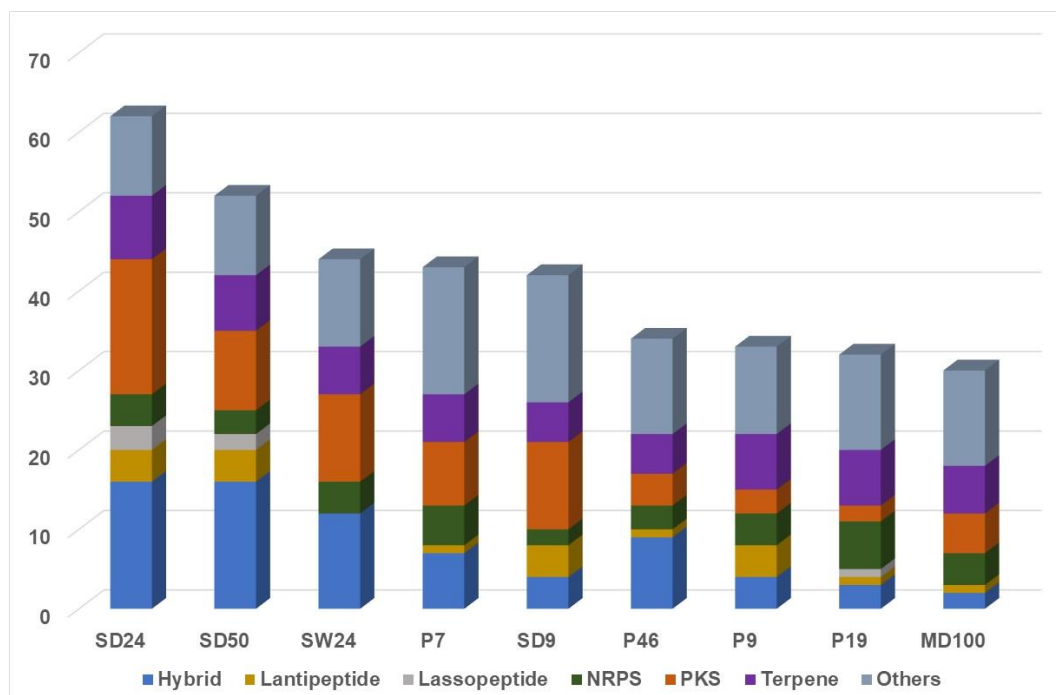


Figure 4.2 Nine high-potential strains containing large number of gene clusters (≥ 30 BGCs).

Draft genome information from the prioritized strains have revealed a lot of BGCs that has the potential to produce bioactive compounds. The larger the number of BGCs present in the genome, the higher the potential for a given strain to produce more diverse secondary metabolites. Therefore, strains with large numbers of BGCs were prioritized for metabolite profiling to increase the chances of finding novel compounds. As the strains isolated are highly similar to known strains, these strains do contain BGCs that are already well characterised and studied. Draft genome information for our case, can act as a dereplication tool to avoid working on highly similar known strains that are already producing a lot of known compounds.

4.3.3 Metabolite profiling of *Streptomyces* sp. P19 and SD50

Genome information can be valuable source of information and a gauge of how many secondary metabolites a strain is capable of producing. However, much of the genetic capacity is ‘cryptic’ as natural products encoded by them are not being produced under laboratory conditions¹⁷⁰. This gap between genetic potential and actual expression of natural products posed a barrier in natural product discovery. This is also evident in our observations. We have tried culturing several strains in different media and culturing conditions for studying of various metabolite profile. Many of the strains does not seem to produce significant metabolites that correspond to its genetic potential. Out of the strains we have studied, *Streptomyces* sp. P19 and SD50 displayed the potential for producing a wide variety of secondary metabolites when cultured under specific conditions.

As discussed in Chapter 3 (**Section 3.3.2**), P19 only produces compounds with strong bioactivities against Gram-positive and Gram-negative bacteria in pharmamedia. Figure 4.3(A) shows the HPLC profile of pharmamedia. Organic compounds from plant extracts (cornsteep powder, oatmeal and cottonseed flour) were extracted from pharmamedia in varying quantities. Figure 4.3(B) shows the HPLC profile of P19 (cultured in pharmamedia). Carefully analysis of HPLC profile against pharmamedia and crude extracts was necessary to ensure that the bioactive compounds were indeed produced by the actinomycete strain. The peak that corresponds to retention time of 44 min in Figure 4.3(B) was tested to be

bioactive against Gram-positive and Gram-negative bacteria and it was produced in high abundance. This peak was absent in the media shown in Figure 4.3(A).

As discussed in Chapter 3 (**Section 3.3.1**), SD50 produces compounds with strong bioactivities against Gram-positive bacteria in ISP2 agar. Figure 4.4(A) shows the HPLC profile of ISP2 agar. The profile looks neat with several peaks eluting out before 10 min. Figure 4.4(B) shows HPLC profile of SD50 (cultured on ISP2 agar). SD50 produces several non-polar compounds that eluted after 40 min. The peak with retention time 41.2 min was tested to be bioactive against Gram positive bacteria and fungus shown in Figure 4.4(B). The peak was absent in the media as shown in Figure 4.4(A).

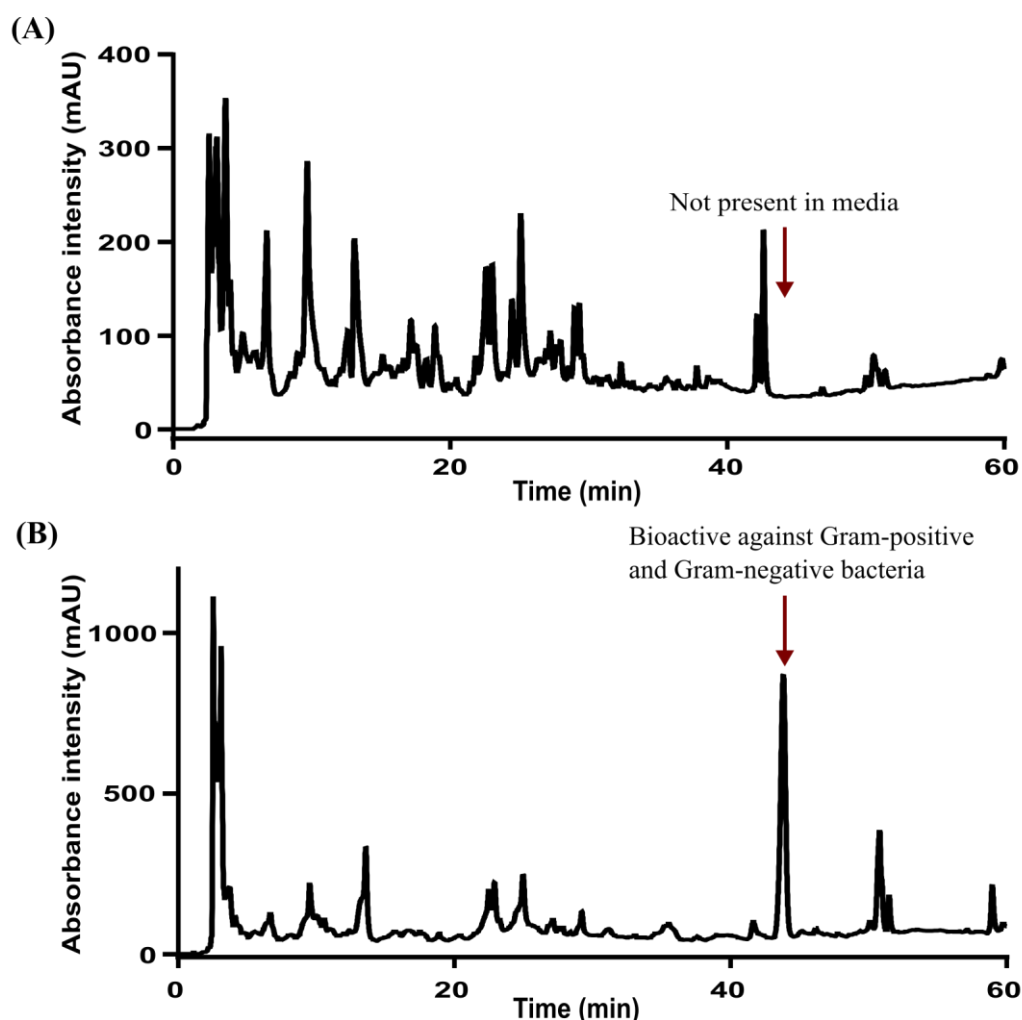


Figure 4.3 HPLC profile of the (A) Negative control (pharmamedia culture medium) and (B) Crude extract of P19 (Wavelength: 220 nm). The peak at 44 min was tested to be bioactive against Gram-positive and Gram-negative bacteria.

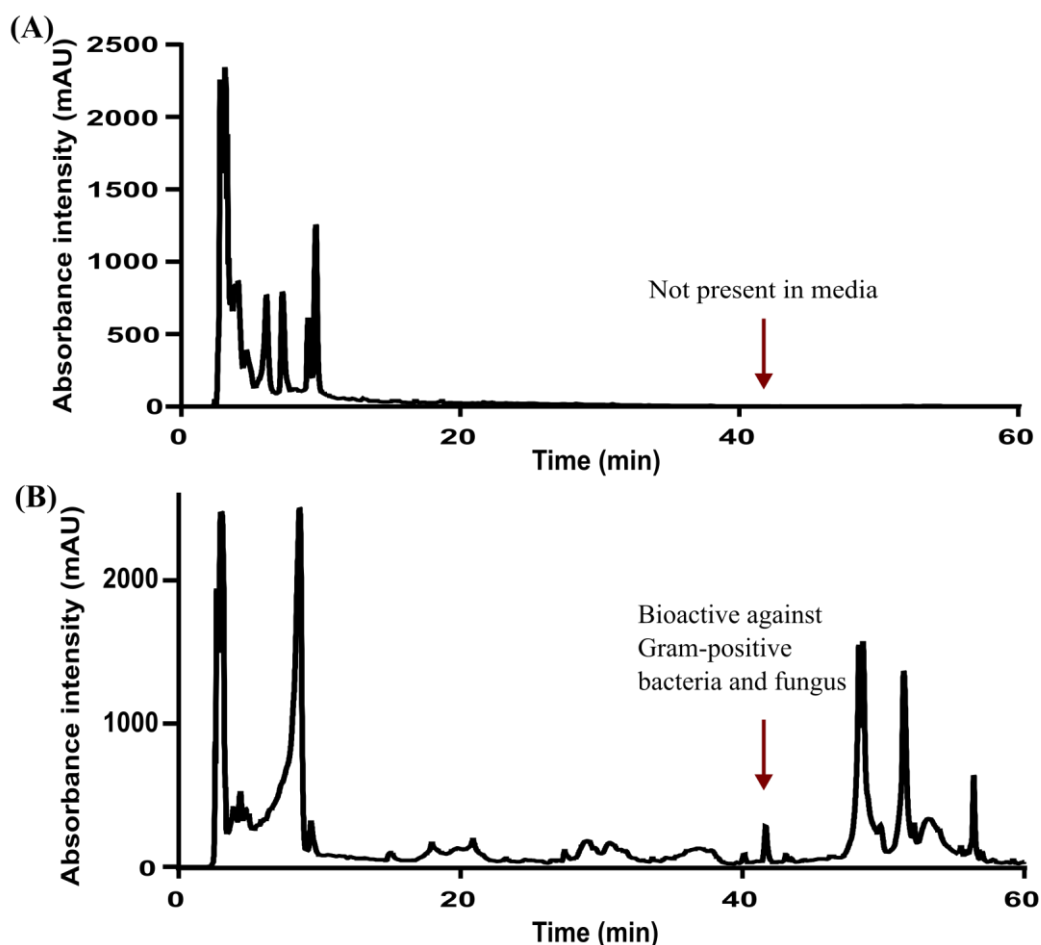


Figure 4.4 HPLC profile of the (A) Negative control (ISP2 agar plate) and (B) Crude extract of SD50 (Wavelength: 284 nm). The peak at 41.2 min was tested to be bioactive against Gram-positive bacteria and fungus.

4.4 Discussion

Development of genome sequencing technologies has revolutionized natural product research¹⁷⁴. Actinomycetes with large genomes (>8 Mbp) is one of the microorganisms that encodes the largest numbers of secondary metabolites. Detailed analysis of biosynthetic gene clusters from genome mining would reveal the potential for uncovering novel natural products. The only complete genome obtained from this study was *Micromonospora* sp. MD118, which is closely related to *Micromonospora aurantiaca* ATCC 27029 and *Micromonospora* sp. L5. Although the percentage similarities based on 16S rDNA identification was 100%, a close comparison of gene clusters with closest strains revealed slight differences in terms of biosynthetic gene clusters. Interestingly, MD118 contains dynemicin, macbecin and teicoplanin which were absent in *Micromonospora*

aurantiaca ATCC 27029 and *Micromonospora* sp. L5. In addition, the homology of known biosynthetic gene clusters are low. This also meant that there are many potential novel clusters to be uncovered from MD118.

Lateral gene transfer (LGT) (also known as horizontal gene transfer) is widely known to have an important role in the evolution of bacterial genomes^{175,176}. Bacteria, unlike eukaryotes, has the ability to acquire genes distantly related organisms. LGT can also significantly change the ecological and pathogenic character of the bacterial strains¹⁷⁶. Similar strains isolated from different environment can contain different sets of BGCs due to the need for LGT of housingkeeping genes¹⁷⁷. This provides an effective strategy for bacteria to exploit new resources that are essential for its survival¹⁷⁸. This could be used to explain the differences in BGCs predicted in MD118 and its closely related strains.

Genome information can also reveal the genetic capacity of the actinomycete strains. From our strain collection, many strains belonging to the genus of *Streptomyces* contains large numbers of gene clusters. Genome approach provided data for identification of known gene clusters. It can be used as a dereplication tool to avoid isolating known compounds from the particular strain. It also revealed several gene clusters that were uncharacterized. These uncharacterized BGCs have huge potential of uncovering novel compounds. However, many genetic capacity is known to be ‘cryptic’ and the compounds encoded by the BGCs are not being expressed under laboratory conditions¹⁷⁰. This gap between genetic potential and secondary metabolites production is also evident in our observations. In view of this limitations, synthetic biology approach has been established for several research groups to expand the chemical diversity of natural products¹⁷⁹. Advances in technology coupled with improvements in bioinformatic tools will eventually help to close this gap and allow a more systematic approach for natural product discovery¹⁷⁹. Although engineering natural product biosynthesis contributed has to natural product discovery, this approach is still difficult due to several reasons¹⁸⁰. Transcription factors, translation factors, protein-protein interactions, self-resistance, cofactor and precursor availability have to be accounted for in the production strain¹⁷⁹.

Organisms that contain the BGCs are might also be difficult to manipulate or cultivate^{181,182}.

Instead of synthetic biology approach, I have adopted OSMAC approach by optimizing culturing conditions and eliciting various secondary metabolites from the prioritized actinomycete strains. Though this approach was adopted due to its simplicity and feasibility within the time frame, it also has some limitations. By focusing on cultivation of strains under different conditions, it is considered a pleiotropic approach that is unlikely to activate all the compounds that are present in the BGCs.

Addition of chemical elicitors such as *N*-acetylglucosamine and sodium butyrate did not help in elicitation of novel compounds from the tested strains. From my experimental observations, it only increased the production of existing peaks found in the HPLC chromatogram. On the other hand, alteration of media components contributed to significant differences in the secondary metabolite profiles of the same strain. Metabolite profiling of several strains were carried out to assess the relations between genetic potential and actual secondary metabolite expression. Many of the strains does not seem to produce significant metabolites that correspond to its genetic potential. From strains we have studied, *Streptomyces* sp. P19 and SD50 displayed high potential for producing a wide variety of secondary metabolites when cultured under specific conditions.

Complete dereplication of secondary metabolite profiling of strains and secondary metabolite BGCs analysis have uncovered several known and new compounds associated with the corresponding gene clusters and prediction of biosynthetic pathways¹⁸³. Due to time constraints, we were unable to do a complete dereplication of secondary metabolite profiling to aid in the discovery of novel compounds. Only the peaks that were responsible for its bioactivity were narrowed down and isolated for further identification and characterisation.

4.5 Conclusion

Post-genomics era brought a lot of benefits especially in natural product research. The reduction in cost of genome sequencing has significantly increased the amount of data for genome mining and discovery of novel biosynthetic pathway. *Micromonospora* sp. MD118 whole genome revealed the potential of uncovering novel compounds as homology to known biosynthetic gene clusters were low. From the strains we have sequenced so far, 9 strains contain 30 or more biosynthetic gene clusters. The data from these strains would provide basis of the project for isolation of novel compounds biosynthesized by uncharacterized biosynthetic gene clusters. Metabolite profiling of *Streptomyces* sp. P19 and SD50 also revealed the potential of producing several compounds in a given condition. The large number of BGCs predicted from antiSMASH also highlighted the genetic capacity to produce a wide variety of secondary metabolites. The bioactive compound isolated from these two strains were identified and characterized in the following chapter.

