EVALUATION OF THE ANTIBIOTICS AND SECONDARY METABOLITES BIOSYNTHETIC POTENTIAL OF *Plantactinospora* sp. KBS50, A RARE MARINE-DERIVED ACTINOMYCETE

By

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ABSTRACT

Rare actinomycetes, especially from the marine environment, have been increasingly recognised as a prolific source of bioactive natural product compounds. In the search of a new source of natural products, a rare marine-derived actinomycete, designated as KBS50, was previously isolated from a beach sediment sample collected from the Santubong area of Sarawak. The aim of the present study was to evaluate the metabolic capabilities of this strain in producing antibiotics and secondary metabolite compounds, by using the traditional antimicrobial screening method as well as using a genome mining approach. Additionally, the One Strain Many Compound (OSMAC) fermentation strategy was utilised to enhance the detection of antimicrobial activities and secondary metabolites from KBS50. The first part of this thesis reports on the identification, characterization and the preliminary evaluation of the antimicrobial activities of KBS50. Based on the molecular identification using a near-complete 16S rRNA gene sequence and phylogenetic analysis, the identity of KBS50 was established as a potentially new species of the *Plantactinospora* genus. The most notable characteristics that clearly differentiate KBS50 from its closest relatives were its tolerance to high salinity of up to 7% NaCl (w/v), its ability to grow at a higher pH (pH 12), as well as its inability to grow at an incubation temperature of 40°C. KBS50 exhibited strong antagonistic activities against selected test microorganisms i.e. the Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and fungi (*Aspergillus niger*, *Ganoderma boninense*, and *Rhizoctonia solani*). The second part of this thesis reports on the sequencing of a complete genome of KBS50 for the detection and identification of secondary metabolite biosynthetic gene clusters. The whole genome sequence was assembled as a single circular chromosome comprises of 6,689,726 nucleotides. Genome annotation and analysis using the RAST server identified a total of 5,820 coding sequences and 56 RNAs. Further analysis using the antiSMASH revealed the presence of a highly diverse type of secondary metabolite biosynthetic gene clusters that include polyketide synthases, nonribosomal peptide synthetases, bacteriocins, lantipeptide/ thiopeptide, terpenes and siderophore, among others. A total of 60 biosynthetic gene clusters were identified from the genome of KBS50 using the antiSMASH analysis. The antibiotics and secondary metabolites production by KBS50 was evaluated further in the final part of this study. To enhance the detection of antibiotics and secondary metabolites, the strain was cultivated using the OSMAC fermentation approach, in which its fermentation broths were

incorporated with different types of biological and chemical elicitors. Antimicrobial screening and high-performance liquid chromatography (HPLC) analysis of the crude extracts recorded an increase in antimicrobial activities as well as increased production of secondary metabolite compounds. The OSMAC fermentation strategy also showed that the production of secondary metabolites by KBS50 was significantly affected by the various cultivation conditions and elicitor elements. This was demonstrated by the increased production of secondary metabolites when the strain was cultivated with 1% NaCl, or with the culture filtrate of *A. niger*. Through a bioassay-guided fractionation, two semi-pure bioactive compounds were successfully isolated and tested to have a strong antimicrobial activity against *S. aureus*. The strongest antimicrobial activity was recorded from compound 3 with the lowest minimum inhibitory concentration at 7.81 µg/ml and the minimum bactericidal concentration of 15.63 µg/ml. Through the classical antimicrobial screening with the combination of OSMAC fermentation strategy, and the identification of biosynthetic gene clusters from its complete genome sequence, *Plantactinospora* sp. KBS50 was found to be a prolific producer of antibiotics and secondary metabolite compounds that may possibly exhibit various biological activities ranging from the antimicrobial, anticancer or iron transport function. Therefore, the data presented in this thesis may facilitate the discovery of potentially novel bioactive compounds from KBS50 for future drug development.

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DECLARATION

I hereby declare that this research entitled "Evaluation of the antibiotics and secondary metabolites biosynthetic potential of *Plantactinospora* sp. KBS50, a rare marine-derived actinomycete" is original and contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of my knowledge contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where work is based on joint research or publications, discloses the relative contributions of the respective workers or authors.

(HOLED ANAK JUBOI)
DATE: 12 December 2017

In my capacity as the Principal Coordinating Supervisor of the candidate's thesis, I hereby certify that the above statements are true to the best of my knowledge.

(ASSOCIATE PROFESSOR DR. PETER MORIN NISSOM)
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CONFERENCE PRESENTATIONS

<u>Oral presentation</u>: Juboi, H., Hwang, S. S., Yeo, T. C., and Nissom, P. M. (2017). A Marine-Derived Rare Actinomycete, *Plantactinospora* sp. KBS50 as a Promising Source for Discovery of New Bioactive Natural Product Compound. International Conference on Natural Products (ICNP) 2017, 15-16 March 2017, Lumut, Perak, Malaysia.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ANOVA	Analysis of Variance
BGCs	Biosynthetic Gene Clusters
BLAST	Basic Local Alignment Search Tool
CDS	Coding Sequences
CFB	Cell-Free Culture Broth
CFU	Colony-Forming Unit
CMC	Carboxymethyl Cellulose
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTAB	Cetyl Trimethylammonium Bromide
DAD	Diode Array Detector
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
HGAP	Hierarchical Genome Assembly Process
HPLC	High-Performance Liquid Chromatography
HTS	High Throughput Screening
ISP	International Streptomyces Project
LB	Luria Broth
LC-MS	Liquid Chromatography–Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MTP	Microtiter Plate
NA	Nutrient Agar
NCBI	National Center for Biotechnology Information
NGS	Next-Generation Sequencing
NMR	Nuclear Magnetic Resonance
NRPS	Nonribosomal Peptide Synthetases
OD	Optical Density
OSMAC	One Strain Many Compound
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PIRG	Percentage Inhibition of Radial Growth

PKS	Polyketide Synthases
RAST	Rapid Annotations Using Subsystems Technology
RNA	Ribonucleic Acid
RO	Reverse Osmosis
SDB	Sabouraud Dextrose Broth
SEM	Standard Error of the Mean
SMA	Skim Milk Agar
SMRT	Single-Molecule Real-Time

PROJECT WORKFLOW

Identification and characterization of *Plantactinospora* sp. KBS50

- Strain cultivation and maintenance.
- Morphological and physiological characterization.
- Strain identification using 16S rRNA for BLAST analysis.
- Phylogenetic tree analysis.