CHAPTER 5: Isolation and identification of bioactive compounds from two prioritized strains

5.1 Introduction

Compound characterisation was done for the bioactive compounds isolated from *Streptomyces* sp. P19 and SD50 to assess its novelty. Minimum inhibitory concentration (MIC) against test organisms MRSA, *E. coli*, *P. aeruginosa* and *C. albicans* were carried out with the two compounds isolated from P19 and SD50. Compound isolated from P19 resembles the known compound echinomycin produced by *Streptomyces echinatus* and *Streptomyces lasaliensis*¹⁸⁴. Echinomycin is an antibiotic that binds to DNA via intercalation of both quinoxaline rings to base pairs^{185,186}. Interestingly, echinomycin is perfectly symmetrical in structure. Both quinoxaline rings allows double-intercalation and unwinding of DNA helix structure to occur efficiently¹⁸⁶. It was known to be bioactive against Gram-positive, Gram-negative bacteria and some viruses¹⁸⁴. Novel 4',5-dihydroxy-7-methoxy-3-methylflavanone isolated from SD50 is an analogue of a known compound (4',5,7-trihydroxy-3-methylfavone) previously isolated from *Streptomyces graminofaciens* BA14348 and *Streptomyces* sp. No. 7528^{187,188}. 4',5,7-trihydroxy-3-methylfavone has been reported to be a potent estrogen receptor that has agonistic activity against MCF-7, estrogen dependent cell line^{187,188}. It also has low toxicity against MCF-7 and HeLa cells¹⁸⁷. 4',5-dihydroxy-7-methoxy-3-methylflavanone has an extra methyl group as compared to 4',5,7-trihydroxy-3-methylfavone. Due to the high resemblance in chemical structure, we investigated the estrogenic properties of 4',5-dihydroxy-7-methoxy-3-methylflavanone using Estrogen Response Element ((ERE)₃-luciferase) reporter assay.

5.2 Materials and methods

5.2.1 Isolation of 4',5-dihydroxy-7-methoxy-3-methylflavanone from *Streptomyces* sp. SD50

Isolation of compound was kindly assisted by Low Zhen Jie (Graduate Student from Assoc Prof Liang's group). The dried extracts were dissolved in optima grade methanol (Fisher Scientific, Singapore) and diluted to 60 – 70% methanol

for separation via semi-preparative reversed phase HPLC using Shimadzu Prominence Preparative HPLC system. Semi-preparative HPLC was conducted using ACE C18-HL (5 μ m, 10 x 250 mm) column for fractionation. Crude extract was resuspended in 40% Methanol. A gradient solvent system of water + 0.1% formic acid: acetonitrile + 0.1% formic acid (60:40), flow rate was 4.7 mL/min and binary conditions with starting mobile phase of acetonitrile + 0.1% formic acid 40% were used for compound isolation. Manual injector sampler was washed with 20 mL of 40% methanol. For each HPLC run, 1.5 mL of crude extract was injected manually. 4',5-dihydroxy-7-methoxy-3-methylflavanone was eluted at 33.5 minutes. Fractions were pooled together and evaporated to dryness. Fractions were stored by first redissolving in 3 mL methanol, 40 μ L was collected into a glass insert for analysis via analytical HPLC. The remaining volume was transferred to a pre-weighed labelled glass vial to be dried using Genevac at low boiling point conditions. Mass of compound were recorded and stored at -20 $^{\circ}$ C freezer.

5.2.2 Isolation of Echinomycin from *Streptomyces* sp. P19 culture

Isolation of compound was kindly assisted by Low Zhen Jie (Graduate Student from Assoc Prof Liang's group). The acetone solution was then concentrated under reduced pressure to a dark brown oil. The dried extracts were dissolved in optima grade methanol (Fisher Scientific, Singapore). Semi-preparative HPLC was conducted using Agilent eclipse xdb-C18 (9.4 x 250 mm, 5 μ m) column for fractionation. P19 crude extract was resuspended in 100% Methanol. A gradient solvent system of water: methanol (60:40), flow rate was 4.7 mL/min and binary conditions with starting mobile phase of 40% Methanol were used for compound isolation. Manual injector sampler was washed with 20 mL of 40% methanol prior to the run. For each HPLC run, 1.5 mL of crude extract was injected manually. Echinomycin was eluted between 27.5 to 32.5 minutes. Fractions were pooled together and evaporated to dryness. Fractions were stored by first redissolving in 3 mL methanol, 40 μ L was collected into a glass insert for analysis via analytical HPLC. The remaining volume was transferred to a pre-weighed labelled glass vial to be dried using Genevac at low boiling point conditions. Mass of compound were recorded and stored at -20 $^{\circ}$ C freezer.

5.2.3 Determination of the structure of the compounds

Mass Spectrometry of compounds from SD50 and P19 were kindly conducted by Dr. Yoganathan S/O Kanagasundaram of Bioinformatics Institute (A*STAR). Compounds were analyzed using Agilent 6500 Q-TOF Liquid Chromatography/Mass Spectrometer (LC/MS) system bearing with electrospray ionization (ESI) source coupled to Agilent 6200 Series TOF. A ZORBAX C18 column was selected for separation. Analyses were performed in ESI positive ion mode. Fragmentor Voltage used was 110V and Collision Energy value was 0.

NMR of echinomycin (compound from P19) was kindly conducted by Gary Ding (Graduate Student, NIE). ^1H and ^{13}C NMR of echinomycin (compound from P19) were recorded using Bruker Avance at 400 MHz. Chemical shifts were expressed as δ (ppm) in relative to chloroform-d (7.24 ppm) and coupling constants (J) were reported in hertz (Hz).

Structure elucidation of 4',5-dihydroxy-7-methoxy-3-methylflavanone (compound from SD50) was kindly conducted by Dr. Yoganathan S/O Kanagasundaram of Bioinformatics Institute (A*STAR). NMR spectrum were kindly provided by Low Zhen Jie (Graduate Student from Assoc. Prof Liang's group) and Ye Hong (Post Doc from Prof Yoon's group). ^1H NMR, ^{13}C NMR, HSQC, HMBC and COSY spectra of 4',5-dihydroxy-7-methoxy-3-methylflavanone were recorded using Bruker Avance at 400 MHz. Chemical shifts were expressed as δ (ppm) in relative to methanol- d_4 ($\delta_{\text{H}} = 3.31$ and 4.78 ppm, $\delta_{\text{C}} = 49.15$ ppm) and coupling constants (J) were reported in hertz (Hz).

5.2.4 Determination of colony forming unit (CFU) for the test organisms

Determination of CFU was carried out to find the correlation between OD₆₀₀ test organisms, MRSA, *E. coli*, *P. aeruginosa* and *C. albicans*, and the microbial number prior to MIC determination¹⁸⁹.

For MRSA, *E. coli* and *P. aeruginosa*:

Fresh colony was picked from the agar plate and inoculated into 5 mL of Muller Hinton Broth (MHB). The liquid cultures were incubated at 37 °C overnight with shaking. 1% of the overnight cultures were subcultured into 5 mL of fresh MHB

and incubated for another 2 – 4 hours until the log phase was achieved. OD₆₀₀ (values <1.0) were measured using UV spectrometer.

For *C. albicans*:

Fresh colony was picked from the agar plate and inoculated into 5 mL of RPMI 1640. The liquid culture was incubated at 30 °C overnight with shaking. 1% of the overnight culture was subcultured into 5 mL of fresh RPMI 1640 media and incubated for another 4 hours until the log phase was achieved. The cells was centrifuged at 1,500 g and washed with fresh RPMI 1640 media twice. Cell pellet was reconstituted in 1 mL of RPMI 1640 and transferred to a 1.5 mL sterile Eppendorf tube. 1/10 of the volume was diluted with water before OD₆₀₀ (values <1.0) were measured using UV spectrometer.

Dilute the test organisms cultured as mentioned with sterile water from 10⁻¹ to 10⁻⁷. 100 µL of each test organisms with different dilutions were spread evenly on MHB agar (for MRSA, *E. coli* and *P. aeruginosa*) and 50% RPMI 1640 agar (for *C. albicans*). Plates were incubated overnight at 37 °C (for MRSA, *E. coli* and *P. aeruginosa*) and 30 °C (for *C. albicans*). After the incubation, colonies were calculated and the CFU was calculated based on the equation: $N = \frac{C \times 10}{10^{-D}}$

Where:

N = cfu/mL

C = number of colonies per plate

D = number of the 1:10 dilution

The relationship between OD₆₀₀ and the microbial number was established based on calculating the plates displaying numbers of colonies with a range between 100 to 400. Average of 3 reproducible results were used for the estimate of 5 x 10⁵ CFU/mL required in determination of MIC.

5.2.5 Determination of minimal inhibitory concentration (MIC)

Broth micro dilution method was carried out in 96 well microtiter plate to determine the MIC of pure compound¹⁸⁹. Concentrations of pure compound was prepared by serial dilution starting from 128 µg/mL to 0.06 µg/mL in a 96 well polypropylene plate (Agilent, Singapore). Test organisms were prepared the same way as mentioned in Section 4.2.5. Final desired inoculum of test organisms

per well was 5×10^5 cfu/mL. 50 μ L of pure compound dissolved in 2% DMSO were added to 50 μ L of cells per well. Growth control containing broth with bacterial inoculum and no compound was included to check for viability of the cells. Sterility control containing broth only was included to check for possible contaminations. Microtiter plate containing test organisms MRSA, *E. coli* and *P. aeruginosa* were incubated at 37 °C static incubator for 16 hours. Microtiter plate containing test organism *C. albicans* was incubated at 30 °C incubator with shaking for 20 hours. The microtiter plates were observed by naked eye and microplate reader for the lowest concentration of the compound that inhibits visible growth of the tested organisms.

5.2.6 Estrogen Response Element (ERE) Response in MCF-7

Experiment was kindly carried out by Dr Amanda Woo (Post Doc from Assoc Prof Valerie Lin's group). 2×10^5 MCF-7 cells were seeded into 35 mm dishes, in phenol-red free DMEM (Nacalai Tesque, Japan) supplemented with 5% DCC-FCS (Dextran-coated charcoal treated fetal calf serum) and 2 mM L-glutamine (Hyclone), were transfected with 500ng ERE-Luc reporter plasmids using Polyethyleneimine (PEI) transfection reagent (Polysciences, Warrington, PA, USA). The cells were then treated with 10 nM 17 β -estradiol (E2) in 0.01% Ethanol, in the presence or absence of 4',5-dihydroxy-7-methoxy-3-methylflavanone (1 μ M or 10 μ M) in 0.01% DMSO. The lysate from the cells were harvested using 1X Reporter Lysis Buffer provided by Luciferase Reporter System (Promega, Madison, WI, USA) and analysed using a microplate luminometer, GloMax [®]-Multi+ Microplate Multimode Reader with Instinct[®] (Promega). The activities of ERE were measured as per manufacturer's protocol and normalised against each sample's protein concentration. Protein concentrations of the lysate were obtained using BCA[™] Protein Assay Kit (Pierce, Rockford, IL), as per manufacturer's protocol.

5.3 Results

5.3.1 Bioactive compound echinomycin from *Streptomyces* sp. P19

Characterisation of compound isolated from *Streptomyces* sp. P19 (99.45% similarities to *Streptomyces tendae* ATCC 19812) was carried out with Electrospray Ionization High-Resolution Mass Spectrometry (ESI-HRMS) and Nuclear Magnetic Resonance (NMR). The bioactive compound extracted from P19 culture broth and biomass was found to be a known compound echinomycin, shown in Figure 5.1(A). The molecular formula of echinomycin was $C_{51}H_{65}N_{12}O_{12}S_2$ confirmed by ^1H and ^{13}C NMR spectra and ESI-HRMS (observed $[M+H]^+$ at m/z : 1101.4300; calculated: 1100.42). The HRMS spectrum can be found in Figure S.1 (**Appendix**). The ^1H and ^{13}C NMR spectra found in Figure S.2 and Figure S.3 (**Appendix**) resembled those that has been reported^{185,190}. Figure 5.1(B) shows the analytical HPLC chromatogram of echinomycin at wavelength of 220 nm and the retention time of the dominant peak was 44.0 minutes. According to literature¹⁸⁴, echinomycin has λ_{max} of 242 and 322 nm and these values were similar to compound from P19 shown in Figure 5.1(C).

Minimum inhibitory concentration (MIC) of echinomycin was carried out on 4 clinical isolates (MRSA, *E. coli*, *P. aeruginosa* and *C. albicans*) and the results are tabulated in Table 5.1. The strong bioactivity observed in P19 crude extract was attributed by echinomycin which has strong activities against both Gram-positive bacteria MRSA (MIC = 0.5 $\mu g/mL$) and Gram-negative bacteria *E. coli* (MIC = 4 $\mu g/mL$) and *P. aeruginosa* (MIC = 32 $\mu g/mL$). Echinomycin does not exhibit bioactivity against *C. albicans*.

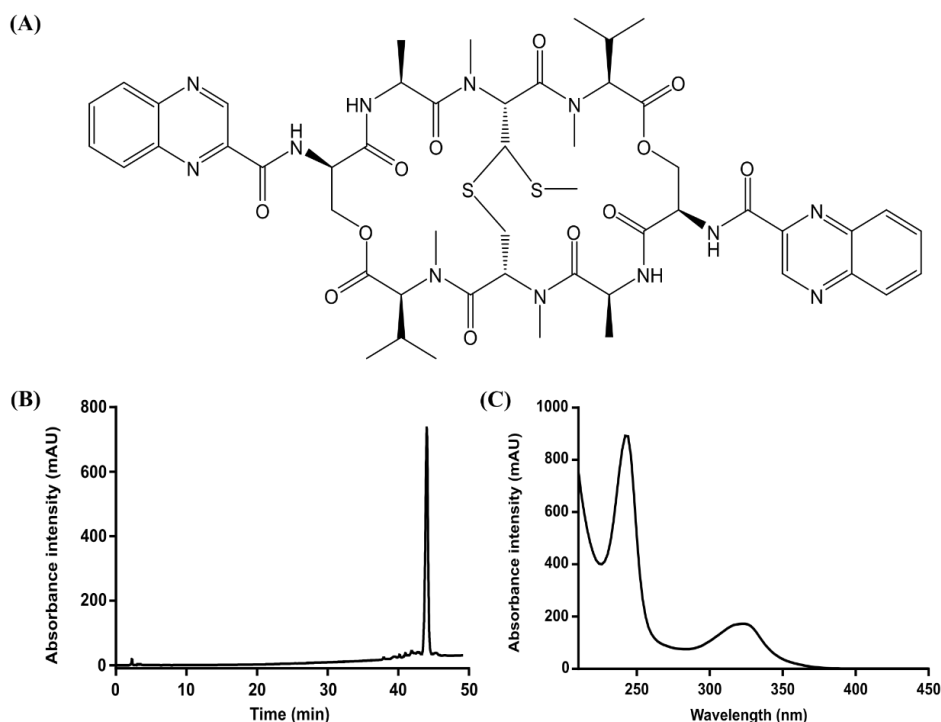


Figure 5.1 (A) NRPS derived echinomycin was extracted from *Streptomyces* sp. P19 culture broth and biomass. (B) HPLC chromatogram of echinomycin at 220 nm and (C) UV spectrum of echinomycin $\lambda_{\text{max}} = 242 \text{ nm}$ and 324 nm .

Table 5.1 Minimum inhibitory concentration (MIC) against 4 clinical isolates.

Test organism	Minimum inhibitory concentration (MIC) ($\mu\text{g/mL}$)
MRSA	0.5
<i>E. coli</i>	4
<i>P. aeruginosa</i>	32
<i>C. albicans</i>	>128

In addition, antiSMASH prediction of P19 genome shown in Table S.18, revealed a NRPS echinomycin BGC with high percentage similarities (94%). This further concludes that P19 can produce echinomycin under certain culturing conditions.

5.3.2 Novel bioactive compound from *Streptomyces* sp. SD50

Characterisation of 4',5-dihydroxy-7-methoxy-3-methylflavanone isolated from *Streptomyces* sp. SD50 (99.72% similarities to *Streptomyces bingchenggensis* BCW-1) was established using Electrospray Ionization High-Resolution Mass Spectrometry (ESI-HRMS) and Nuclear Magnetic Resonance (NMR) found in Figure S.5 - Figure S.9 (**Appendix**). Assignment of protons and carbons were tabulated in Table 5.2. 4',5-dihydroxy-7-methoxy-3-methylflavanone, shown in

Figure 5.2(A), was found to be an analogue of a known compound (4',5,7-trihydroxy-3-methylflavanone)^{187,188}. The molecular formula was C₁₇H₁₆O₅ confirmed by ¹H and ¹³C NMR spectra and ESI-HRMS (observed [M+H]⁺ at m/z: 301.1081; calculated: 300.10). Figure 5.2(B) shows the analytical HPLC chromatogram of 4',5-dihydroxy-7-methoxy-3-methylflavanone at wavelength of 284 nm and the retention time of the peak was 26 minutes. 4',5-dihydroxy-7-methoxy-3-methylflavanone has λ_{max} of 218, 288 and 332 (sh) nm shown in Figure 5.2(C).