Preliminary screening and profiling of the biological activities of *Plantactinospora* sp. KBS50

- Screening for proteolytic and glucanase activities.
- Preliminary screening of antagonistic activities.
- Preliminary screening of antimicrobial activities of secondary metabolites.
- HPLC profiling of secondary metabolites produced by KBS50.

Identification of secondary metabolite biosynthetic capabilities of *Plantactinospora* sp. KBS50 through genome sequencing and analysis

- Extraction and purification of genomic DNA.
- Genome sequencing using the PacBio SMRT Sequencing technology.
- *De novo* sequence assembly and genome annotation.
- Identification and analysis of secondary metabolites biosynthetic gene clusters (BGCs).

Enhancement of the antimicrobial activity and secondary metabolites detection from *Plantactinospora* sp. KBS50 using the OSMAC approach

- Cultivation of KBS50 using fermentation media supplemented with chemical and biological elicitors using the OSMAC strategy.
- Antimicrobial screening of crude extracts
- Comparative secondary metabolites profiling using HPLC to determine the effect of chemical and biological elicitors towards secondary metabolites production

Bioassay-guided fractionation for the isolation of bioactive secondary metabolite compounds

- Production of antimicrobial compounds in 2 litre fermentation.
- Bioassay-guided fractionation for isolation and purification of bioactive compounds.
- Antimicrobial screening to determine the MIC and MBC of the isolated compounds.

Introduction and Literature Review

1.1.   Introduction

The emergence of antibiotic-resistant pathogens is becoming a major health concern globally. This situation is aggravated further by the declining number of new drug candidates to combat infectious diseases caused by these pathogens. According to Lewis (2013), most of the antibiotics currently in use today are natural products that were discovered during the sixties or their derivatives thereof. It was estimated that more than one-third of the pathogenic bacteria are no longer susceptible to the existing antibiotics, thus causing a high rate of death among the patients infected with the antibiotics-resistant pathogens (Bérdy 2012). At the same time, the number of new antibiotics or other drug candidates discovered in recent years remain very low (Boucher et al. 2009). This is indeed a worrying situation that, if left unresolved, could have an undesirable consequence on the supply of effective treatment for infectious diseases and may even lead to a total widespread of the drug-resistance pathogens. Hence, there is an urgent need to screen and discover new sources of natural products for drugs development.

Unfortunately, discovering new drug candidates is not easy especially with the high rate of the rediscovery of known compounds. This is one of the main reason for the slow discovery rate of new drug candidates from natural products (Bérdy 2005, 2012; Boucher et al. 2009; Lam 2007). In addition, the high investment required for natural product screening and the greater emphasis on combinatorial chemistry had driven many major pharmaceutical companies to abandon natural product research during the nineties. Notwithstanding that, the last decades have seen an improved interest in natural product research and development (Harvey 2008). The current trend in the natural product research, particularly the increased emphasis on rare actinomycetes from unique environments such as the marine ecosystem (Lam 2006; Subramani & Aalbersberg 2012; Zotchev 2012), and advances in genome mining strategy (Challis 2008; Zerikly & Challis

2009; Ziemert et al. 2016; Zotchev et al. 2012) could potentially accelerate the drug discovery process once again.

Screening of rare actinomycetes from the less-explored environment and from the biodiversity-rich region such as Sarawak might be the key to the discoveries of novel bioactive compounds. Sarawak is the home for diverse types of forest ecosystems that are rich in biological resources for natural product screening (Yeo et al. 2014). At present, the natural product discovery from the microbial sources in Malaysia is still scarce although various studies have shown that the Malaysian ecosystem contains diverse groups of actinomycetes with promising biological activities (Jeffrey 2008; Jeffrey & Halizah 2014; Muramatsu 2008; Muramatsu et al. 2011; Numata & Nimura 2003; Tan et al. 2002; Yeo et al. 2014). Considering that various groups of actinomycetes including the rare genera can be found in Sarawak (Yeo et al. 2014), there is a huge prospect for the discovery of novel natural product compounds from the local isolates.

Based on these premises, this research project was undertaken to assess the metabolic capabilities of a rare actinomycete strain isolated from the marine-associated environment in Sarawak. This project leveraged on the classical approach to natural product screening coupled with the genome mining approach to evaluate the antibiotics production and the biosynthetic potential of the unique actinomycete strain. The findings from this study reflect on the importance of genome mining strategy to complement the classical approach in natural product bioprospecting from microbial sources. Through this project, the value of rare actinomycetes from the marine-associated environment in Sarawak would be further recognised as a potential source of novel natural product compounds and hence may contribute to the discovery of new antibiotics and other drug candidates beneficial for human beings.

1.2. Literature Review

1.2.1. Overview of natural product drugs discovery

Naturally-derived molecules known as natural products are produced by the living organisms, including plants, animal and microorganism. The major source of natural products from the microbial world are fungi, actinomycetes and myxobacteria, which produces secondary metabolite compounds that are rich in structural diversity and biological activities (Cragg & Newman 2013). Secondary metabolites produced by these microorganisms have been the major source of bioactive compounds with extensive medical and pharmaceutical application. These include the antibiotics, antiparasitic agents, anticancer, and immunosuppressive agents, among others. Since the unintentional discovery of penicillin in the 1920s by Alexander Fleming and its subsequent development as the first antibiotics in the 1940s, microorganisms have been studied extensively for their capability to produce bioactive natural product compounds and continuously providing diverse chemical structures for the drug discovery screening program (Demain 2014).

Drug discoveries were at its peak during the 1960s as the results of extensive screenings of natural products. Unfortunately, the discovery rate of new natural product compounds has declined sharply in the nineties (Bérdy 2012; Cragg & Newman 2013). The major reasons for this trend were the high rate of the rediscovery of already-known compounds and the decline of research activities in drug discovery from natural products by major pharmaceutical companies (Bérdy 2005, 2012; Boucher et al. 2009; Lam 2007). In addition, the increased use of high throughput screening (HTS) method necessitated the prevalent use of combinatorial chemistry to provide a large number of compounds in the shorter time frame for screening. The traditional approaches in drug discovery, which rely extensively on bioprospecting large number of microbial strains, were considered inefficient and costly (Bérdy 2012; Genilloud et al. 2011), and could not provide sufficient input for the HTS. However, despite a large number of synthetic compounds generated through the combinatorial chemistry for the HTS platform, it has failed to deliver on its initial prospect of delivering many new bioactive compounds as potential drug candidates (Lewis 2013).

The increasing needs for novel bioactive compounds that could not be fulfilled through combinatorial chemistry resulted in a renewed interest in natural product screening. One of the main reason for this is owing to the realisation that compounds discovered from natural products have clear advantages over combinatorial chemistry with regards to their vast structural diversity, biological activity and their potential use as templates for developing synthetic or semi-synthetic derivatives (Lam 2007). The advancement in genetics and genomics further revealed that the biosynthetic potential of these microorganisms is actually much greater than previously recognised (Challis 2008). Considering that the vast majority of existing drugs were discovered from the screening of soil-derived cultivable microorganism, which represents only a small proportion of the total microorganism in the soil, there is still a huge prospect of finding novel natural product compounds from the microorganisms (Demain 2014).

The recent advances in natural product screening may potentially propel the discovery rate of new drug candidates to new heights. Technological advancements that are especially beneficial to natural product screening include the genome mining and modern analytical techniques. Genome mining is viewed as a revolutionary approach to drug discovery and quickly becoming one of the valuable tools for studying the biosynthetic potential of microorganisms (Perić-Concha & Long 2003; Ziemert et al. 2016). Genome mining was made possible with the rapid development of DNA sequencing technologies coupled with sophisticated computational methodologies (Medema & Fischbach 2015). Likewise, the analytical techniques have evolved from the basic form of thin layer chromatography to the more sophisticated techniques including the high-performance liquid chromatography (HPLC), gas chromatography, mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy (Ravali et al. 2011; Siddiqui et al. 2017). The liquid chromatography technique when coupled with mass spectrometry (LC-MS) is a highly sensitive, fast and accurate method for natural product analysis especially for compound dereplication, identification and to some extend the chemical structure elucidation (Bouslimani et al. 2014).

The most interesting development in analytical technique in recent years is perhaps the molecular networking which was developed based on the application of tandem high-resolution mass spectrometry (Frank et al. 2008; Watrous et al. 2012). Molecular networking technique could provide a link between the biosynthetic gene clusters (BGCs)

identified through genome mining to their respective secondary metabolite products (Duncan et al. 2015; Klitgaard et al. 2015; Nguyen et al. 2013; Trautman & Crawford 2016). Molecular networking can also be a powerful tool for compound dereplication strategy (Duncan et al. 2015; Yang et al. 2013). These technological and analytical advances that complement classical screening method could potentially increase the rate of discovery of novel bioactive compounds from natural products sources.

1.2.2. General features of actinomycetes

Actinomycetes are a group of Gram-positive bacteria with filamentous morphology and high G+C content genomic DNA. The word actinomycete is a general term used to describe the groups that belong to the order *Actinomycetales* (Adegboye & Babalola 2013), which consist of many suborder, families and genera with diverse morphological characteristics. Although many of the suborder under the order *Actinomycetales* has been elevated into orders (Zhi et al. 2009), the term is still widely used but no longer refer to the order *Actinomycetales* specifically, but wider groups across many order under the class *Actinobacteria*. The term is also often used interchangeably with actinobacteria. Actinomycetes are ubiquitous in nature and can be found in various habitats including terrestrial, freshwater, marine, and plants (Ul-Hassan & Wellington 2009).

Because of their unique morphological characteristics on an agar plate, most actinomycetes can be easily recognised and differentiated from other types of bacteria. Some of the unique characteristics include the filamentous hyphae with extensive branching and the production of spores on the aerial or the substrate hyphae (Lechevalier & Lechevalier 1967). The spore structures, their shape and the spore chain arrangements are important morphological characteristics for identification and classification of actinomycetes (Li et al. 2016). These basic morphological characteristics on agar media are keys to their preliminary identification during isolation for strain dereplication. The identification of actinomycetes, similarly with other bacteria, are based on the polyphasic approach which is a combination of comprehensive tests involving the phenotypic and genotypic methods (Vandamme et al. 1996). A simple molecular identification based on the 16S rRNA gene sequence and phylogenetic analysis can be done to provide a rapid identification of actinomycetes up to the genus level (Muramatsu 2008).

Actinomycetes are a very good producer of enzymes with cellulolytic, proteolytic, and amylolytic activities, among others (Prakash et al. 2013). They play important biological functions including nitrogen fixation, biological control agents, and the degradation of organic materials in nutrient cycling (Chaudhary et al. 2013). Actinomycetes are also one of the microbial sources of pigments for natural colourants (Tuli et al. 2015). Although most actinomycetes are not known to be pathogenic, some species including the member of *Nocardia*, *Actinomadura*, *Gordona*, and *Rhodococcus*, among others, can cause serious clinical diseases in human and animals, especially in the immunocompromised host (Mcneil & Brown 1994). Because of their well-known capability to produce enzymes, antibiotics and other high-value compounds with medicinal properties, actinomycetes are an important source of natural products for the pharmaceutical and industrial applications.

1.2.3. Natural product drugs discovery from actinomycetes

Actinomycetes have been the subject of intensive research in drug discovery screening program around the world due to their ability to produce bioactive secondary metabolite compounds. Secondary metabolites are the products of secondary metabolism which are generally not essential for the growth of the producing organism but may provide advantages in adaptation and survival in the natural environment (Adegboye & Babalola 2013). Many secondary metabolites discovered from actinomycetes are medically and economically important for humans especially the antibiotics which are being used to combat illnesses resulted from microbial infection as well as for agricultural purposes.

According to Demain (2014), about 32% from more than 20,000 bioactive compounds discovered from microorganism were produced by actinomycetes. Various genera of actinomycetes including the *Streptomyces*, *Micromonospora*, *Actinoplanes*, *Saccharopolyspora* and *Amycolatopsis* are well-known producers of bioactive secondary metabolite compounds (Solanki et al. 2008). The vast majority of antibiotics discovered from actinomycetes were produced by the *Streptomyces* genus (de Lima Procópio et al. 2012; Watve et al. 2001).