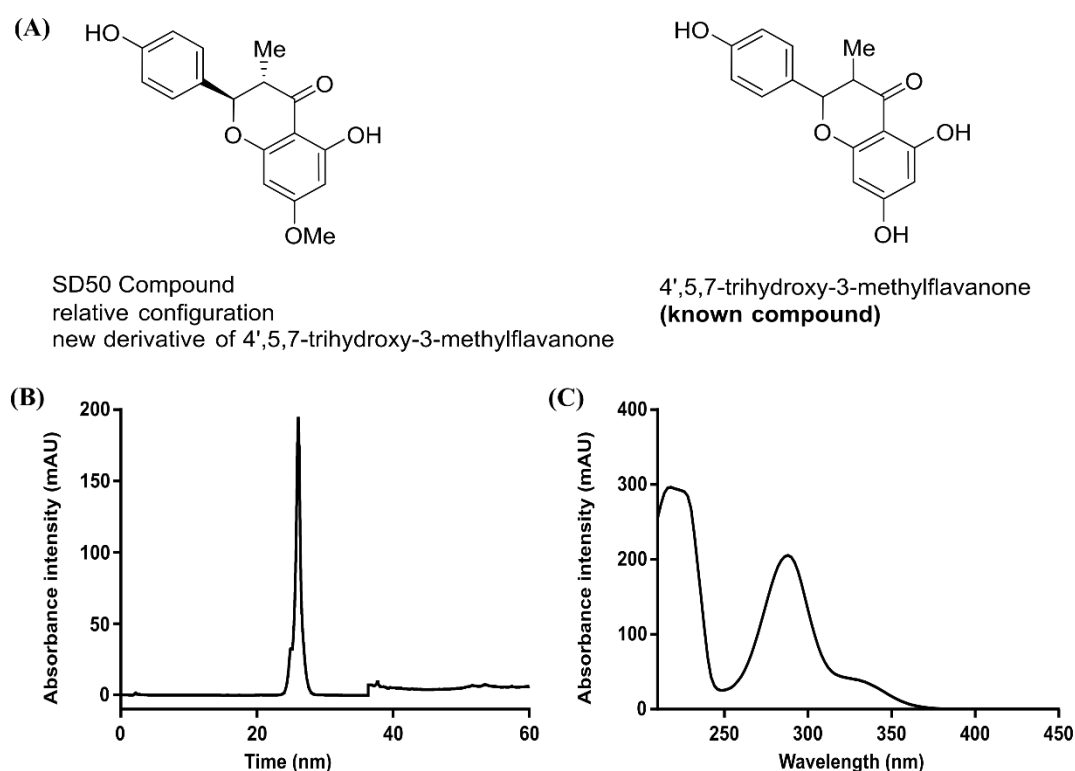
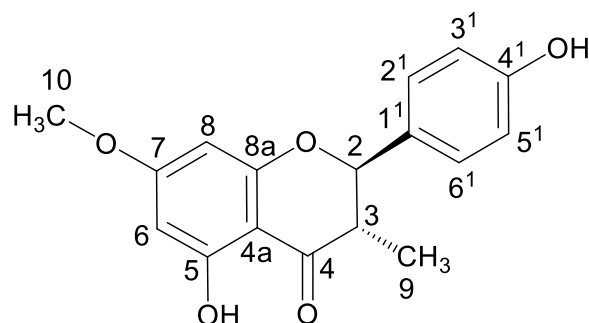


Figure 5.2 (A) 4',5-dihydroxy-7-methoxy-3-methylflavanone, new analogue of 4',5,7-trihydroxy-3-methylflavone (known compound) with a difference of a methyl group, was extracted from well sporulated *Streptomyces* sp. SD50 ISP2 agar plates. (B) HPLC chromatogram of 4',5-dihydroxy-7-methoxy-3-methylflavanone at 284 nm and (C) UV spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone at λ_{max} = 218 nm, 288 nm and 332 (sh) nm.

Table 5.2 CD₃OD-*d*₄ NMR data for 4',5-dihydroxy-7-methoxy-3-methylflavanone.



No.	δ_H	δ_C
2	4.91 d (3.0)	82.3
3	3.02 dq (3.3, 7.0)	46.2
4	-	200.4
4a	-	103.3
5	-	165.1
6	6.00 d (2.0)	95.7
7	-	169.2
8	5.95 d (2.0)	94.6
8a	-	164.4
9	0.93 d (6.8)	10.3
10	3.34 d (8.0) ^a	56.2
C1'	-	130.2
C2', C6'	7.27 d (8.3)	129.1
C3', C5'	6.82 d (8.3)	116.3
C4'	-	159.2

^a From HBM C correlation

Minimum inhibitory concentration (MIC) of 4',5-dihydroxy-7-methoxy-3-methylflavanone was carried out on 4 clinical isolates (MRSA, *E. coli*, *P. aeruginosa* and *C. albicans*) and the results are tabulated in Table 5.3. Interestingly, it has bioactivity toward Gram-positive bacteria MRSA (MIC = 28 – 30 μ g/mL) and fungus *C. albicans* (MIC = 26 – 28 μ g/mL). It is rare for a compound to contain features that target both prokaryotic and eukaryotic cells. However, it does not exhibit bioactivity against Gram-negative bacteria *E. coli* and *P. aeruginosa*.

Table 5.3 Minimum inhibitory concentration (MIC) against 4 clinical isolates.

Test organism	Minimum inhibitory concentration (MIC) (μ g/mL)
MRSA	28 – 30
<i>E. coli</i>	>128
<i>P. aeruginosa</i>	>128
<i>C. albicans</i>	26 – 28

4',5-dihydroxy-7-methoxy-3-methylflavanone was identified to have similar chemical structure as 4',5,7-trihydroxy-3-methylflavanone (also known as WS-7528 or BE-14348B) which has specific estrogenic properties^{187,188}. When 4',5,7-trihydroxy-3-methylflavanone was included to the growth medium containing estrogen-responsive human breast cancer MCF-7 cells, it was able to induce the cell growth¹⁸⁸. Therefore, it is plausible that 4',5-dihydroxy-7-methoxy-3-methylflavanone could possess either estrogenic or anti-estrogenic effect given the high resemblance in chemical structure. Estrogenic property of 4',5-dihydroxy-7-methoxy-3-methylflavanone was investigated using an Estrogen Response Element ((ERE)₃-luciferase) reporter assay using MCF-7 cells. MCF-7 cells were treated with natural estrogen 17 β -estradiol (E2), 4',5-dihydroxy-7-methoxy-3-methylflavanone under two different concentrations (1 μ M or 10 μ M), or both. The results were shown in Figure 5.3. It had enhanced the transcriptional activity of ER in MCF-7 to similar degree as E2 under both concentrations. However, to achieve similar transactivity as E2, the compound concentration used was 100 – 1000 times higher than E2. Furthermore, the combined treatment of both the compound and E2 did not further enhance the transcriptional activity of ER in MCF-7 cells. This suggests that the estrogenic effect of the compound might not be more potent than E2.

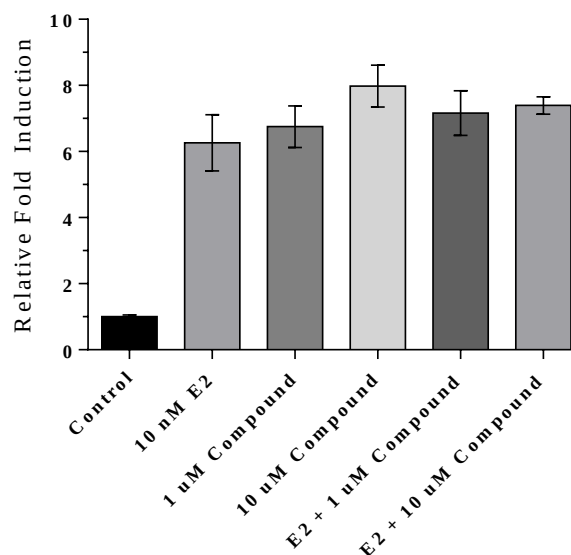


Figure 5.3 MCF-7 cells transfected with 500 ng ERE-LUC reporter plasmids were treated with 10 nM E2 (in 0.01% Ethanol) and/or varying concentrations of compound 4',5-dihydroxy-7-methoxy-3-methylflavanone (1 μ M and 10 μ M in 0.01% DMSO) for 24 hours.

5.4 Discussion

Bioactivity screening were popular option back in 1940s when the Waksman platform was striving and churning out a lot of new antibiotics⁶⁹. With the huge repertoire of natural products identified from soil derived microorganisms, bioassay guided approach in current times may lead to isolation of known compound. This was evident in the case for echinomycin compound being isolated from *Streptomyces* sp. P19 culture broth and biomass. From bioactivity assays as discussed in Chapter 3, it was rare to find a particular strain that is able to produce such strong bioactivity against both Gram-positive and Gram-negative bacteria. Hence, isolation of this pure compound from P19 was carried out before genome information was made available. Isolation and characterisation of this bioactive compound revealed the identity to be a known compound echinomycin. This was further confirmed with the 94% similarity with echinomycin biosynthetic gene cluster.

A novel flavanone compound was successfully isolated from SD50 agar plates. Flavanone compound is usually synthesized by Type 3 PKS found in plants¹⁹¹. This gene cluster was not found in antiSMASH prediction for SD50 draft genome. The biosynthetic mechanism of 4',5-dihydroxy-7-methoxy-3-methylflavanone remains unknown. 4',5-dihydroxy-7-methoxy-3-methylflavanone has interesting activity that targets both prokaryotes and eukaryotes. It is uncommon for compounds to have features to target both groups of microorganisms as the mode of action for bacterial and fungal cells are different. It is also an analogue of a known compound, 4',5,7-trihydroxy-3-methylflavanone (also known as WS-7528 or BE-14348B) that has estrogenic activity of on immature rats¹⁸⁸. 4',5,7-trihydroxy-3-methylflavanone has agonistic activity towards MCF-7 and has the potential to be used in treatment of estrogen deficient syndromes like osteoporosis^{187,188}. With only a difference of methyl group attached to the hydroxyl at the 7th position, similar agonistic activity towards MCF-7 cells was also observed from 4',5-dihydroxy-7-methoxy-3-methylflavanone.

Selective estrogen receptor modulators (SERMs) are compounds that interact with intracellular estrogen receptors as estrogen agonists and anatagonists¹⁹². SERMs has been studied as treatment for hormone-responsive cancer and

osteoporosis¹⁹². As 4',5-dihydroxy-7-methoxy-3-methylflavanone exhibits estrogenic activities, there is a potential for developing it into SERMs.

5.5 Conclusion

Bioactive compound isolated from *Streptomyces* sp. P19 was known compound echinomycin. This was one of the limitations that is unavoidable from bioassay guided approach. A novel bioactive 4',5-dihydroxy-7-methoxy-3-methylflavanone with estrogenic activity was isolated from *Streptomyces* sp. SD50 whereby biosynthetic mechanism is currently unknown. There is a potential for 4',5-dihydroxy-7-methoxy-3-methylflavanone to be developed into estrogen receptor modulators for the treatment of hormone-responsive diseases.

Conclusion and Future Outlook

In conclusion, this thesis provided insights of the microbial diversity from 2 locations in Singapore. In attempt to recover rare actinomycetes whose isolation frequency are much lower, a wide range of specialised media recipes and different isolation procedures were included in this study. A total of 193 bacterial strains were isolated, including 152 actinomycete strains. The highest number of actinobacteria were found to be affiliated with the genus *Streptomyces* (59.07%), followed by *Micromonospora* (16.06%), *Verrucosipora* (1.04%), *Jishengella* (0.52%), *Pseudonocardia* (0.52%), *Isophtericola* (0.52%), *Mycobacterium* (0.52%) and *Microbacterium* (0.52%).

In-situ cultivation mentioned in the thesis revealed a large number of non-actinomycetes. This brings a need for reassessing the method for isolation of actinomycetes. Selective *in-situ* isolation of actinomycetes could be further explored by the microbial trap method as mentioned previously⁵³. Microbial trap has similar setup as compared to diffusion chamber but the principle is different. Microbial trap does not contain pre-inoculated agar matrix and the membrane pore size of 0.2 μm , allows actinomycetes to penetrate into the inner space and form colonies. Both methods are based on the expectation that diffusion through membranes will establish conditions inside the device that closely mimic the natural conditions. This would then allow the previously unculturable bacteria to get access to the critical growth factors supplied by neighboring species for its propagation.

As it is not possible to study every single strain in detail, strain prioritization by bioactivity assays and genome sequencing provided some clues for us to proceed. Bioactivity assays and genome sequencing information have displayed both their strengths and weaknesses in the attempt for uncovering novel compounds. Bioactivity assays are fast and cheap way to screen the 152 actinomycete strains in a reasonable time frame. Therefore, bioactivity assays have been selected as our first strain prioritization method to shortlist strains for genome sequencing. There were also other factors such as interesting morphology or 16S identity which also play a role in strain selection. Intrigued by the strong bioactivities against *P. aeruginosa*, *E. coli* and MRSA, it led to the isolation of known

compound echinomycin from *Streptomyces* sp. P19. Bioassay guided approach has this drawback of rediscovery of known compounds.

Genome mining can be used as a dereplication tool by comparing the biosynthetic gene clusters with the known biosynthetic gene clusters. Therefore, with careful planning and genome analysis of the subsequent strains containing large number of gene clusters, we managed to isolate a novel compound (4',5-dihydroxy-7-methoxy-3-methylflavanone) from *Streptomyces* sp. SD50. Not only did 4',5-dihydroxy-7-methoxy-3-methylflavanone exhibit interesting bioactivities that targets both bacterial and fungal cells, it also possesses estrogenic properties.

4',5-dihydroxy-7-methoxy-3-methylflavanone is the first novel compound that our group has discovered. This is a big step forward in our natural product discovery research. The platform and foundations that have been laid out and the experiences we have accumulated throughout the years would be invaluable for other PhD students to continue. Though this marks the end of my PhD journey, I believe this is only the beginning for our natural product discovery team to uncover more novel compounds and biosynthetic pathways from the strain collection that I have contributed.

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Appendix

Table S.1 Table of isolates (strains) with its closest match computed by EzBioCloud database.

No.	Isolates	Closest match based on 16S rDNA gene sequencing	Similarity (%)	Coverage (%)
1	SE1	<i>Streptomyces exfoliatus</i> NBRC 13191	100.00	60.50
2	SE2	<i>Streptomyces omiyaensis</i> NBRC 13449	100.00	42.90
3	SE3	<i>Streptomyces griseosporus</i> NBRC 13458	99.55	61.70
4	SW1	<i>Micromonospora siamensis</i> TT2-4	99.64	100.00
5	SW3	-		
6	SW5	<i>Micromonospora fulviviridis</i> DSM 43906	99.78	63.80
7	SW9	<i>Micromonospora oryzae</i> CP2R9-1	100.00	42.70
8	SW10	<i>Micromonospora chalcea</i> DSM 43026	100.00	63.40
9	SW15	<i>Jishengella endophytica</i> 202201	99.44	100.00
10	SW16	<i>Verrucosipora sonchi</i> NEAU-QY3	99.16	100.00
11	SW19	<i>Micromonospora chalcea</i> DSM 43026	100.00	63.10
12	SW24	<i>Streptomyces yogyakartensis</i> NBRC 100779	99.59	100.00
13	SD1	-		
14	SD2	-		
15	SD3	<i>Streptomyces capoamus</i> JCM 4734	99.88	56.90
16	SD4	-		
17	SD5	-		
18	SD7	-		
19	SD8	<i>Streptomyces longwoodensis</i> DSM 41677	99.78	61.80
20	SD9	-		
21	SD10	-		
22	SD11	-		
23	SD17	-		
24	SD18	-		
25	SD19	-		
26	SD21	-		
27	SD24	<i>Streptomyces bingchenggensis</i> BCW-1	99.59	100.00
28	SD25	-		
29	SD26	-		
30	SD29	-		

31	SD32	<i>Streptomyces collinus</i> Tu365	99.10	100.00
32	SD33	-		
33	SD35	<i>Streptomyces cyslabdanicus</i> K04-0144	99.17	100.00
34	SD37	-		
35	SD38	-		
36	SD40	-		
37	SD41	-		
38	SD42	-		
39	SD46	-		
40	SD48	<i>Streptomyces lannensis</i> TA4-8	100.00	100.00
41	SD49	-		
42	SD50	<i>Streptomyces bingchenggensis</i> BCW-1	99.72	100.00
43	SD52W	-		
44	SD52Y	-		
45	SD55	-		
46	SD60	-		
47	SD67	-		
48	SD70	<i>Micromonospora chaiyaphumensis</i> MC5-1	99.44	100.00
49	SD72	-		
50	SD73	-		
51	SD74	-		
52	SD75	-		
53	SD77	<i>Streptomyces sanglieri</i> NBRC 100784	99.86	100.00
54	SD83	-		
55	SD84	-		
56	SD85	<i>Streptomyces hiroshimensis</i> NBRC 3839	99.51	100.00
57	SD86	-		
58	SD90	-		
59	SD91	-		
60	SD92	-		
61	SD93	-		
62	MD35	-		
63	MD37	<i>Micromonospora endolithica</i> DSM 44398	100.00	67.00
64	MD38	-		
65	MD41	-		
66	MD42	-		
67	MD43	-		
68	MD44	<i>Micromonospora sediminicola</i> SH2-13	99.88	58.90
69	MD58	-		
70	MD60	-		
71	MD62	<i>Streptomyces albogriseolus</i> NRRL B-1305	100.00	61.70
72	MD63	<i>Streptomyces griseorubens</i> NBRC 12780	100.00	60.70
73	MD64	<i>Streptomyces diastaticus</i> subsp. <i>ardesiacus</i> NRRL B-1773	99.78	61.60
74	MD65	<i>Streptomyces griseoincarnatus</i> LMG 19316	100.00	100.00
75	MD66	<i>Streptomyces sanyensis</i> 219820	99.67	63.40
76	MD68	-		