The discovery of streptomycin in 1944 from the *Streptomyces griseus* (Schatz et al. 1944) marked the onset of actinomycetes as one of the primary source of antibiotics and other bioactive compounds. Streptomycin was the first antibiotics used to treat tuberculosis.

Since then, there were numerous other antibiotics discovered from actinomycetes including chloramphenicol, nystatin, erythromycin, vancomycin and gentamycin, which possesses broad spectrum or narrow spectrum antimicrobial activities against pathogenic bacteria, yeast or fungi (de Lima Procópio et al. 2012; Solecka et al. 2012).

The secondary metabolites produced by the actinomycetes are diverse in chemical structures that include the major classes of antibiotics such as ß-lactams, aminoglycosides, glycopeptides, macrolides, tetracyclines, polyenes and anthracyclines (Adegboye & Babalola 2013; Solecka et al. 2012). These secondary metabolites exhibit the various mechanism of actions such as the inhibition of cell wall synthesis, protein biosynthesis, DNA replication, RNA synthesis, and interfering with other essential cellular functions (de Lima Procópio et al. 2012).

1.2.4. Classical approach to natural product screening and isolation

The traditional or classical approach to natural product bioprospecting from microbial sources require the collection of environmental samples for strain isolation, followed by the fermentation and extraction of secondary metabolite compounds. A collection of microbial extracts is then screened in a bioassay to identify extracts with 'hits', upon which the bioactive pure compounds will be isolated and purified through a series of bioassay-guided fractionation for subsequent structural elucidation and characterization. Throughout the years, this basic principle of drug discovery remains unchanged, although various improvements have been made along the way to provide better screening efficiency and sensitivity at a much higher throughput (Gray et al. 2012).

The success of the classical approach was evident judging from the large number of new antibiotics that have been discovered from the microbial sources (Baltz 2006). However, the high rate of the rediscovery of known compounds from actinomycetes had resulted in the diminishing returns on natural product drugs discovery. The classical approach which involves the screening of a large number of actinomycetes strains or other microbial sources is costly and has a very small success rate in delivering new compounds (Genilloud et al. 2011).

To minimize the rediscovery rate and to increase the chances of finding new compounds, the classical approach was refocused to the screening of actinomycetes from unique and under-explored or unexplored environmental habitats such as the marine environment (Bredholt et al. 2008; Fiedler et al. 2005; Hong et al. 2009; Jensen et al. 2005; Lam 2006), higher plants (Qin et al. 2009a; Strobel & Daisy 2003) and desert (Ding et al. 2013; Harwani 2013; Takahashi et al. 1996), as well as screening the taxonomically diverse group of actinomycetes with greater emphasis on the rare genera (Genilloud et al. 2011; Lazzarini et al. 2000; Tiwari & Gupta 2012).

1.2.5. Rare actinomycetes

Rare actinomycetes generally have low isolation frequencies as compared to the common actinomycetes (Tiwari & Gupta 2012). In general term, rare actinomycetes may refer to all the non-*Streptomyces* species which are generally more difficult to cultivate during the isolation process (Tiwari & Gupta 2012, 2013). Some of these rare actinomycetes genera include *Micromonospora*, *Actinoplanes*, *Amycolatopsis*, *Dactylosporangium*, *Nonomuraea*, and *Thermomonospora*, among others (Hayakawa 2008). However, not all of the rare actinomycetes are difficult to cultivate, although some modifications to the conventional isolation method are usually required to increase their isolation frequency. For example, *Actinoplanes* and *Micromonospora* are the major groups of cultivable soil actinomycetes after *Streptomyces* which have been isolated extensively for natural product screening (Lazzarini et al. 2000). Similar to other common actinomycetes group, rare actinomycetes can also be found in the various type of ecosystems including terrestrial and aquatic environment (Tiwari & Gupta 2012). Many new genera and species are continuously being discovered in recent years from the marine environments, forest soils, peat swamps, and plant tissues, among others (Tiwari & Gupta 2013).

The conventional isolation method of which serially diluted samples are plated on isolation agar media often yield the fast-growing filamentous *Streptomyces* and other microorganisms. Therefore, the isolation process for the rare actinomycetes usually involves physical or chemical pretreatment method to eliminate common actinomycetes or enrichment method to improve the isolation frequencies of the non-streptomycetes species (Hayakawa 2008). Some of these methods may include pretreatment of the soil samples at a high temperature of 120°C, or pretreatment using 1.5% phenol or chloramine

T, while the enrichment may involve rehydration and centrifugation, or baiting using pollen grains (Hayakawa 2008). The pretreatment or enrichment methods are necessary to suppress the growth of the unwanted microorganism and to allow the slow-growing rare actinomycetes to proliferate on the isolation agar plates. Isolation also often requires specialised agar formulated to enhance the growth and recovery of particular genera of rare actinomycetes (Hayakawa 2008; Hayakawa & Nonomura 1987; Tiwari & Gupta 2012).

Rare actinomycetes have been increasingly isolated and screened for natural products, resulted in the discoveries of novel compounds (Jose & Jebakumar 2013; Tiwari & Gupta 2014). Although most of the antibiotics discovered from actinomycetes were produced by *Streptomyces* species, some antibiotics were only discovered from the non-*Streptomyces* species (Baltz 2006). The fact that the isolation frequency of rare actinomycetes is much lower than *Streptomyces* could be the main reason for the smaller percentage of novel compounds discovered from the rare actinomycetes group.

To increase the success rate of new compounds discovery, Baltz (2006) recommended that the screening program should exclude most common actinomycetes and *Streptomyces* species. Understanding the key morphological features of actinomycetes can as well increases the success rate in isolating the rare actinomycetes from the environmental samples. Feature, such as spore morphology, can be used to differentiate between *Streptomyces* species and the much rarer group such as the many members of *Micromonosporaceae*. By lowering the frequency of common actinomycetes while increasing the number of rare actinomycetes during isolation, there is a higher chance of finding potentially novel species of rare and unknown actinomycetes (Hayakawa 2008). Thus, the likelihood of discovering new natural product compounds from these rare actinomycetes is more likely to increase, while at the same time, reducing the possibility of rediscovering known compound frequently associated with common actinomycetes.

Overall, there has been a noticeable increase of bioactive compounds discovered from rare actinomycetes. According to Berdy (2005), over 2,500 bioactive compounds were discovered from rare actinomycetes by the year 2003 as compared to about only 50 known compounds in the year 1970. Based on the survey carried out by Lazzarini et al. (2000), about 16% of the total of over eight thousands antibiotics produced by microorganism

was discovered from the rare actinomycetes. The prolific producer of bioactive compounds among the rare actinomycetes genera includes *Micromonospora*, *Actinoplanes*, *Saccharopolyspora* and *Amycolatopsis* (Solanki et al. 2008). They produce some of the antibiotics currently available in the market including rifamycins, erythromycin, vancomycin and gentamycin (Tiwari & Gupta 2012). With over 200 genera (Tiwari & Gupta 2012), the rare actinomycetes group represent a significant source of potentially novel bioactive secondary metabolite compounds for drug discovery.

1.2.6. Marine actinomycetes

Actinomycetes isolated from the marine environment was once thought to be originated from the terrestrial environment from which the spores were washed off to the ocean (Goodfellow & Haynes 1984, cited in Jensen et al. 2005). However, the discoveries of novel actinomycetes from the marine samples (Magarvey et al. 2004; Maldonado et al. 2005; Mincer et al. 2002; Tian et al. 2009) provided the evident that specific group or species of actinomycetes are naturally adapted to the marine ecosystems and they may represent new taxa easily differentiated from their terrestrial counterparts.

The examples of actinomycetes genera exclusively isolated from marine environments include *Salinispora* (Maldonado et al. 2005) and *Marinispora* (Kwon et al. 2006). Common characteristics between these marine actinomycetes are their requirement of seawater for growth (Subramani & Aalbersberg 2012). Some marine actinomycetes are also belong to known genera, for example *Streptomyces* (Huang et al. 2016; Moran et al. 1995), *Micromonospora* (Phongsopitanun et al. 2015; Veyisoglu et al. 2016) and *Verrucosispora* (Supong et al. 2013), of which their members are commonly found from the terrestrial environments.

Marine ecosystem represents an underexploited source of rare actinomycetes species for natural product drug discovery. Due to the extreme differences in conditions between the marine and terrestrial environments, it is estimated that marine actinomycetes are capable of producing unique secondary metabolite compounds with important biological activities (Lam 2006). In fact, actinomycetes from the marine ecosystem have been increasingly recognised in recent years as an alternative source of novel natural product drug candidates. Many efforts have been made to explore the marine actinomycetes that

resulted in the discovery of novel compounds with various biological activities including antibacterial, antifungal, and anticancer (Solanki et al. 2008). These compounds include salinosporamide A (Feling et al. 2003), abyssomycin C (Bister et al. 2004) and caboxamycin (Nigh et al. 2004). According to Solanki (2008), many of these compounds have unique chemical structures from which the synthesis of new compounds for drug development may be based upon.

Marine actinomycetes have been recovered from various type of samples including marine water, sediments, sponges and seaweeds (Genilloud et al. 1994; Mincer et al. 2002; Pham et al. 2014; Zhang et al. 2008). However, sample collection, strain isolation and cultivation of marine actinomycetes from deep-sea sediment or organisms often require specialised equipment and techniques (Subramani & Aalbersberg 2012; Zotchev 2012). Isolation and cultivation techniques remain the major obstacle in recovering many of the marine actinomycetes as the number of cultivable species are far less than what has been estimated using culture-independent or metagenomic approach (Zotchev 2012). Other marine-associated environments such as the intertidal zones and mangrove forest also present high diversity of actinomycetes as another source of bioactive secondary metabolite compounds for drug discovery (Amrita et al. 2012; Azman et al. 2015; Xu et al. 2014).

1.2.7. Screening for antimicrobial compounds

Antimicrobial compounds produced by the actinomycetes may inhibit the growth of other microorganisms through the various mode of actions including the inhibition of DNA replication or by blocking the synthesis of RNA, cell wall or protein (de Lima Procópio et al. 2012). Not all antimicrobial compounds are equal in term of its bioactivity spectrum, as certain antibiotics can only kill Gram-positive bacteria but not Gram-negative, while other antibiotics may kill both (Coates et al. 2002). In general, the Gram-negative bacteria are more resistance to antimicrobial compounds as compared to Gram-positive bacteria. The differences are largely due to their cell wall components and functions which could restrict the uptake of antimicrobial compounds inside the cells, thus limiting the destructive effect of the compound (Coates et al. 2002). The antimicrobial activities of an antimicrobial agent can be detected using the bioassay screening. In a drug discovery pipeline, preliminary antimicrobial screening is an important initial step to identify the

potential producer of antibiotics from a large collection of microbial isolates. The secondary metabolites production with antimicrobial properties from the selected strains can be evaluated further in the secondary screening.

There are a number of bioassay methods that can be employed for the preliminary screening including perpendicular streak method (also known as cross streak method) and agar overlay method. In perpendicular streak method, the actinomycete strain is streaked on agar medium as a single line across the agar. After incubation for a few days, the strain will produce antimicrobial compound(s) which will diffuse into the agar. Bacterial test strains are then inoculated as a single streak perpendicular to the actinomycete culture. Antagonistic activity can be observed directly after further incubation, whereby the growth of test strains along the streaking line nearest to the actinomycete might be inhibited (Velho-Pereira & Kamat 2011). In agar overlay method, the actinomycete strain is spot inoculated onto agar medium. After several days of incubation, another layer of soft agar containing a standardised suspension of the test strain is then added. Further incubation will results in the inhibition area around the actinomycete colony if the antimicrobial compound is present (Gebreyohannes et al. 2013).

In secondary screening, agar diffusion method using either agar-well diffusion or disk diffusion can be employed to evaluate the presence of antimicrobial compounds in the spent fermentation broth or the organic crude extracts of actinomycetes (Balouiri et al. 2016; Valgas et al. 2007). In both methods, a standardised suspension of test strain from an overnight culture is inoculated onto agar medium. Paper disk impregnated with the crude extract suspension is then placed onto the agar surface. For agar-well method, the crude extract suspension or the spent fermentation broth will be introduced directly into the agar well. The antimicrobial compound will diffuse into the surrounding agar and inhibit the growth of test strain, producing the inhibition diameter around the agar well or the paper disk. Agar disk diffusion method is also used as a standard method to evaluate the susceptibility of bacterial pathogens towards antimicrobial drugs (Jenkins & Schuetz 2012).