77	MD69	-		
78	MD70	<i>Streptomyces sundarbansensis</i> MS1/7	99.86	100.00
79	MD71	<i>Streptomyces thermolineatus</i> DSM 41451	99.89	63.80
80	MD72	<i>Streptomyces sundarbansensis</i> MS1/7	99.86	100.00
81	MD73	-		
82	MD75	<i>Streptomyces gardneri</i> NBRC 12865	100.00	35.20
83	MD76	<i>Streptomyces puniceus</i> NRRL ISP-5058	100.00	61.50
84	MD77	<i>Streptomyces pseudogriseolus</i> NBRC 12902	100.00	61.20
85	MD78	<i>Streptomyces lusitanus</i> NBRC 13464	100.00	62.50
86	MD79	<i>Streptomyces sanyensis</i> 219820	100.00	62.10
87	MD80	<i>Streptomyces puniceus</i> NRRL ISP-5058	100.00	63.30
88	MD83	-		
89	MD84	<i>Streptomyces sanyensis</i> 219820	99.64	57.70
90	MD85	<i>Streptomyces thermocarboxydus</i> DSM 44293	99.93	97.70
91	MD94	-		
92	MD95	-		
93	MD100	<i>Streptomyces coelicoflavus</i> NBRC 15399	100.00	100.00
94	MD101	<i>Streptomyces puniceus</i> NRRL ISP-5058	100.00	58.40
95	MD102	<i>Streptomyces violascens</i> ISP 5183	100.00	93.80
96	MD104	-		
97	MD106	-		
98	MD108	-		
99	MD109	-		
100	MD114	-		
101	MD115	-		
102	MD116	-		
103	MD117	-		
104	MD118	<i>Micromonospora aurantiaca</i> ATCC 27029	100.00	100.00
105	MD120	<i>Micromonospora tulbaghia</i> TVU1	100.00	64.30
106	MD121	-		
107	MD122	<i>Verrucosipora andamanensis</i> SP03-05	100.00	35.60
108	MD123	-		
109	MD124	<i>Micromonospora chalcea</i> DSM 43026	100.00	35.40
110	MD130	-		
111	MD132	-		
112	MD135	-		
113	MD138	-		
114	MI1	<i>Streptomyces sanyensis</i> 219820	99.77	61.20
115	MI2	<i>Micromonospora aurantiaca</i> ATCC 27029	100.00	100.00
116	MI3	<i>Isopterocola chiayiensis</i> 06182M-1	99.12	62.90
117	MI4	<i>Cohaesibacter haloalkalitolerans</i> JC131	99.29	70.20
118	MI5	-		
119	MI6	-		
120	MI7	-		
121	MI8	-		
122	MI9	-		

123	MI10	-		
124	MI11	-		
125	MI12	-		
126	MI13	-		
127	MI14	-		
128	MI15	<i>Pseudomonas aeruginosa</i> JCM 5962	99.64	57.50
129	MI16	<i>Pseudomonas aeruginosa</i> JCM 5962	99.65	58.40
130	MI17	<i>Pseudomonas aeruginosa</i> JCM 5962	99.48	66.40
131	MI18	-		
132	MI19	-		
133	MI20	-		
134	MI21	-		
135	MI22	-		
136	MI23	-		
137	MI24	-		
138	MI25	-		
139	MI26	-		
140	MI27	-		
141	MI28	-		
142	MI29	-		
143	MI30	-		
144	MI31	-		
145	MI32	-		
146	MI33	-		
147	MI34	-		
148	MI35	<i>Erthrobacter nanhaisediminis</i> T30	99.90	72.30
149	MI36	-		
150	MI37	-		
151	MI38	-		
152	MI39	-		
153	MI40	-		
154	MI41	<i>Pseudomonas aeruginosa</i> JCM 5962	99.56	62.00
155	MI42	<i>Enhydrobacter aerosaccus</i> LMG 21877	99.88	57.40
156	P1	<i>Streptomyces cyslabdanicus</i> K04-0144	99.03	100.00
157	P3	-		
158	P4	-		
159	P5	-		
160	P6	-		
161	P7	<i>Streptomyces longwoodensis</i> DSM 41677	100.00	99.40
162	P9	<i>Streptomyces enissocaesilis</i> NRRL B-16365	100.00	100.00
163	P10	<i>Streptomyces rameus</i> LMG 20326	100.00	100.00
164	P11	-		
165	P12	-		
166	P14	-		
167	P15	-		
168	P16	-		

169	P17	<i>Streptomyces fradiae</i> NBRC 3439	99.44	100.00
170	P18	-		
171	P19	<i>Streptomyces tendae</i> ATCC 19812	99.45	100.00
172	P20	<i>Streptomyces cinereospinus</i> NBRC 15397	99.87	54.10
173	P21	-		
174	P22	-		
175	P23	-		
176	P24	<i>Streptomyces iakyrus</i> NRRL ISP 5482	100.00	62.80
177	P25	-		
178	P26	-		
179	P27	-		
180	P28	-		
181	P29	<i>Pseudonocardia carboxydvorans</i> Y8	99.85	94.50
182	P30	<i>Methylobacterium aquaticum</i> DSM 16371	99.88	58.50
183	P31	-		
184	P32	<i>Mycobacterium chubuense</i> DSM 44219	99.32	51.20
185	P34	<i>Microbacterium laevaniformans</i> DSM20140	99.23	27.20
186	P36	-		
187	P41	-		
188	P42	-		
189	P46	<i>Streptomyces malachitospinus</i> NBRC 101004	99.03	100.00
190	P51	-		
191	P52	-		
192	P53	-		
193	P54	<i>Streptomyces hawaiiensis</i> NBRC 12784	99.52	100.00

∴ 16S rDNA gene sequences not identified

Table S.2 Strains isolated from different sampling locations, pre-treatment methods and isolation media.

No.	Isolates	Coordinates of sample	Pre-treatment method	Medium
1	SE1	1°26'41.6"N 103°43'27.8"E	Surface sterilization	ISP3 gellan gum
2	SE2	1°26'41.6"N 103°43'27.8"E	Surface sterilization	SW ISP3 bacto agar
3	SE3	1°26'41.6"N 103°43'27.8"E	Surface sterilization	SW ISP3 bacto agar
4	SW1	1°26'58.6"N 103°43'12.8"E	Wet heat	SW gellan gum
5	SW3	1°26'58.6"N 103°43'12.8"E	Wet heat	SW gellan gum
6	SW5	1°26'58.6"N 103°43'12.8"E	Wet heat	SW ISP3 bacto agar
7	SW9	1°26'58.6"N 103°43'12.8"E	Wet heat	SW ISP3 bacto agar
8	SW10	1°26'58.6"N 103°43'12.8"E	Wet heat	SW ISP3 bacto agar
9	SW15	1°26'58.6"N 103°43'12.8"E	Phenol	SW ISP3 bacto agar
10	SW16	1°26'58.6"N 103°43'12.8"E	Phenol	Nutrient poor gellan gum
11	SW19	1°26'58.6"N 103°43'12.8"E	Wet heat	Humic acid agar
12	SW24	1°26'58.6"N 103°43'12.8"E	Microwave	SW ISP3 bacto agar
13	SD1	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
14	SD2	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
15	SD3	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
16	SD4	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
17	SD5	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
18	SD7	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
19	SD8	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
20	SD9	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
21	SD10	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
22	SD11	1°26'41.5"N 103°43'19.3"E	Wet heat	Humic acid agar
23	SD17	1°26'41.5"N 103°43'19.3"E	Wet heat	Soil extract agar
24	SD18	1°26'41.5"N 103°43'19.3"E	Wet heat	Soil extract agar
25	SD19	1°26'41.5"N 103°43'19.3"E	Wet heat	Soil extract agar
26	SD21	1°26'41.5"N 103°43'19.3"E	Wet heat	Soil extract agar
27	SD24	1°26'41.5"N 103°43'19.3"E	Wet heat	Soil extract agar
28	SD25	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 gellan gum
29	SD26	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 gellan gum
30	SD29	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 gellan gum
31	SD32	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 gellan gum
32	SD33	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 bacto agar
33	SD35	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 bacto agar
34	SD37	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 bacto agar
35	SD38	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 bacto agar
36	SD40	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 bacto agar
37	SD41	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 gellan gum
38	SD42	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 gellan gum
39	SD46	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 gellan gum
40	SD48	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 gellan gum
41	SD49	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 bacto agar
42	SD50	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 bacto agar

43	SD52W	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 bacto agar
44	SD52Y	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 bacto agar
45	SD55	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 bacto agar
46	SD60	1°26'41.5"N 103°43'19.3"E	Phenol	Soil extract agar
47	SD67	1°26'41.5"N 103°43'19.3"E	Wet heat	ISP3 bacto agar
48	SD70	1°26'41.5"N 103°43'19.3"E	Phenol	Soil extract agar
49	SD72	1°26'41.5"N 103°43'19.3"E	Phenol	Soil extract agar
50	SD73	1°26'41.5"N 103°43'19.3"E	Phenol	Soil extract agar
51	SD74	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 gellan gum
52	SD75	1°26'41.5"N 103°43'19.3"E	Phenol	ISP3 gellan gum
53	SD77	1°26'41.5"N 103°43'19.3"E	Microwave	ISP3 gellan gum
54	SD83	1°26'41.5"N 103°43'19.3"E	Microwave	ISP3 bacto agar
55	SD84	1°26'41.5"N 103°43'19.3"E	Microwave	ISP3 bacto agar
56	SD85	1°26'41.5"N 103°43'19.3"E	Microwave	ISP3 bacto agar
57	SD86	1°26'41.5"N 103°43'19.3"E	Microwave	Soil extract agar
58	SD90	1°26'41.5"N 103°43'19.3"E	Microwave	Soil extract agar
59	SD91	1°26'41.5"N 103°43'19.3"E	Microwave	Humic acid agar
60	SD92	1°26'41.5"N 103°43'19.3"E	Microwave	Humic acid agar
61	SD93	1°26'41.5"N 103°43'19.3"E	Microwave	Humic acid agar
62	MD35	1°26'38.5"N 103°43'30.4"E	Microwave	Arginine glycerol salt agar
63	MD37	1°26'38.5"N 103°43'30.4"E	Microwave	Arginine glycerol salt agar
64	MD38	1°26'38.5"N 103°43'30.4"E	Microwave	HV gellan gum
65	MD41	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
66	MD42	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
67	MD43	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
68	MD44	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
69	MD58	1°26'38.5"N 103°43'30.4"E	Microwave	Arginine glycerol salt agar
70	MD60	1°26'38.5"N 103°43'30.4"E	Microwave	Arginine glycerol salt agar
71	MD62	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMP
72	MD63	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMP
73	MD64	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMP
74	MD65	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMP
75	MD66	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
76	MD68	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
77	MD69	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
78	MD70	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
79	MD71	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
80	MD72	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
81	MD73	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
82	MD75	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
83	MD76	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
84	MD77	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
85	MD78	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Starch casein agar
86	MD79	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Gauze's synthetic media No. 1

87	MD80	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Gauze's synthetic media No. 1
88	MD83	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Gauze's synthetic media No. 1
89	MD84	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Gauze's synthetic media No. 1
90	MD85	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Gauze's synthetic media No. 1
91	MD94	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Arginine glycerol salt agar
92	MD95	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	Arginine glycerol salt agar
93	MD100	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
94	MD101	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
95	MD102	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
96	MD104	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
97	MD106	1°26'38.5"N 103°43'30.4"E	Desiccation	Starch casein
98	MD108	1°26'38.5"N 103°43'30.4"E	Microwave	Arginine glycerol salt agar
99	MD109	1°26'38.5"N 103°43'30.4"E	Microwave	Arginine glycerol salt agar
100	MD114	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
101	MD115	1°26'41.2"N 103°43'36.1"E	Microwave	SM3 agar
102	MD116	1°26'41.2"N 103°43'36.1"E	Microwave	SM3 agar
103	MD117	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
104	MD118	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
105	MD120	1°26'38.5"N 103°43'30.4"E	Desiccation	SM3 agar
106	MD121	1°26'38.5"N 103°43'30.4"E	Desiccation	SM3 agar
107	MD122	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
108	MD123	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
109	MD124	1°26'38.5"N 103°43'30.4"E	Microwave	Starch casein agar
110	MD130	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
111	MD132	1°26'38.5"N 103°43'30.4"E	Microwave	Starch casein agar
112	MD135	1°26'38.5"N 103°43'30.4"E	Microwave	SM3 agar
113	MD138	1°26'38.5"N 103°43'30.4"E	Microwave	HV gellan gum
114	MI1	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
115	MI2	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
116	MI3	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
117	MI4	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
118	MI5	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
119	MI6	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
120	MI7	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
121	MI8	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
122	MI9	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
123	MI10	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
124	MI11	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
125	MI12	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
126	MI13	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
127	MI14	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
128	MI15	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
129	MI16	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar

130	MI17	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
131	MI18	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
132	MI19	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
133	MI20	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
134	MI21	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
135	MI22	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
136	MI23	1°26'41.2"N 103°43'36.1"E	Microwave	SMS agar
137	MI24	1°26'41.2"N 103°43'36.1"E	Desiccation	SMS agar
138	MI25	1°26'41.2"N 103°43'36.1"E	Desiccation	SMS agar
139	MI26	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
140	MI27	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
141	MI28	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
142	MI29	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
143	MI30	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
144	MI31	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
145	MI32	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMS agar
146	MI33	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMS agar
147	MI34	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMS agar
148	MI35	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
149	MI36	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
150	MI37	1°26'38.5"N 103°43'30.4"E	Microwave	SMS agar
151	MI38	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
152	MI39	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMS agar
153	MI40	1°26'38.5"N 103°43'30.4"E	Calcium carbonate	SMS agar
154	MI41	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
155	MI42	1°26'38.5"N 103°43'30.4"E	Desiccation	SMS agar
156	P1	1°24'21.3"N 103°57'22.8"E	None	Arginine glycerol salt agar
157	P3	1°24'21.3"N 103°57'22.8"E	None	Arginine glycerol salt agar
158	P4	1°24'21.3"N 103°57'22.8"E	None	Humic acid agar
159	P5	1°24'21.3"N 103°57'22.8"E	None	Humic acid agar
160	P6	1°24'21.3"N 103°57'22.8"E	None	Humic acid agar
161	P7	1°24'21.3"N 103°57'22.8"E	None	Humic acid agar
162	P9	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
163	P10	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
164	P11	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
165	P12	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
166	P14	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
167	P15	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
168	P16	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
169	P17	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
170	P18	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
171	P19	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
172	P20	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
173	P21	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1
174	P22	1°24'21.3"N 103°57'22.8"E	None	Gauze's synthetic No. 1

175	P23	1°24'21.3"N 103°57'22.8"E	Desiccation	SW ISP3
176	P24	1°24'21.3"N 103°57'22.8"E	Desiccation	SW ISP3
177	P25	1°24'21.3"N 103°57'22.8"E	Desiccation	SW ISP3
178	P26	1°24'21.3"N 103°57'22.8"E	Desiccation	SW ISP3
179	P27	1°24'21.3"N 103°57'22.8"E	Desiccation	SW ISP3
180	P28	1°24'21.3"N 103°57'22.8"E	Desiccation	Arginine glycerol salt agar
181	P29	1°24'21.3"N 103°57'22.8"E	Desiccation	Arginine glycerol salt agar
182	P30	1°24'21.3"N 103°57'22.8"E	Desiccation	HV gellan gum
183	P31	1°24'21.3"N 103°57'22.8"E	Desiccation	Gauze's synthetic No. 1
184	P32	1°24'21.3"N 103°57'22.8"E	Desiccation	Gauze's synthetic No. 1
185	P34	1°24'21.3"N 103°57'22.8"E	Desiccation	Gauze's synthetic No. 1
186	P36	1°24'21.3"N 103°57'22.8"E	Desiccation	Gauze's synthetic No. 1
187	P41	1°24'21.3"N 103°57'22.8"E	Desiccation	Arginine glycerol salt agar
188	P42	1°24'21.3"N 103°57'22.8"E	Enrichment & re- centrifugation	SW ISP3
189	P46	1°24'21.3"N 103°57'22.8"E	Enrichment & re- centrifugation	SW ISP3
190	P51	1°24'21.3"N 103°57'22.8"E	Enrichment & re- centrifugation	Gauze's synthetic No. 1
191	P52	1°24'21.3"N 103°57'22.8"E	Enrichment & re- centrifugation	Gauze's synthetic No. 1
192	P53	1°24'21.3"N 103°57'22.8"E	Enrichment & re- centrifugation	Gauze's synthetic No. 1
193	P54	1°24'21.3"N 103°57'22.8"E	Enrichment & re- centrifugation	Gauze's synthetic No. 1

Table S.3 Cross streak antibacterial assay against *S. aureus* ATCC14775, *B. subtilis* 168 and *P. aeruginosa* PA01.