Another important assay often performed to evaluate the susceptibility of a microbial test strain towards an antimicrobial compound is the minimum inhibitory concentration or MIC (Andrews 2001). For the determination of MIC, agar disk diffusion method or broth

dilution method can be employed. The definition of MIC is the minimum or the lowest concentration of an antimicrobial compound (pure antibiotic or crude extract) that can impede the "visible growth" of the test microorganism after it was incubated overnight in the presence of the antimicrobial compound (Andrews 2001). Subsequently, the minimum bactericidal concentration (MBC) or minimum fungicidal concentration can be performed to determine the nature of the growth inhibition caused by the antimicrobial compound, as to whether it was bactericidal/fungicidal or bacteriostatic/fungistatic (Balouiri et al. 2016).

1.2.8. DNA sequencing technology

The development of DNA sequencing technology started with the chain-termination method or widely known as Sanger sequencing, which was developed by Frederick Sanger in 1977 (Liu et al. 2012). Since then, DNA sequencing technology has improved tremendously with the introduction of an automatic sequencing machine based on Sanger sequencing, followed by the development of second-generation or next-generation sequencing (NGS) technologies that enabled higher sequencing throughput at a reduced cost (Buermans & den Dunnen 2014).

There are several NGS technologies currently available including Roche 454, SOLiD, HiSeq, and PGM system which uses different sequencing mechanism, and thus has their own advantages and disadvantages (Buermans & den Dunnen 2014; Liu et al. 2012; Mardis 2008). In recent years, third generation sequencing technologies have emerged that include the single-molecule real-time (SMRT) sequencing (Rhoads & Au 2015) and nanopore sequencing (Feng et al. 2015; Jain et al. 2016). The similarities between these two platforms that differentiate them from NGS are that the sequencing process does not require any PCR amplification and the signal is detected in real time (Liu et al. 2012). The NGS and third-generation sequencing technology have a wide range of applications including metagenomics, transcriptomics, whole genome resequencing or *de novo* sequencing and epigenomics (Buermans & den Dunnen 2014; Liu et al. 2012; Mardis 2008).

1.2.9. Microbial genome sequencing

The first complete genome of a bacteria, *Haemophilus influenza*, was successfully sequenced in 1995 using the shotgun sequencing strategy (Fleischmann et al. 1995). A similar strategy was then used to sequence many other microorganisms (Fraser et al. 2000). Ten years later, there were about 300 sequenced microbial genomes available publicly (Binnewies et al. 2006). During that period, microbial genomes were primarily sequenced using the Sanger sequencing technology (Forde & O'Toole 2013). The NGS platforms were only started to emerge from 2005 onward (Liu et al. 2012). Ever since, the number of sequenced microbial genomes has increased dramatically for the past 12 years with about 100,000 genome sequences (bacteria and archaea) available in the public database (NCBI 2017).

It is predicted that the number of microbial genomes sequences will continue to increase and could reach millions within the next 10 years with the rapid advances in sequencing technology and as more efforts being put into the genome sequencing (Medema & Fischbach 2015). However, according to Land et al. (2015), most of the bacterial genomes deposited into NCBI GenBank database are not complete assemblies. These sequences, primarily generated using the NGS platforms, are available either as draft or contigs. For microbial genome sequencing, SMRT sequencing (also known as PacBio sequencing) have few advantages over other NGS technologies in that it generates longer sequencing reads with the average of more than 10 kb. Longer reads are relatively easier to assemble into a single contig to generate a complete genome sequence and therefore is better suited for the *de novo* sequencing application where no reference genome is available (Rhoads & Au 2015). SMRT sequencing is also particularly useful for sequencing of the genome with high GC content (Koren et al. 2013; Rhoads & Au 2015; Shin et al. 2013).

Genome sequencing and assembly are based on the concept of shotgun sequencing strategy, whereby smaller-sized DNA fragments representing different areas of the whole genome are sequenced and then reconstructed into longer stretches of DNA pieces by overlapping the reads using computer algorithm (Ekblom & Wolf 2014). During the initial step, short reads are assembled into contigs which subsequently joined to produce longer scaffolds. With most NGS platform, the short reads are more difficult to realign due to the shorter overlapping regions between the reads (Ekblom & Wolf 2014).

Depending on the coverage of the sequencing and the presence of repeats, the final assembly may contain gaps which may need to be filled (Ekblom & Wolf 2014; Koren & Phillippy 2015).

Sequence assembly is a computer-intensive task as there could be millions of reads to be assembled. This requires a considerable amount of computing resources, time and bioinformatics knowledge. With the longer reads from SMRT sequencing and the improvements in sequence assembly algorithm, the task of assembling a complete microbial genome is getting easier, faster and can even be automated (Koren & Phillippy 2015). For a microbial genome that is relatively small, the assembly may produce a single contig representative of the bacterial chromosome. Obtaining a long contig or a complete genome sequence is vital for downstream analysis such as annotation (Ekblom & Wolf 2014). Once a complete microbial genome is obtained, the annotation can be performed using the Rapid Annotations using Subsystems Technology (RAST) server (Aziz et al. 2008; Overbeek et al. 2005, 2014) to provide a meaningful information and interpretation of the genetic information available in the genome sequence.

1.2.10. Secondary metabolites biosynthesis in actinomycetes

In fungi and bacteria including the actinomycetes, secondary metabolite compounds such as polyketides and nonribosomal peptides are synthesised through metabolic pathways involving large, multi-domain enzymes (Anzai et al. 2003; Mootz & Marahiel 1997; Schwecke et al. 1995; Shen 2003). The enzyme complexes for the biosynthesis of polyketides and nonribosomal peptides are known as polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), respectively. These enzymes are encoded by the secondary metabolite BGCs that contain a series of genes arranged adjacent to each other in the chromosomal DNA. The BGCs encode necessary components for the synthesis of secondary metabolites, including the core enzyme modules system and other accessory proteins (Medema & Fischbach 2015). The biosynthesis of secondary metabolites is a highly regulated process that involves transcriptional regulators and transporters which are usually encoded by other genes adjacent to the BGCs (Adegboye & Babalola 2013).