No.	Isolates	Zone of inhibition (mm)		
		<i>S. aureus</i> ATCC 14775	<i>B. subtilis</i> 168	<i>P. aeruginosa</i> PA01
1	SE1	0	10	0
2	SE2	12	6	0
3	SE3	8	50	0
4	SW1	0	8	0
5	SW3	0	0	0
6	SW5	0	0	0
7	SW9	0	0	0
8	SW10	8	12	0
9	SW15	0	0	0
10	SW16	19	21	0
11	SW19	0	0	0
12	SW24	62	72	0
13	SD1	0	19	0
14	SD2	31	23	0
15	SD3	38	59	0
16	SD4	23	17	0
17	SD5	0	15	0
18	SD7	14	6	0
19	SD8	21	21	0
20	SD9	0	0	0
21	SD10	29	21	0
22	SD11	4	11	0
23	SD17	0	0	0
24	SD18	13	11	0
25	SD19	0	0	0
26	SD21	20	20	0
27	SD24	55	53	0
28	SD25	11	16	0
29	SD26	0	0	0
30	SD29	29	23	0
31	SD32	39	51	0
32	SD33	0	0	0
33	SD35	0	15	0
34	SD37	25	10	0
35	SD38	17	15	0
36	SD40	0	0	0
37	SD41	23	17	0
38	SD42	38	48	0
39	SD46	55	60	0
40	SD48	0	15	0
41	SD49	12	10	0
42	SD50	48	50	0

43	SD52W	14	23	0
44	SD52Y	22	26	0
45	SD55	15	17	0
46	SD60	9	7	0
47	SD67	18	35	0
48	SD70	39	44	0
49	SD72	0	0	0
50	SD73	0	0	0
51	SD74	20	26	0
52	SD75	0	0	0
53	SD77	22	26	0
54	SD83	43	43	0
55	SD84	0	0	0
56	SD85	38	13	0
57	SD86	31	31	0
58	SD90	0	0	0
59	SD91	0	0	0
60	SD92	21	23	0
61	SD93	0	0	0

Table S.4 Overlay assay against *S. aureus* ATCC14775, *B. subtilis* 168, *E. coli* K12 and *P. aeruginosa* PA01.

No.	Isolates	Zone of inhibition (mm)			
		<i>S. aureus</i> ATCC 14775	<i>B. subtilis</i> 168	<i>E. coli</i> K12	<i>P. aeruginosa</i> PA01
1	SE1	2	0	0	0
2	SE2	0	0	0	0
3	SE3	12	7	0	0
4	SW1	0	0	0	0
5	SW3	2	0	0	0
6	SW5	0	0	0	0
7	SW9	20	16	0	0
8	SW10	0	0	0	0
9	SW15	0	0	0	0
10	SW16	4	11	0	0
11	SW19	0	0	0	0
12	SW24	0	1	0	0
13	SD1	0	0	0	0
14	SD2	9	12	0	0
15	SD3	0	0	1	0
16	SD4	0	0	0	0
17	SD5	0	0	0	0
18	SD7	12	7	12	0
19	SD8	2	1	1	0
20	SD9	0	0	0	0
21	SD10	4	3	3	0
22	SD11	0	0	0	0
23	SD17	0	1	0	0
24	SD18	0	0	0	0
25	SD19	0	0	0	0
26	SD21	0	1	0	0
27	SD24	0	0	0	0
28	SD25	0	0	0	0
29	SD26	1	1	1	0
30	SD29	0	14	0	0
31	SD32	18	22	16	0
32	SD33	1	1	7	0
33	SD35	0	0	0	0
34	SD37	3	4	4	0
35	SD38	0	0	0	0
36	SD40	0	0	2	0
37	SD41	8	11	13	0
38	SD42	1	14	0	0
39	SD46	0	0	0	0
40	SD48	0	0	0	0
41	SD49	0	0	0	0

42	SD50	0	0	0	0
43	SD52W	0	0	0	0
44	SD52Y	0	1	0	0
45	SD55	0	0	0	0
46	SD60	0	0	0	0
47	SD67	25	45	0	0
48	SD70	45	45	11	1
49	SD72	0	0	0	0
50	SD73	0	0	0	0
51	SD74	0	4	10	0
52	SD75	6	12	8	0
53	SD77	5	4	15	0
54	SD83	0	0	0	0
55	SD84	0	0	0	0
56	SD85	0	0	0	0
57	SD86	0	0	0	0
58	SD90	0	0	2	0
59	SD91	0	0	0	0
60	SD92	1	0	0	0
61	SD93	0	0	0	0

Table S.5 Overlay assay against MRSA, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *C. albicans*.

No.	Isolates	Zone of inhibition (mm)				
		MRSA	<i>K. pneumoniae</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
1	MD35	0	0	0	0	0
2	MD37	37	10	3	0	0
3	MD38	0	0	0	0	0
4	MD41	0	0	0	0	0
5	MD42	0	0	0	0	0
6	MD43	0	0	0	0	0
7	MD44	0	0	0	0	0
8	MD58	0	0	0	0	0
9	MD60	0	0	0	0	0
10	MD62	0	0	0	0	0
11	MD63	21	0	0	0	0
12	MD64	5	0	0	0	0
13	MD65	28	3	4	0	0
14	MD66	0	0	0	0	0
15	MD68	0	0	0	0	0
16	MD69	0	0	0	0	0
17	MD70	6	0	0	0	0
18	MD71	0	0	0	0	0
19	MD72	0	0	0	0	0
20	MD73	0	0	0	0	0
21	MD75	7	0	0	0	0
22	MD76	10	0	0	0	0
23	MD77	18	0	0	0	0
24	MD78	0	0	0	0	0
25	MD79	0	0	0	0	0
26	MD80	0	0	0	0	0
27	MD83	0	0	0	0	0
28	MD84	0	0	0	0	0
29	MD85	0	0	0	0	0
30	MD94	0	0	0	0	0
31	MD95	0	0	0	0	0
32	MD100	25	0	23	0	0
33	MD101	0	0	0	0	0
34	MD102	0	0	0	0	21
35	MD104	0	0	0	0	0
36	MD106	0	0	0	0	0
37	MD108	0	0	0	0	0
38	MD109	0	0	0	0	0
39	MD114	0	0	0	0	0
40	MD115	0	0	0	0	0
41	MD116	0	0	0	0	0

42	MD117	0	0	0	0	0
43	MD118	38	10	16	4	0
44	MD120	2	0	0	0	0
45	MD121	0	0	0	0	0
46	MD122	3	0	0	0	0
47	MD123	0	0	0	0	0
48	MD124	3	0	0	0	0
49	MD130	0	0	0	0	0
50	MD132	0	0	0	0	0
51	MD135	0	0	0	0	0
52	MD138	0	0	0	0	0
53	MI1	0	0	0	0	0
54	MI2	0	0	0	0	0
55	MI3	0	0	0	0	0
56	P1	5	0	0	0	0
57	P3	3	0	1	0	0
58	P4	12	0	2	0	0
59	P5	0	0	0	0	0
60	P6	0	0	0	0	0
61	P7	0	0	0	0	0
62	P9	17	9	7	0	16
63	P10	0	0	0	0	3
64	P11	0	0	0	0	0
65	P12	0	0	0	0	0
66	P14	2	0	0	0	0
67	P15	0	0	0	0	0
68	P16	0	0	0	0	0
69	P17	0	0	0	0	4
70	P18	20	0	0	0	0
71	P19	13	0	0	0	0
72	P20	0	0	0	0	0
73	P21	0	0	0	0	0
74	P22	25	0	0	0	0
75	P23	0	0	0	0	0
76	P24	6	0	0	0	0
77	P25	0	0	0	0	0
78	P26	0	0	0	0	0
79	P27	5	0	0	0	0
80	P28	0	0	0	0	0
81	P29	0	0	0	0	8
82	P31	0	0	0	0	0
83	P32	0	0	0	0	0
84	P34	0	0	0	0	0
85	P36	0	0	0	0	0
86	P42	0	0	0	0	0
87	P46	6	3	5	1	0

88	P51	0	0	0	0	0
89	P52	0	0	0	0	0
90	P53	0	0	0	0	0
91	P54	0	0	0	0	5

Table S.6 Isolates were cultured in GYM fermentation media. Crude extracts from isolates were extracted both in broth (ethyl acetate extraction) and biomass (acetone extraction). The table tabulates the results of microtiter plate antibacterial assay against MRSA, *E. coli* and *P. aeruginosa*.

No.	Isolates	Percentage inhibition (%)					
		MRSA		<i>E. coli</i>		<i>P. aeruginosa</i>	
		EA	Acetone	EA	Acetone	EA	Acetone
1	P1	40.0±19.6*	7.5±0.1	28.7±18.2	35.6±0.2	15.5±0.9	17.6±2.0
2	P3	33.1±10.2	13.5±4.4	36.1±13.8	21.1±4.1	8.9±6.8	-4.3±6.4
3	P4	2.1±2.9	11.9±0.6	8.7±4.7	6.1±1.1	-6.7±4.9	-4.5±2.6
4	P5	34.7±20.0	19.6±5.6	30.1±15.7	33.9±2.0	9.7±2.2	7.8±9.1
5	P6	32.5±20.0	29.0±0.9	33.6±10.3	29.6±3.9	7.6±1.4	25.6±12.6
6	P7	37.8±8.2	9.2±5.2	35.8±10.0	35.9±0.2	13.3±3.2	-6.0±7.5
7	P9	31.8±8.9	12.7±2.0	6.1±16.6	-17.5±1.0	3.8±7.2	-13.7±3.1
8	P10	9.6±4.1	18.9±15.7	48.3±0.3*	38.9±1.6	6.5±4.3	8.0±2.7
9	P11	49.0±4.1*	19.0±0.1	18.8±10.1	37.3±3.9	4.5±6.3	-1.8±5.8
10	P12	44.9±6.3*	22.2±10.3	40.8±3.6*	36.7±1.9	9.9±1.3	-4.6±16.4
11	P14	48.9±14.9*	17.6±5.2	41.6±3.6*	37.3±1.7	14.1±9.1	13.8±3.4
12	P15	30.3±2.1	36.7±1.1	18.1±1.0	9.9±0.6	-1.4±2.9	-7.8±1.6
13	P16	5.1±4.6	36.1±3.7	22.5±5.0	23.4±18.8	4.5±0.4	4.1±1.8
14	P17	40.2±3.9*	33.7±10.5	26.2±0.1	8.4±1.4	-2.4±4.8	-2.5±3.8
15	P18	74.0±2.8*	34.1±9.6	36.3±1.9	6.2±0.2	1.7±7.7	-10.5±4.9
16	P19	37.3±7.2	39.2±0.7	30.8±10.2	11.2±3.1	-1.3±7.3	-5.9±2.3
17	P20	46.4±13.6*	29.0±0.3	25.4±1.2	7.3±3.7	-1.2±2.4	5.9±2.6
18	P21	37.6±5.2	29.9±3.5	11.5±8.1	-5.6±19.0	3.9±5.7	-8.3±4.5
19	P22	37.5±3.1	34.4±1.5	29.0±8.0	14.2±3.1	4.1±0.6	11.3±0.4
20	P23	46.0±1.6*	40.8±3.7*	35.6±0.1	17.9±0.2	2.3±6.1	-0.8±1.2
21	P24	9.9±1.8	20.6±2.8	11.1±0.9	7.7±0.2	-0.7±3.2	-44.0±4.1
22	P25	12.3±5.9	20.7±2.2	11.8±0.3	6.8±1.9	7.0±11.5	-17.0±0.1
23	P26	32.9±1.1	20.3±4.7	20.0±12.8	33.9±0.9	3.0±4.0	-1.2±6.8
24	P27	28.9±1.1	17.2±1.2	23.6±0.8	35.2±0.7	-0.4±2.1	3.7±5.1
25	P28	36.8±2.8	12.6±3.6	28.9±0.9	31.6±0.1	7.7±3.6	3.0±4.1
26	P29	30.2±3.0	0.9±6.9	19.8±4.4	34.1±0.3	10.0±5.5	-6.0±4.3
27	P31	39.2±0.4	51.3±6.6*	46.2±3.6*	44.3±0.9*	9.7±5.1	1.0±8.5
28	P32	38.6±0.3	46.5±3.5*	26.2±0.4	41.8±2.5*	8.5±11.7	1.9±9.8
29	P34	32.9±3.4	37.7±4.3	19.0±10.9	18.8±1.1	-10.4±1.1	-13.2±2.5
30	P36	30.3±0.9	36.4±1.9	23.9±12.9	17.3±5.6	-5.5±2.0	-11.1±7.9
31	P42	26.3±4.8	32.0±1.9	33.3±13.6	4.8±9.2	2.5±3.1	2.0±6.0
32	P46	73.8±0.2*	27.1±0.5	46.1±1.8*	36.7±5.9	73.8±0.8*	19.8±16.8
33	P51	27.5±0.7	25.6±0.9	39.6±1.5	22.1±2.4	-2.7±6.8	9.1±16.9
34	P52	39.3±1.7	23.5±0.2	46.7±2.6*	16.0±8.3	15.4±9.4	12.6±11.9
35	P53	41.7±0.1*	32.3±6.8	46.3±0.8*	15.7±12.9	38.4±9.4	22.2±11.9
36	P54	39.2±0.2	26.4±1.5	41.3±0.42*	13.4±5.9	44.2±2.7*	5.9±3.9

*represents at least 40% percentage inhibition

Table S.7 Isolates were cultured in Pharmamedia fermentation media. Crude extracts from isolates were extracted both in broth (ethyl acetate extraction) and biomass (acetone extraction). The table tabulates the results of microtiter plate antibacterial assay against MRSA, *E. coli* and *P. aeruginosa*.

No.	Isolates	Percentage inhibition (%)					
		MRSA		<i>E. coli</i>		<i>P. aeruginosa</i>	
		EA	Acetone	EA	Acetone	EA	Acetone
1	P1	9.3±1.3	62.7±6.0*	42.1±2.8*	15.7±1.3	19.5±8.4	31.0±6.0
2	P3	49.8±3.2*	66.5±6.8*	42.4±1.6*	33.4±0.4	26.1±8.6	5.3±1.3
3	P4	94.2±1.4*	92.7±0.6*	36.6±6.5	1.6±1.3	0.1±14.6	-4.7±4.7
4	P5	-6.7±7.2	17.0±6.5	48.4±1.2*	28.2±0.9	18.0±8.8	24.0±6.7
5	P6	36.6±2.3	38.2±7.9	43.1±2.5*	15.5±2.9	19.8±7.0	37.4±1.2
6	P7	38.5±0.3	42.3±5.8*	43.8±2.0*	18.5±2.2	19.2±7.7	40.8±2.3*
7	P9	27.0±9.8	24.7±1.1	35.5±2.9	38.2±1.7	-8.4±0.0	8.3±1.8
8	P10	9.0±2.1	45.8±0.5*	47.8±1.7*	20.8±0.7	20.0±6.4	44.9±3.9*
9	P11	99.9±0.6*	102.8±0.3*	41.4±2.2*	29.0±3.9	-5.1±19.6	-13.3±10.1
10	P12	50.1±0.1*	105.4±0.9*	48.5±2.1*	26.1±1.0	22.0±6.6	28.1±0.2
11	P14	31.8±13.9	-11.6±7.4	45.2±2.9*	19.8±1.5	34.3±9.7	32.9±1.3
12	P15	40.8±5.1*	28.6±2.1	39.2±1.9	33.0±1.0	14.2±4.4	14.8±0.5
13	P16	17.1±0.1	15.8±5.5	40.7±0.8*	39.7±1.1	23.6±4.9	4.7±7.8
14	P17	16.5±1.2	12.3±5.2	43.5±1.3*	39.4±0.7	18.2±0.6	0.0±35.9
15	P18	102.9±0.7*	91.5±0.7*	64.4±3.6*	30.3±5.2	-4.5±6.8	-35.7±1.4
16	P19	107.6±0.2*	105.5±0.0*	100.3±0.5*	99.4±0.5*	117.8±6.7*	98.9±1.1*
17	P20	14.0±0.1	-3.8±16.4	43.7±0.7*	32.2±0.1	0.2±6.2	-0.5±2.9
18	P21	34.1±3.4	27.2±1.8	39.3±5.4	21.4±3.5	-6.2±3.6	2.8±2.0
19	P22	105.6±1.5*	107.7±21.1*	43.2±0.3*	37.6±0.4	12.0±1.4	-6.6±3.7
20	P23	21.0±7.0	20.7±15.6	41.5±0.6*	43.6±2.0*	7.3±8.6	-4.3±5.6
21	P24	37.7±0.1	-14.5±6.7	40.1±1.3*	29.2±2.4	8.0±3.1	10.3±6.0
22	P25	75.0±0.6*	61.6±0.3*	40.0±2.2*	40.6±3.9*	-2.7±19.6	-18.2±10.1
23	P26	67.6±22.9*	28.8±1.3	45.1±5.8*	30.4±0.4	2.1±18.5	-20.5±0.3
24	P27	37.9±5.8	-28.0±4.9	50.8±1.4*	34.0±0.6	-7.4±16.3	-23.3±4.8
25	P28	43.6±9.4*	64.2±19.65*	43.3±2.2*	31.7±2.3	-6.6±15.8	-28.6±0.4
26	P29	22.8±15.3	20.6±1.0	45.6±0.4*	41.1±0.3*	-5.4±13.6	-0.8±5.2
27	P31	55.0±0.6*	19.3±1.0	47.9±1.5*	18.2±0.2	-10.6±10.1	-30.1±0.3
28	P32	6.9±20.8	17.0±0.2	46.2±1.2*	32.5±0.5	13.0±0.5	17.0±0.2
29	P34	8.3±2.4	55.3±4.6*	42.8±1.0*	43.2±1.3*	30.5±14.3	21.2±4.4
30	P36	80.4±1.2*	44.1±1.7*	53.6±0.5*	16.5±3.0	-17.1±5.7	-34.6±7.3
31	P42	50.0±1.8*	44.5±1.0*	44.9±3.6*	30.5±5.6	-11.9±6.2	15.0±3.5
32	P46	40.0±2.0*	26.3±0.5	50.3±0.4*	27.9±1.8	-7.7±2.5	-24.1±6.4
33	P51	1.7±13.8	12.9±6.2	43.4±0.0*	36.9±2.2	-6.8±3.3	-2.1±3.0
34	P52	-8.8±1.5	14.3±0.2	44.4±1.0*	32.1±3.7	-17.5±0.5	-40.7±5.0
35	P53	11.4±0.3	39.5±0.6	46.4±1.3*	10.5±3.6	6.9±0.5	-0.2±1.6
36	P54	9.2±2.8	38.6±0.2	45.0±0.4*	16.8±0.8	12.0±1.5	-13.6±2.3

*represents at least 40% percentage inhibition

Table S.8 Isolates were cultured in GYM and Pharmamedia fermentation media. Crude extracts from isolates were extracted both in broth (ethyl acetate extraction) and biomass (acetone extraction). The table tabulates the results of microtiter plate antifungal assay against *C. albicans*.

No.	Isolates	Percentage inhibition (%) against <i>C. albicans</i>			
		GYM Media Extracts		Pharmamedia Extracts	
		EA	Acetone	EA	Acetone
1	P1	18.0±4.2	-2.5±1.3	89.2±0.6*	29.6±7.8
2	P3	22.5±2.9	13.5±6.5	12.3±3.6	8.3±3.6
3	P4	8.2±1.1	-6.2±4.5	7.2±3.1	-4.5±0.7
4	P5	22.0±1.8	11.3±5.9	8.8±1.6	-9.6±16.3
5	P6	9.5±11.9	4.1±3.5	0.5±11.1	9.4±12.3
6	P7	20.3±8.2	7.3±5.8	12.0±7.9	4.5±5.7
7	P9	1.7±2.2	-10.9±1.5	9.8±2.3	0.3±4.7
8	P10	11.5±5.0	-15.1±0.3	20.6±1.8	5.7±1.1
9	P11	12.1±5.7	45.9±3.8*	25.4±4.0	104.8±2.4*
10	P12	17.1±3.3	6.7±4.2	-26.6±1.7	12.8±7.0
11	P14	19.8±3.8	17.5±8.7	14.9±3.6	13.4±6.9
12	P15	17.8±2.0	54.1±4.4*	7.8±1.6	-20.5±4.0
13	P16	17.5±1.6	36.8±2.8	9.4±6.5	0.2±2.8
14	P17	15.6±4.0	16.8±1.7	-1.9±2.3	-16.9±6.5
15	P18	14.6±5.8	25.0±2.1	8.5±2.5	-2.6±4.1
16	P19	10.8±10.3	31.2±3.6	-5.7±1.5	-32.9±1.9
17	P20	9.9±8.5	26.8±3.2	8.1±4.9	-32.9±1.1
18	P21	4.5±5.7	-4.6±1.8	13.5±4.1	8.2±8.3
19	P22	33.8±2.8	98.1±0.2*	57.3±1.6*	102.3±0.2*
20	P23	11.7±2.8	12.3±0.5	8.3±5.4	-26.4±1.8
21	P24	5.9±5.5	24.1±5.5	17.3±17.7	-22.1±3.1
22	P25	5.9±0.5	28.2±2.3	5.5±3.0	-30.5±0.4
23	P26	26.0±7.5	13.4±6.5	-6.2±4.0	11.1±2.5
24	P27	26.5±6.0	22.0±5.6	13.6±2.2	16.9±2.5
25	P28	27.0±10.6	23.3±3.0	29.4±2.5	58.0±3.6*
26	P29	28.8±3.3	26.3±1.2	3.1±3.4	13.1±2.8
27	P31	26.4±3.6	24.3±5.1	-22.1±9.9	8.9±0.4
28	P32	31.7±2.0	14.8±7.4	-21.1±2.9	0.4±3.9
29	P34	28.2±1.8	29.0±3.9	-7.1±2.1	11.3±1.2
30	P36	21.5±7.9	1.6±4.6	-21.3±4.5	31.0±3.4
31	P42	22.1±4.1	22.8±3.5	-14.1±2.4	12.6±0.5
32	P46	8.0±5.2	-14.2±0.9	15.8±5.4	8.9±2.5
33	P51	35.9±1.7	30.8±1.6	-4.6±4.5	10.6±1.4
34	P52	13.6±5.7	19.5±9.2	-15.42±6.3	9.3±0.9
35	P53	20.5±8.9	5.9±1.8	-9.9±5.0	21.3±3.4
36	P54	24.2±3.5	19.5±3.1	-21.2±9.4	8.5±0.9

*represents at least 40% percentage inhibition

Table S.9 Anti-biofilm assay against *P. aeruginosa* PA01.

No.	Isolates	OD ₆₀₀ of biofilm	Percentage inhibition (%)
1	SE1	0.3108±0.02	61.2±7.9*
2	SE2	0.3384±0.03	50.2±11.3*
3	SE3	0.3635±0.02	40.2±6.4*
4	SW1	0.5470±0.03	-33.0±10.1
5	SW3	0.4205±0.08	17.5±32.8
6	SW5	0.3344±0.03	51.8±13.3*
7	SW9	0.3990±0.07	26.0±27.0
8	SW10	0.3553±0.08	43.4±33.2*
9	SW15	0.3417±0.12	48.9±49.9*
10	SW16	0.3074±0.04	62.5±14.2*
11	SW19	0.3084±0.04	62.1±17.2*
12	SW24	0.4267±0.06	15.0±22.1
13	SD1	0.3419±0.04	48.8±14.5*
14	SD2	0.3661±0.06	39.2±22.3
15	SD3	0.3506±0.02	45.3±8.4*
16	SD4	0.3725±0.04	36.6±17.1
17	SD5	0.3581±0.02	42.3±9.4*
18	SD7	0.3969±0.08	26.9±30.4
19	SD8	0.3968±0.06	26.9±22.4
20	SD9	0.2422±0.03	88.5±12.5*
21	SD10	0.4304±0.06	13.5±24.3
22	SD11	0.4098±0.04	21.7±14.2
23	SD17	0.3258±0.06	55.2±24.2*
24	SD18	0.4187±0.11	18.2±43.1
25	SD19	0.3683±0.05	38.3±21.0
26	SD21	0.3712±0.07	37.1±26.9
27	SD24	0.3880±0.03	30.4±12.9
28	SD25	0.4384±0.08	10.3±32.7
29	SD26	0.4024±0.06	24.7±24.2
30	SD29	0.3730±0.04	36.4±16.3
31	SD32	0.3242±0.02	55.9±6.7*
32	SD33	0.3063±0.02	63.0±6.8*
33	SD35	0.2616±0.03	80.8±13.5*
34	SD37	0.3494±0.06	45.8±22.4*
35	SD38	0.3408±0.01	49.2±5.8*
36	SD40	0.4310±0.06	13.3±24.6
37	SD41	0.4338±0.07	12.2±29.5
38	SD42	0.4164±0.07	19.1±28.6
39	SD46	0.3560±0.06	43.2±24.0*
40	SD48	0.2589±0.04	81.9±16.8*
41	SD49	0.3222±0.07	56.7±27.3*
42	SD50	0.3620±0.04	40.8±16.6*
43	SD52W	0.3316±0.02	52.9±7.9*
44	SD52Y	0.4226±0.08	16.6±33.3
45	SD55	0.5432±0.08	-31.4±32.3
46	SD60	0.4205±0.10	17.5±40.8
47	SD67	0.3452±0.05	47.5±19.2*
48	SD70	0.3387±0.03	50.1±13.8*
49	SD72	0.3409±0.05	49.2±20.4*
50	SD73	0.3392±0.05	49.9±21.8*
51	SD74	0.3794±0.01	33.9±5.2
52	SD75	0.3597±0.04	41.7±14.2*

53	SD77	0.3362±0.07	51.1±27.0*
54	SD83	0.4186±0.11	40.5±10.3*
55	SD84	0.4208±0.11	-2.6±26.7
56	SD85	0.3061± 0.02	63.1±6.3*
57	SD86	0.3925±0.08	28.6±30.6
58	SD90	0.333±0.02	52.2±8.7*
59	SD91	0.3205±0.02	57.3±9.5*
60	SD92	0.4279±0.08	14.5±29.9
61	SD93	0.3875±0.05	30.6±19.7

*represents at least 40% percentage inhibition

Table S.10 antiSMASH-predicted BGCs for *Micromonospora* sp. MD118

Cluster No.	Type descriptor	Position		Most similar known cluster (% similarity)
		From	To	
1	Terpene	2432	45282	-
2	Type 3 PKS	1547269	1588330	Alkyl-O-dihydrogeranyl-methoxyhydroquinones (71%)
3	Phosphonate	1683110	1723967	Gentamicin (7%)
4	NRPS	1730905	1790422	Dynemicin (13%)
5	Terpene	2194495	2215421	Sioxanthin (100%)
6	Terpene	2295055	2316020	Phosphonoglycans (3%)
7	Type 1 PKS-NRPS	4467023	4697756	Macbecin (43%)
8	NRPS-Siderophore-Type 1 PKS-Other KS	4758985	4913021	Dynemicin (23%)
9	Type 1 PKS-NRPS	5013021	5080660	Bleomycin (12%)
10	Terpene	5552330	5573532	Nocathiacin (4%)
11	Type 2 PKS	5767757	5810328	Xantholipin (16%)
12	Oligosaccharide-Terpene-NRPS	5982942	6054675	Lobosamide (13%)
13	NRPS	6101525	6162762	Lobosamide (6%)
14	Type 3 PKS-Other KS-NRPS	6236774	6364472	Teicoplanin (26%)
15	Bacteriocin-Terpene	6582319	6611897	Lymphostin (38%)
16	Terpene	6749616	6761541	Apramycin (6%)

Table S.11 antiSMASH-predicted BGCs for *Streptomyces* sp. SD24

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Ectoine	Ectoine (100%)
2	Terpene	Colabomycin (9%)
3	Type 1 PKS	Borrelidin (9%)
4	NRPS	Enduracidin (8%)
5	Bacteriocin	-
6	Bacteriocin-Lantipeptide-Type 1 PKS	Tetronasin (5%)
7	Linaridin-Siderophore	Desferrioxamine B (100%)
8	Other	Echosides (88%)
9	Type 1 PKS	Nanchangmycin (45%)
10	Terpene	2-methylisoborneol (100%)
11	Other	Echosides (11%)
12	NRPS	Bleomycin (6%)
13	Terpene	Hopene (53%)
14	Lasso peptide	Chaxapeptin (42%)
15	Type 2 PKS	Spore pigment (83%)
16	Thiopeptide-NRPS	Landepoxcin (16%)
17	Butyrolactone	-
18	Type 1 PKS	Nanchangmycin (81%)
19	Other KS	Acarviostatin (25%)
20	NRPS	Stenothricin (22%)
21	Terpene	-
22	Other KS-Type 1 PKS-NRPS	Maklamicin (6%)
23	Terpene	Actinomycin (21%)
24	Bacteriocin	Leinamycin (2%)
25	Type 1 PKS	Herbimycin (13%)
26	Type 1 PKS	Herboxidiene (3%)
27	Nucleoside	Toyocamycin (40%)
28	Lantipeptide	-
29	Type 1 PKS-NRPS	Thuggacin (15%)
30	Other KS-NRPS	Himastatin (8%)
31	Terpene	Rabelomycin (4%)
32	NRPS	-
33	Oligosaccharide-NRPS-Type 2 PKS-Trans AT PKS-Other KS	Saquayamycin Z / galtamycin B (47%)
34	Type 1 PKS	Epoxomicin (25%)
35	Lantipeptide	SBI 06990 alpha / SBI 06989 beta (100%)
36	Lantipeptide	-
37	Lasso peptide	-
38	Type 1 PKS-NRPS	Sch47554 / Sch47555 (5%)
39	Type 1 PKS	SF2575 (8%)
40	Type 1 PKS	Meilingmycin (31%)
41	Trans AT PKS-Type 1 PKS-NRPS	Oxazolomycin (39%)
42	Type 1 PKS	Kedarcidin (1%)
43	Terpene	Geosmin (100%)
44	Type 1 PKS	Fosfazinomycin (32%)
45	Lasso peptide	A54145 (5%)
46	Arylpolyene-Ladderane	WS9326 (22%)
47	Lantipeptide	SapB (75%)
48	NRPS	Telomycin (8%)
49	Type 1 PKS	Meilingmycin (9%)
50	Other KS	Heterocyst (28%)
51	Type 1 PKS-NRPS	Meilingmycin (10%)
52	Type 1 PKS	Tetronasin (15%)
53	Siderophore	-
54	Type 1 PKS-NRPS	A54145 (8%)
55	Type 1 PKS-NRPS	Meilingmycin (14%)
56	Type 1 PKS	ECO-02301 (17%)
57	Type 1 PKS	Tetrocarcin A (15%)

58	Type 1 PKS	Herboxidiene (4%)
59	Terpene-Ladderane-Trans AT PKS-Other KS-NRPS	Pentalenolactone (52%)
60	Type 1 PKS	Lasalocid (20%)
61	Type 1 PKS-NRPS	WS9326 (22%)
62	Trans AT PKS-Type 1 PKS-Siderophore- NRPS	Lobophorin (16%)

Table S.12 antiSMASH-predicted BGCs for *Streptomyces* sp. SD50

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Type 1 PKS	Nanchangmycin (75%)
2	Siderophore	-
3	Ectoine	Ectoine (100%)
4	Other KS	Acarviostatin (25%)
5	Lantipeptide	SapB (75%)
6	Lantipeptide	SBI 06990 alpha / SBI 06989 beta (100%)
7	Lantipeptide	-
8	Type 2 PKS-Oligosaccharide-Other KS	Saquayamycin Z / galtamycin B (47%)
9	NRPS	WS9326 (22%)
10	Arylpolyene-Ladderane	WS9326 (25%)
11	Lasso peptide	A54145 (5%)
12	Terpene	Colabomycin (9%)
13	Nucleoside	Toyocamycin (40%)
14	Type 1 PKS	Meridamycin (23%)
15	Type 1 PKS	Meilingmycin (53%)
16	Type 1 PKS-NRPS	Meilingmycin (22%)
17	Bacteriocin-Lantipeptide-Type 1 PKS	Tetronasin (5%)
18	Siderophore-Linaridin	Desferrioxamine B (100%)
19	Other	Echosides (88%)
20	Terpene	Rabelomycin (4%)
21	Siderophore	-
22	Type 1 PKS	Borrelidin (9%)
23	NRPS	Enduracidin (8%)
24	Bacteriocin	-
25	Type 2 PKS	Spore pigment (83%)
26	Lasso peptide	Chaxapeptin (42%)
27	Terpene	Hopene (76%)
28	Thiopeptide-Type 1 PKS-NRPS	Clarexpoicin (26%)
29	Terpene	Geosmin (100%)
30	Butyrolactone	-
31	Ladderane-NRPS	Skylamycin (16%)
32	Other KS-NRPS	Clorobiocin (7%)
33	Type 1 PKS-NRPS	Thuggacin (15%)
34	Other	Laspartomycin (4%)
35	Terpene	-
36	Other KS-Type 1 PKS-NRPS	Maklamicin (6%)
37	Terpene	Actinomycin (21%)
38	Bacteriocin	Leinamycin (2%)
39	Type 1 PKS	Herbimycin (13%)
40	Type 1 PKS	Herboxidiene (4%)
41	Type 1 PKS-NRPS	Rimocidin (18%)
42	Type 1 PKS	Nanchangmycin (51%)
43	Type 1 PKS-NRPS	Sch 47554 / Sch 47555 (5%)
44	Type 1 PKS	Lasalocid (54%)
45	Other KS-Terpene-Trans AT PKS-Type 1 PKS-NRPS	Pentalenlactone (52%)
46	Type 1 PKS	Kedarcidin (1%)
47	Trans AT PKS-NRPS	Oxazolomycin (45%)
48	NRPS	-
49	Type 1 PKS-NRPS	Tetrocarcin A (24%)
50	Terpene	2-methylisoborneol (100%)
51	Other	Echosides (11%)
52	Lantipeptide	-