Remarkably, the PKS and NRPS, two most studied secondary metabolite biosynthetic systems, produce a large number of structurally and functionally diverse natural product compounds exhibiting various biological activities including antibiotics, antiparasitic, antitumor, biosurfactant and immunosuppressive properties (Adegboye & Babalola 2013; Mootz & Marahiel 1997; Staunton & Weissman 2001). The vast chemical diversity of compounds produced by these biosynthetic pathways are the results of the multi-step assembly line of the highly modular enzymes. Structural variations in the enzyme complex are the result of the arrangement of different functional units or modules that catalyse different chemical reaction during the elongation of polyketides or polypeptides chain (Cane & Walsh 1999).

Over the past two decades, our basic understanding of the secondary metabolites biosynthetic pathways has improved tremendously as the results of an extensive amount of studies to decode the molecular mechanism and enzymology involves in the biosynthesis processes. This knowledge has enabled *in silico* identification of the biosynthetic genes, as well as the prediction of the structural features and physicochemical properties of secondary metabolites compounds produced through the enzymatic pathways encoded by the genes (Zerikly & Challis 2009).

1.2.11. Genome mining for natural product discovery

Before the genomic era, identification of biosynthetic genes from actinomycetes relies on PCR-based screening. This method allows initial evaluation of the biosynthetic potential of actinomycetes, upon which the metabolically capable strains can be selected and exploited for the discovery of novel secondary metabolite compounds. PCR primers were designed to target conserved region of the core domain in the BGCs, such as the genes that encode for keto-synthase and acyltransferase domain of the PKS type-I (Ayuso-Sacido & Genilloud 2005; Ostash et al. 2005), the adenylation domain of the NRPS (Ayuso-Sacido & Genilloud 2005), or the phosphoenolpyruvate mutase enzyme (*pepM*) of the phosphonate biosynthetic pathways (Ju et al. 2015), among others.

While PCR-based screening could detect the presence of biosynthetic genes, the primers used were designed based on the conserved regions of DNA, thus limiting the use from detection of less conserved gene sequences or novel genes (Ayuso-Sacido & Genilloud 2005). This limitation can be overcome through a genome-wide screening of BGCs. One notable example of the application of PCR-based screening on a large scale was demonstrated by the work of Ju and colleagues (Ju et al. 2015). The research group screened about 10,000 actinomycetes strains for the presence of phosphonate biosynthetic genes and narrow down the potential producer of phosphonate to a few hundred strains. Subsequently, the presence of *pepM* was confirmed from the analysis of the draft genomes of these actinomycetes. Further dereplication of the biosynthetic pathways and screening of the strains with potential novel BGCs led to the discovery of novel phosphonate natural product compounds with antimicrobial activities (Ju et al. 2015).

Genome mining showed that actinomycetes have much larger capacity to produce bioactive secondary metabolites than previously recognised. *Streptomyces coelicolor* was among the first actinomycetes having its complete genome sequence analysed for BGCs. It was learnt that the strain has over 20 BGCs responsible for the production of known and unknown secondary metabolites which was more than the number of natural product compounds that has been isolated from this species under the conventional screening method (Bentley et al. 2002; Challis 2008). Genome mining of other actinomycetes also revealed similar observation (Ikeda et al. 2003; Oliynyk et al. 2007; Omura et al. 2001;

Udwary et al. 2007). The actinomycetes genome contains an average of around 20 gene clusters per actinomycete strain (Farnet & Zazopoulos 2005; Van Lanen & Shen 2006).

Our ability to sequence a complete genome of actinomycetes and other microorganisms, in general, has a big impact on microbial genomics and provided new insights into natural product research (Bode & Müller 2005; Van Lanen & Shen 2006). Coupled with sophisticated bioinformatics tools and a large amount of ever increasing sequence data in the public databases, genome mining strategy has revolutionised the natural product discovery to further revealed the true metabolic potential of the microorganisms (Challis 2008; Ziemert et al. 2016; Zotchev et al. 2012). The wealth of genetic information obtained from genome mining will further improve our understanding of the secondary metabolites biosynthesis by the actinomycetes, enabling genetic manipulation and expression of the biosynthetic genes and consequently increase our chances in finding novel compounds from these prolific microorganisms for drug development. After all, the capability of actinomycetes to produce secondary metabolite compounds could only be limited by its genetic capacity (Bode et al. 2002).

1.2.12. Bioinformatics tools for genome mining

Recent years has seen a rapid development of comprehensive software and databases for genome mining application. Bioinformatics tools such as antiSMASH (Medema et al. 2011), ClustScan (Starcevic et al. 2008), and CLUSEAN (Weber et al. 2009) are among the tools that can be used to automatically identify and analyse various types of BGCs from the DNA sequence of microorganism. Prior to the creation of these bioinformatics tools, genome mining was done manually (Weber & Kim 2016).

The antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) was claimed to be a comprehensive platform for the detection and identification of secondary metabolites BGCs (Blin et al. 2017), and is capable of identifying up to 44 different types of secondary metabolites genes (Weber & Kim 2016). The antiSMASH tool is able to predict the substrate specificity of PKS and NRPS modules in the BGCs, enabling core structure prediction of the putative secondary metabolite product of the biosynthetic pathway encoded by the genes. Since the inception of its first version, antiSMASH has evolved with many improvements and integration with various algorithms to further enhance its

functionality and application for genome mining (Blin et al. 2013, 2017; Weber et al. 2015).

1.2.13. Strategies for activation of silent biosynthetic gene clusters

With the increasing number of genome sequences available from actinomycetes and other microorganisms, many more novel BGCs will continue to be discovered. The major challenges in this endeavour would be the isolation of the compounds produced through the biosynthetic pathway encoded by these BGCs, as only a small proportion of the potential metabolite compounds are usually detected in their fermentation products using the traditional screening approach (Bentley et al. 2002; Udwary et al. 2007). These biosynthetic genes were called 'cryptic' or 'silent', as no known metabolites associated with it have been isolated (Challis 2008). Poor or no gene expression may result in insufficient quantity or the nonexistence of the metabolic product for analytical detection (Laureti et al. 2011).