Table S.13 antiSMASH-predicted BGCs for *Streptomyces* sp. SW24

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Terpene	Hopene (61%)
2	Type 1 PKS-NRPS	Paenibactin (83%)
3	Bacteriocin	-
4	Type 1 PKS	Incednine (8%)
5	Butyrolactone	-
6	Type 1 PKS	Herboxidiene (4%)
7	Type 1 PKS	Aldgamycin (10%)
8	Type 2 PKS	Spore pigment (75%)
9	Type 1 PKS	ECO-02301 (32%)
10	NRPS	Frankiamicin (21%)
11	Terpene	-
12	Terpene	2-methylisoborneol (100%)
13	Other KS	Galbonolides (30%)
14	Terpene	Desotamide (9%)
15	Other	Echosides (100%)
16	Ladderane-Arylpolyene-NRPS	Skylamycin (38%)
17	Type 1 PKS-NRPS	Tetrocarcin (17%)
18	Terpene	-
19	Ectoine	Ectoine (100%)
20	Siderophore	Desferrioxamine B (100%)
21	Bacteriocin-Terpene	Carotenoid (63%)
22	NRPS	Gobichelin (16%)
23	NRPS	Coelichelin (100%)
24	Terpene	Oxazolomycin (6%)
25	Bacteriocin-Lantipeptide-NRPS	Mannopeptimycin (7%)
26	Type 1 PKS	Elaiophylin (66%)
27	Type 1 PKS	Salinomycin (26%)
28	Siderophore	-
29	Type 1 PKS	Telomycin (17%)
30	Type 1 PKS	Daptomycin (10%)
31	Siderophore	-
32	Other	A47934 (14%)
33	Arylpolyene-Ladderane	Skylamycin (20%)
34	NRPS	Ochronotic pigment (75%)
35	Type 3 PKS-Type 1 PKS-NRPS	Feglymycin (84%)
36	Type 1 PKS	Pactamycin (16%)
37	Indole	7-prenylisatin (40%)
38	Butyrolactone	-
39	Hserlactone-Type 1 PKS-NRPS	Concanamycin A (25%)
40	Type 1 PKS-Arylpolyene-Ladderane	Skylamycin (18%)
41	Type 1 PKS	Tetrocarcin A (11%)
42	Type 1 PKS-NRPS	Amphotericin (35%)
43	Type 3 PKS-Type 1 PKS-Lasso peptide	Nigericin (88%)
44	Type 1 PKS-NRPS	Lasalocid (13%)

Table S.14 antiSMASH-predicted BGCs for *Streptomyces* sp. P7

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Terpene-Siderophore	Kosinostatin (3%)
2	Terpene	Hopene (15%)
3	Type 3 PKS	-
4	Type 1 PKS	E-492 / E-975 (100%)
5	Other KS	A33853 (8%)
6	Siderophore	-
7	Type 1 PKS	Daptomycin (14%)
8	Siderophore	Grincamycin (8%)
9	Ectoine-Type 1 PKS	Salinomycin (24%)
10	Type 1 PKS	Streptazone E (66%)
11	Type 2 PKS	Spore pigment (66%)
12	Type 1 PKS-NRPS	Splenocin (31%)
13	Terpene	Isorenieratene (36%)
14	Lantipeptide	Sch47554 / Sch47555 (5%)
15	Melanin	Melanin (100%)
16	Butyrolactone	-
17	Other	Borrelidin (4%)
18	Terpene-Butyrolactone-Other KS	A-500359s (5%)
19	Ectoine	Ectoine (100%)
20	Indole	-
21	Type 3 PKS-Type 1 PKS	Pyrrolomycin (20%)
22	NRPS	Streptolydigin (13%)
23	NRPS	-
24	Terpene	Albaflavenone (100%)
25	Type 1 PKS	-
26	Terpene	2-methylisoborneol (100%)
27	Siderophore	Desferrioxamine B (100%)
28	NRPS	-
29	Butyrolactone	Lactonamycin (3%)
30	Type 1 PKS	Pyralomicin (11%)
31	Terpene	Hopene (69%)
32	Butyrolactone	Lactonamycin (3%)
33	Melanin	Melanin (71%)
34	Type 1 PKS	Oligomycin (38%)
35	Terpene-Butyrolactone	Gamme-butyrolactone (100%)
36	Other	-
37	Bacteriocin	Informatipeptin (28%)
38	Type 3 PKS-Terpene	Furaquinocin A (34%)
39	Terpene	Isorenieratene (36%)
40	NRPS	-
41	Other KS	-
42	Bacteriocin	-
43	NRPS	-

Table S.15 antiSMASH-predicted BGCs for *Streptomyces* sp. SD9

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Trans AT PKS	-
2	Terpene	Hopene (92%)
3	NRPS	Cyclomarin (8%)
4	Terpene	Allylmalonyl-CoA (20%)
5	Phosphonate-Ladderane	Fosfazinomycin (7%)
6	Bacteriocin	-
7	NRPS	Meridamycin (15%)
8	Type 2 PKS	Granaticin (35%)
9	Siderophore	-
10	Ladderane	Asukamycin (4%)
11	Other	-
12	Bacteriocin	-
13	Terpene	-
14	Lantipeptide	-
15	Other	Stenothricin (13%)
16	Siderophore	-
17	Lantipeptide	Sch47554 / Sch47555 (20%)
18	Siderophore	Desferrioxamine B (100%)
19	Type 1 PKS	-
20	Bacteriocin	Informatipeptin (42%)
21	Lantipeptide	Lipopeptide 8D1-1 (6%)
22	Ectoine	Ectoine (100%)
23	Melanin-Terpene	Melanin (71%)
24	Other	-
25	Bacteriocin	-
26	Type 2 PKS	Spore pigment (83%)
27	Terpene	-
28	Type 1 PKS	ECO-02301 (32%)
29	Type 3 PKS	Herboxidiene (8%)
30	Melanin	Istamycin (5%)
31	Terpene	Albaflavenone (100%)
32	Trans AT PKS	Cycloheximide / actiphenol (22%)
33	Other KS-Type 1 PKS-NRPS	A33853 (30%)
34	Type 1 PKS	Divergolide (17%)
35	Siderophore	-
36	Butyrolactone-NRPS	Scabichelin (100%)
37	Lantipeptide	-
38	Type 1 PKS	-
39	Type 1 PKS	-
40	Other KS	-
41	Type 1 PKS	PM100117 / PM100118 (26%)
42	Other KS	-

Table S.16 antiSMASH-predicted BGCs for *Streptomyces* sp. P46

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Terpene	Pentalenolactone (64%)
2	Bacteriocin-Type 3 PKS	Alkylresorcinol (100%)
3	Other	Zorbamycin (4%)
4	Bacteriocin	Informatipeptin (57%)
5	Type 1 PKS	RK-682 (100%)
6	Terpene	Cyclooctatin (75%)
7	Terpene	Hopene (100%)
8	Type 3 PKS	-
9	Siderophore	-
10	Butyrolactone-Terpene	Gamma-butyrolactone (100%)
11	Bacteriocin-Oligosaccharide	Meilingmycin (5%)
12	Type 2 PKS-Type 1 PKS	Pyrrolomycin (45%)
13	Siderophore	-
14	NRPS-Type 1 PKS-Other KS	Naphthyridinomycin (14%)
15	Terpene	Albaflavenone (100%)
16	Type 2 PKS	Spore pigment (83%)
17	Butyrolactone	-
18	Lantipeptide	-
19	Siderophore	Desferrioxamine B (100%)
20	Type 1 PKS-NRPS	SCO-2138 (50%)
21	Type 3 PKS	SCO-2138 (21%)
22	Ectoine	Ectoine (100%)
23	Other	-
24	Other	-
25	Other	Livipeptin (66%)
26	Terpene-Type 3 PKS-Cyanobactin	Furaquinocin A (100%)
27	Melanin-Terpene	Melanin (42%)
28	Type 1 PKS-NRPS	Ralsolamycin (40%)
29	Butyrolactone	Griseoviridin / viridogrisein (8%)
30	Nucleoside	Nikkomycin (100%)
31	Terpene	Anasatrienin (7%)
32	Type 3 PKS	-
33	NRPS	C-1027 (7%)

Table S.17 antiSMASH-predicted BGCs for *Streptomyces* sp. P9

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Type 2 PKS-Other KS	Fluostatin (33%)
2	Type 2 PKS-Other KS	Candicidin (33%)
3	Type 1 PKS-NRPS	Antimycin (100%)
4	Type 3 PKS	Herboxidiene (8%)
5	Indole	Antimycin (20%)
6	Terpene	Hopene (100%)
7	Terpene	Lysolipin (4%)
8	Lantipeptide	SapB (75%)
9	Siderophore	Grincamycin (8%)
10	NRPS	Calcium-dependent antibiotic (75%)
11	Lantipeptide	A54145 (5%)
12	Ectoine	Ectoine (100%)
13	Type 1 PKS	Vicenistatin (70%)
14	Type 2 PKS	Spore pigment (66%)
15	Melanin	Melanin (60%)
16	NRPS	Phosphonoglycans (3%)
17	Lantipeptide	Incednine (2%)
18	Bacteriocin	-
19	Terpene	Albaflavenone (100%)
20	NRPS	Coelichelin (100%)
21	Lantipeptide-NRPS	Coelibactin (100%)
22	Lantipeptide	SapB (100%)
23	Siderophore	-
24	Terpene	-
25	Siderophore	Desferrioxamine B (100%)
26	Terpene	Carotenoid (54%)
27	Bacteriocin	Informatipeptin (57%)
28	Terpene	Versipelostatin (5%)
29	Butyrolactone	Lactonamycin (5%)
30	NRPS	Streptothricin (100%)
31	Indole	7-prenylisatin (100%)
32	Other	Clavams (12%)
33	Terpene	Isorenieratene (85%)

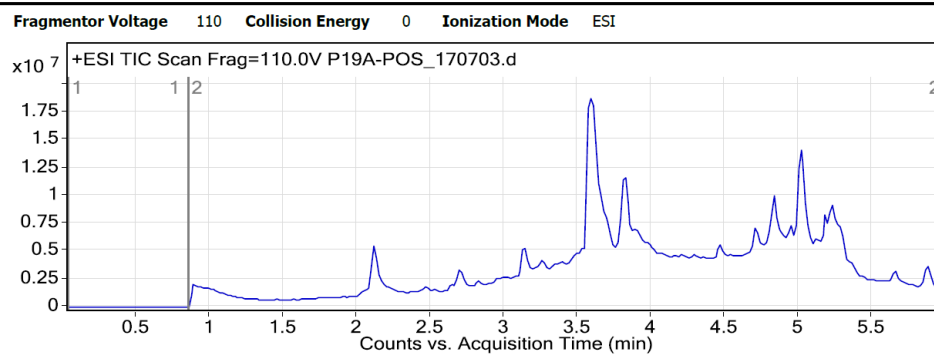
Table S.18 antiSMASH-predicted BGCs for *Streptomyces* sp. P19

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Lasso peptide	-
2	NRPS	-
3	Terpene	2-methylisoborneol (100%)
4	Terpene	SCO-2138 (28%)
5	Bacteriocin	Informatipeptin (57%)
6	Melanin	Melanin (57%)
7	NRPS	Phosphonoglycans (3%)
8	Siderophore	Lividomycin (6%)
9	Terpene	Hopene (92%)
10	Melanin	Melanin (80%)
11	Ectoine	Ectoine (100%)
12	Butyrolactone	Friulimicin (6%)
13	NRPS	Friulimicin (18%)
14	Terpene	Isorenieratene (100%)
15	Siderophore	-
16	NRPS	FD-594 (8%)
17	Bacteriocin	-
18	Terpene	Daptomycin (4%)
19	Type 3 PKS	Herboxidiene (7%)
20	NRPS	Coelichelin (63%)
21	Terpene	Albaflavenone (100%)
22	Siderophore	Desferrioxamine B (100%)
23	Type 1 PKS-NRPS	Soraphen (38%)
24	Butyrolactone	Neocarzinostatin (4%)
25	Butyrolactone	Fosfazinomycin (10%)
26	NRPS	Echinomycin (94%)
27	Butyrolactone-Type 2 PKS-Ectoine-Type 1 PKS-NRPS	Kosinostatin (49%)
28	Butyrolactone	Methylenomycin (9%)
29	Type 2 PKS-Type 1 PKS-NRPS	Antimycin (100%)
30	Type 3 PKS	Rishirilide B (10%)
31	Terpene	-
32	Lantipeptide	SBI 06990 alpha / SBI -6989 beta (75%)

Table S.19 antiSMASH-predicted BGCs for *Streptomyces* sp. MD100

Cluster No.	Type descriptor	Most similar known cluster (% similarity)
1	Terpene	Isorenieratene (100%)
2	Type 1 PKS	Undecylprodigiosin (100%)
3	Siderophore	-
4	Type 3 PKS	Herboxidiene (8%)
5	Terpene	Hopene (100%)
6	Indole	Antimycin (20%)
7	NRPS	Echinomycin (66%)
8	Bacteriocin	-
9	Terpene	Herboxidiene (2%)
10	Terpene	Carotenoid (54%)
11	Siderophore	Desferrioxamine B (100%)
12	Type 2 PKS	Spore pigment (66%)
13	Siderophore	-
14	NRPS	Coelichelin (100%)
15	Butyrolactone	Lactonamycin (8%)
16	Other	Lomaiviticin (44%)
17	Terpene	2-methylisoborneol (100%)
18	Bacteriocin	Informatipeptin (57%)
19	Trans AT PKS-NRPS	Oxazolomycin (15%)
20	Lantipeptide	Kanamycin (3%)
21	Ectoine	Ectoine (100%)
22	Trans AT PKS	Kanamycin (4%)
23	Trans AT PKS-NRPS	-
24	NRPS	Friulimicin (21%)
25	Terpene	Albaflavenone (100%)
26	Melanin	Melanin (60%)
27	Bacteriocin	-
28	Butyrolactone	Griseoviridin / viridogrisein (5%)
29	NRPS	A54145 (6%)
30	Type 2 PKS	Medermycin (11%)

User Chromatograms



User Spectra

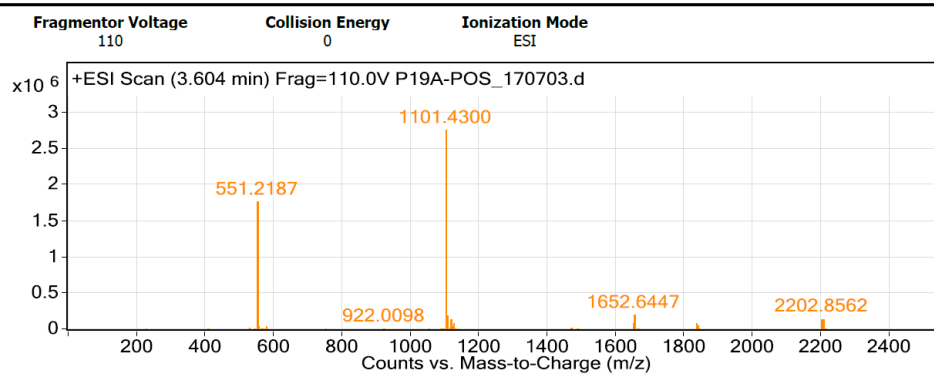


Figure S.1 ESI-HRMS spectrum of echinomycin.

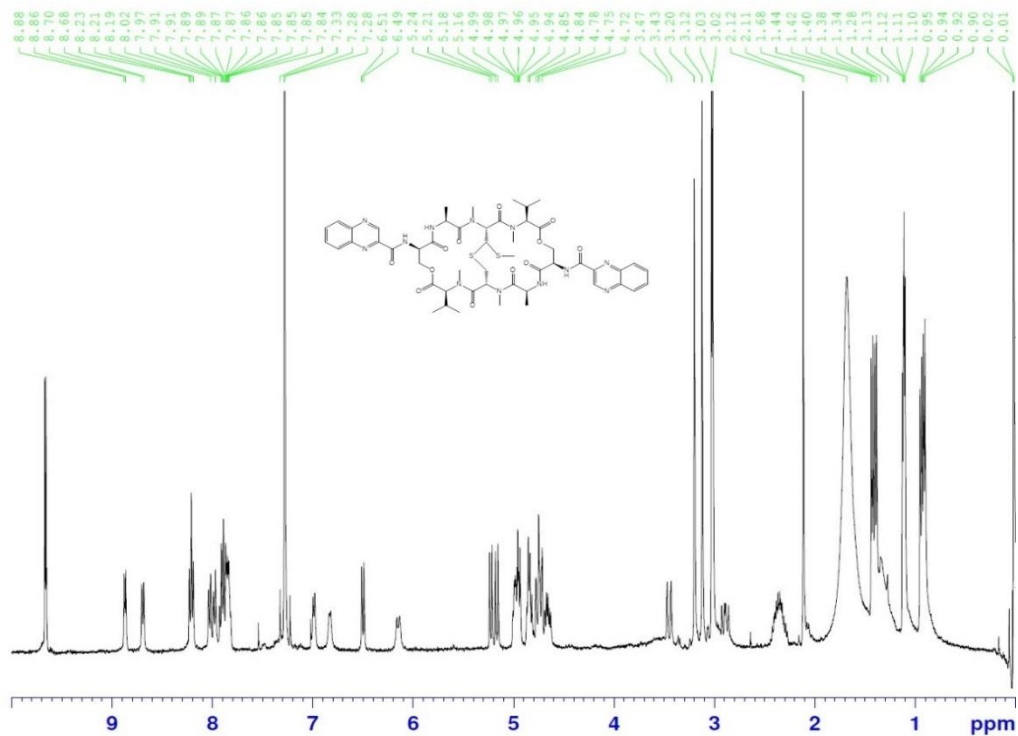


Figure S.2 ^1H NMR spectrum of echinomycin in $\text{CD}_3\text{OD}-d_4$, 400 MHz.

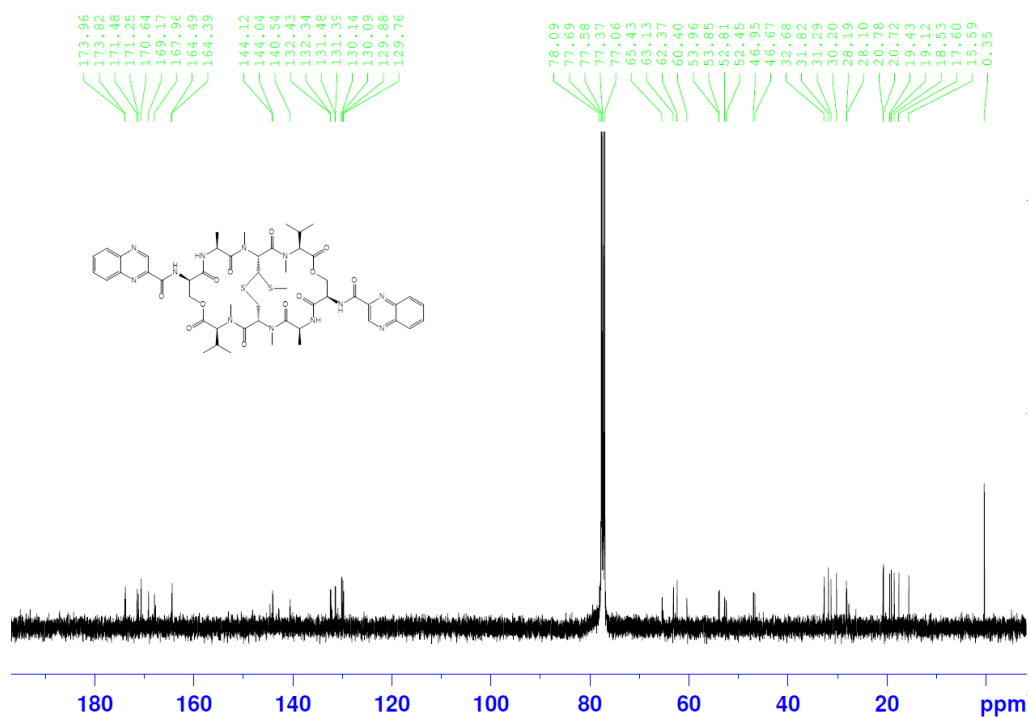
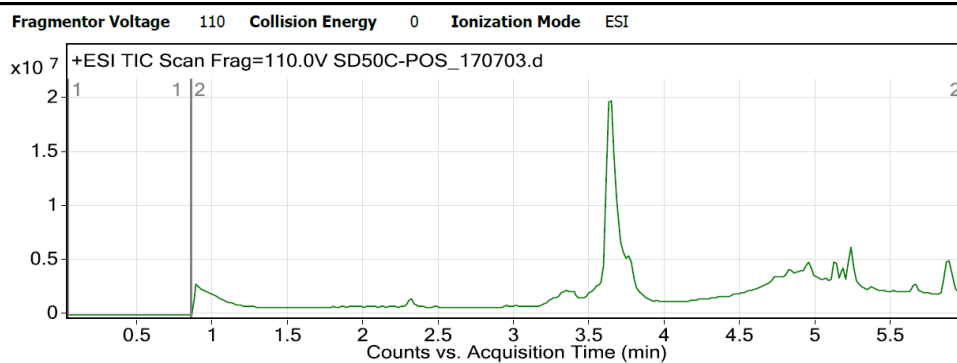


Figure S.3 ^{13}C NMR spectrum of echinomycin in $\text{CD}_3\text{OD}-d_4$, 400 MHz.

User Chromatograms



User Spectra

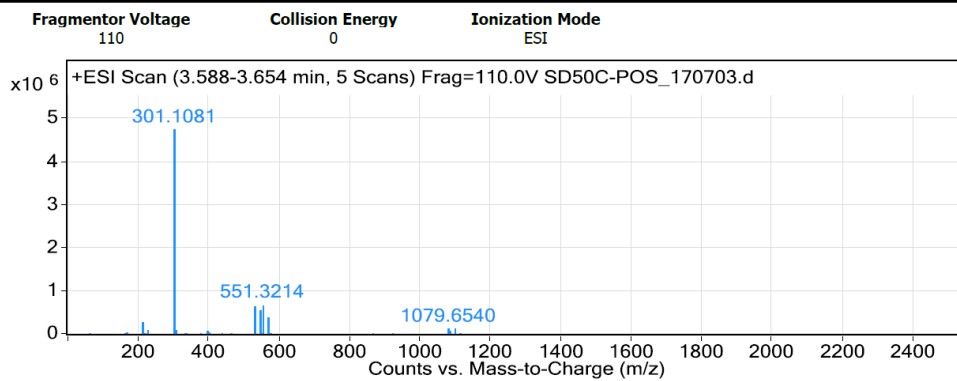


Figure S.4 ESI-HRMS spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone.

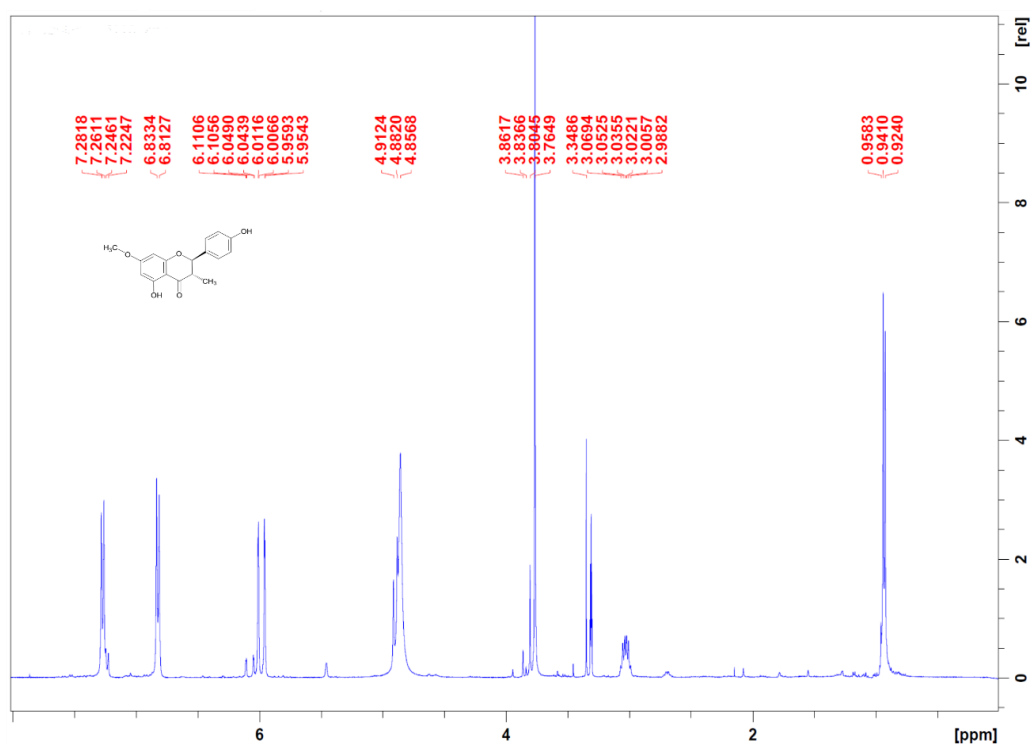


Figure S.5 ¹H NMR spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone in CD₃OD-*d*₄, 400 MHz.

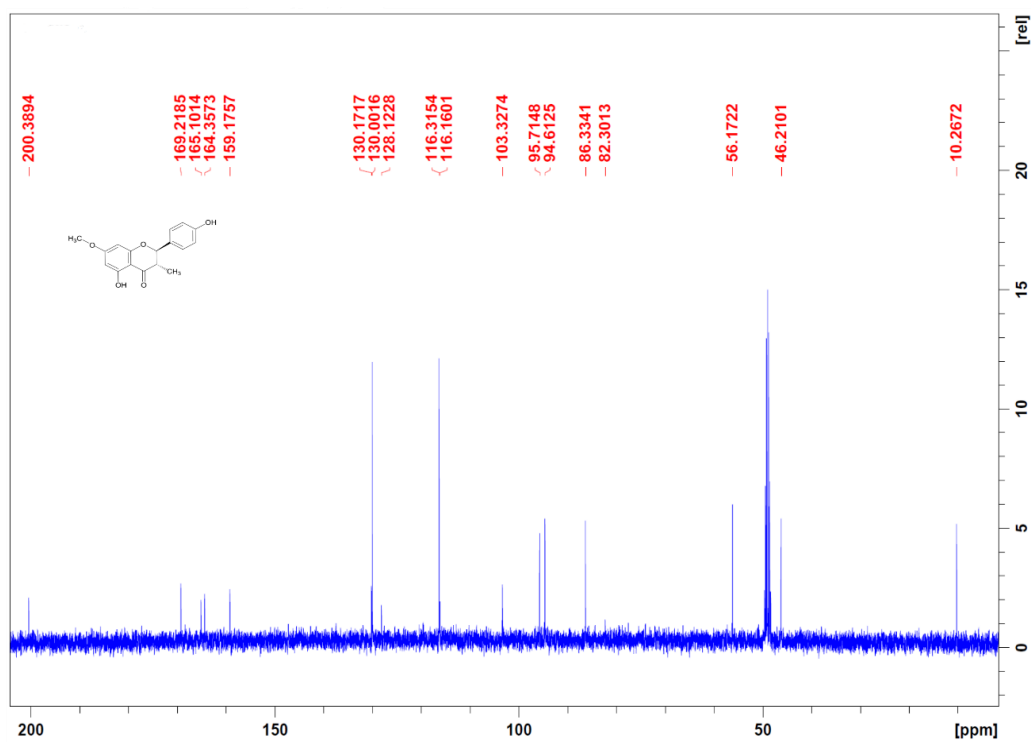


Figure S.6 ¹³C NMR spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone in CD₃OD-*d*₄, 400 MHz.

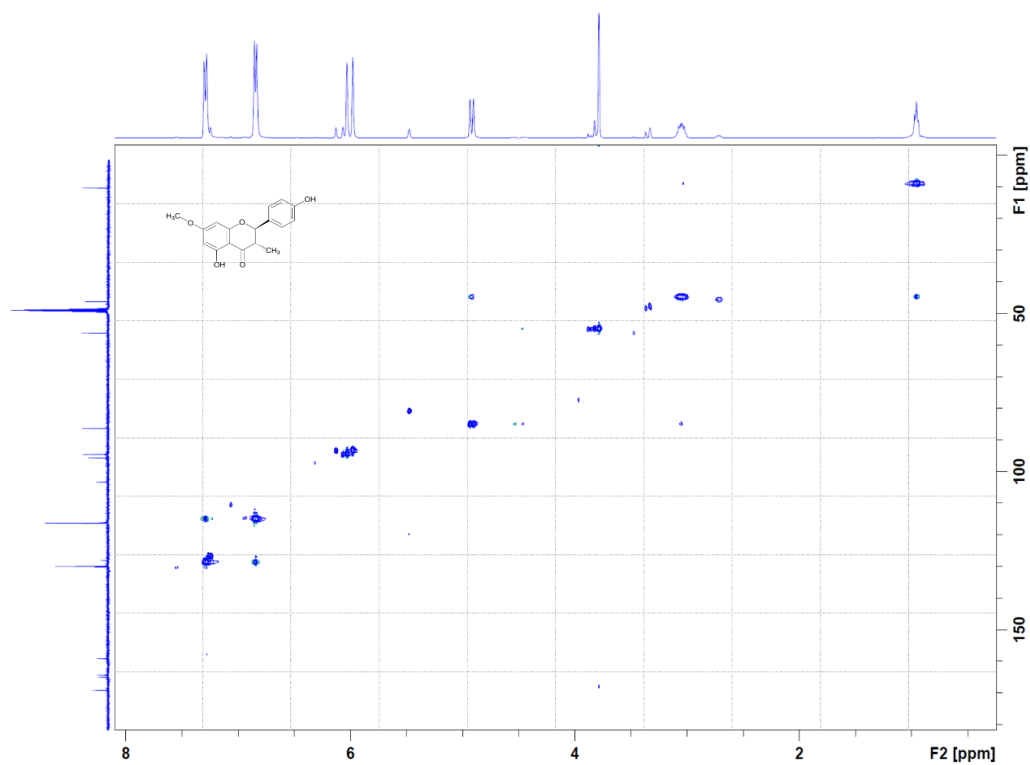


Figure S.7 HSQC spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone in $\text{CD}_3\text{OD}-d_4$, 400 MHz.

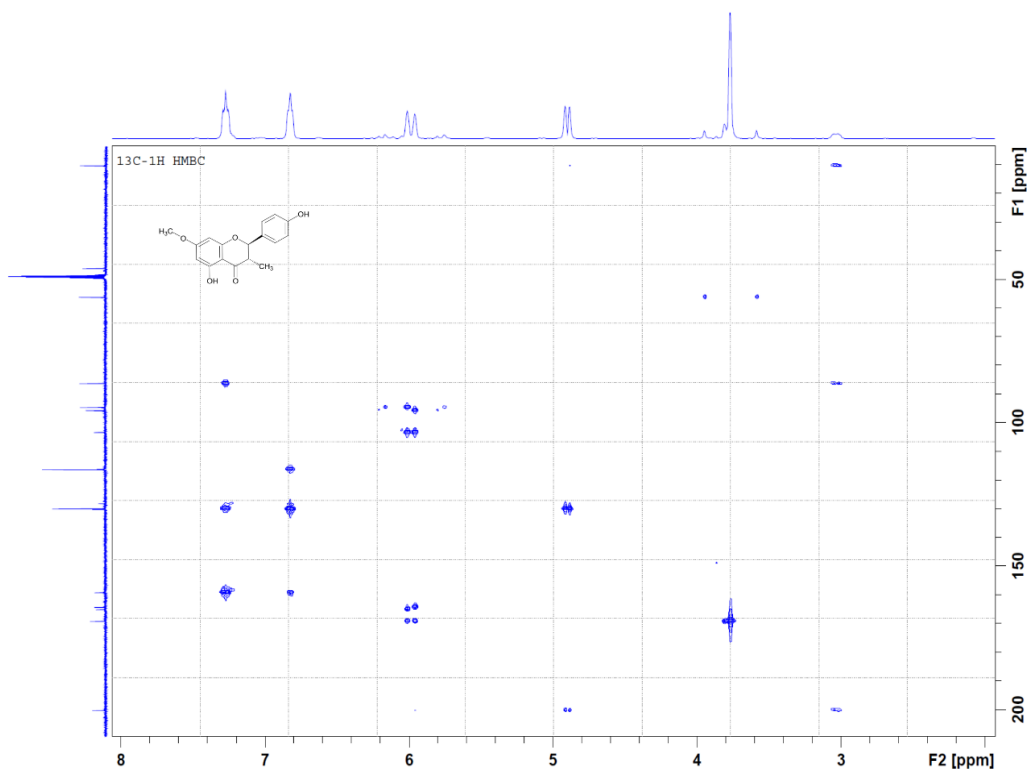


Figure S.8 HMBC spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone in $\text{CD}_3\text{OD}-d_4$, 400 MHz.

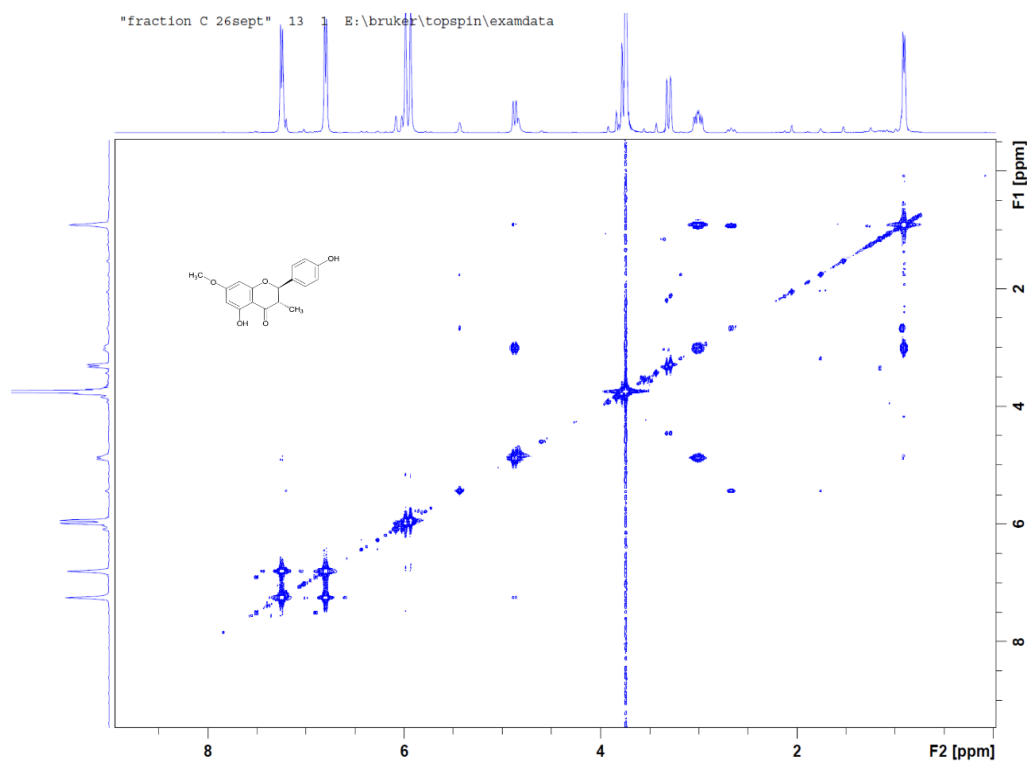


Figure S.9 COSY spectrum of 4',5-dihydroxy-7-methoxy-3-methylflavanone in $\text{CD}_3\text{OD}-d_4$, 400 MHz.