Following genome mining and identification of secondary metabolites BGCs, several strategies can be employed to unleash the hidden metabolic potential of actinomycetes. These include systematic variations of growth conditions, elicitation of stress response using chemical and physical factors, manipulation of regulatory mechanism that control the expression of the BGCs, ribosomal engineering and heterologous expression (Baltz 2016; Choi et al. 2015; Ochi & Hosaka 2013; Rutledge & Challis 2015; Zerikly & Challis 2009).

The utilisation of genome mining to uncover the BGCs and the various strategies to activate the silent BGCs has led to the discoveries of novel natural product compounds as well as facilitated the elucidation of their biosynthetic pathways. As an example, the identification of modular PKS type 1 gene cluster from *Streptomyces ambofaciens* ATCC 23877 and the constitutive expression of a pathway-specific regulator within the gene cluster led to the discovery of stambomycins (Laureti et al. 2011). In another example, Hayashi et al. (2014) discovered silent gene cluster encoding for thiopeptide biosynthesis from *Streptomyces lactacystinaeus* through genome mining. Heterologous expression of the gene cluster in *Streptomyces lividans* TK23 resulted in the production of lactazoles.

Ribosomal engineering was also successfully used to activate silent gene clusters for the biosynthesis of fredericamycin A from a marine *Streptomyces* species (Zhang et al. 2015). Through media optimisation experiment, the production of the anticancer compound by the mutant strain was increased significantly. A comprehensive review of the application of ribosomal engineering to activate silent BGCs can be found here (Ochi & Hosaka 2013).

1.2.14. One Strain Many Compound (OSMAC) approaches

Genome mining has shown that actinomycetes have the genetic capacity to produce more compounds that what it can produce in standard cultivation condition (Ikeda et al. 2003; Oliynyk et al. 2007; Omura et al. 2001; Udwary et al. 2007). However, during cultivation, only small number of these secondary metabolites compound can be detected, as demonstrated by the work done on *Streptomyces coelicolor* (Bentley et al. 2002; Challis 2008). This could be due to the amount of compound produced is very small that it escapes detection, or the biosynthetic genes are not active when cultivated at the selected culture condition or media composition (Reen et al. 2015). Therefore, the discovery of many compounds from a single strain may be possible if the strain were subjected to conducive cultivation conditions to trigger the biosynthesis of the secondary metabolites.

Under this pretext, the term "One Strain Many Compounds" or OSMAC was coined by Bode et al. (2002) to describe an approach that takes advantage of the biosynthetic capabilities of the secondary metabolites-producing strains including actinomycetes and fungi. This strategy can complement the classical approach of natural product discovery and does not require the application of the more technical and laborious genetic engineering techniques.

The OSMAC approach has been used to unselectively enhance the production of compounds from both fungi and actinomycetes strain through manipulation of various cultivation parameters (Bills et al. 2008; Bode et al. 2002; Hewage et al. 2014; Hussain et al. 2017; Martín 2004; Wang, QX et al. 2013). The aim is to provide nutritional and/or physical variation between different cultivation conditions which could potentially activate or increase the expression of biosynthetic genes in the strain's genome.

Parameters that could be manipulated include media composition, aeration rate, pH, temperatures, and the addition of enzyme inhibitors (Bode et al. 2002).

The production of secondary metabolites can also be enhanced by the use of co-culture or mixed-culture technique in which two microorganisms are grown together in the same media (Abdelmohsen et al. 2015), or the pseudo-mixed culture in which spent medium component from one bacterium is added to the culture medium of another bacterium, as demonstrated in research work of Watanabe et al. (1982). The use of dead cells of another microorganism such as *Bacillus subtilis* and *Staphylococcus aureus* or culture filtrate of fungi had also been shown to increase the production of secondary metabolites (Luti & Mavituna 2011; Luti & Yonis 2013; Wang, D et al. 2013).

Another approach that can be used is using chemical elicitor such as dimethyl sulfoxide (Chen et al. 2000) or scandium, a rare earth element (Kawai et al. 2007; Ochi et al. 2014). Subjecting the producer strain to physiological and chemical stress during cultivation may elicit stress response and consequently induce secondary metabolite biosynthesis (Yoon & Nodwell 2014).

Although OSMAC approach has been used successfully for the discovery of secondary metabolites in actinomycetes and fungi (Bills et al. 2008; Hewage et al. 2014; Wang, QX et al. 2013; Bode et al. 2002), there is still no guarantee that all the interesting biosynthetic genes could be expressed under the selected culture conditions (Chiang et al. 2011). The success of this method also might vary from genera or species (Spraker & Keller 2014; Vandermolen et al. 2013). In general, OSMAC is a random approach and therefore it is dependent upon the researcher to formulate a various set of fermentation conditions in order to find new natural product compounds (Bode et al. 2002). The OSMAC approach can be used following a genome mining of an actinomycete to awaken the silent biosynthetic genes and to increase the diversity of secondary metabolite compounds in the extracts for subsequent bioactivity screening and chemical analysis.

1.3. Project background and description

This research project is part of an ongoing natural product bioprospecting program at the Sarawak Biodiversity Centre (SBC). SBC as a research agency established by the Sarawak State Government is actively doing research on natural product bioprospecting from Sarawak's rich biological resources (Yeo et al. 2014). For the past few years, our group has focused on collecting diverse types of rare actinomycetes through a systematic exploration of unique environmental habitats, combined with the selective isolation of morphologically unique actinomycetes. The isolation process involves morphological observation of colonies directly on isolation agar plates and selectively purify the colonies of interest that putatively belongs to the rare actinomycetes group. This isolation strategy has led to a collection of taxonomically diverse actinomycetes strains which could be screened for the discovery of novel natural product compounds.

An effort was made to isolate actinomycetes from the marine-associated environment through a collection of beach sediment samples from the Santubong area in Kuching, Sarawak in the year 2012. A number of actinomycetes were isolated and preliminarily identified based on short (500-600 bp) 16S rRNA gene sequences (unpublished data). One of the actinomycete strain, designated as KBS50, was putatively identified as a potential member of a rare actinomycetes genus *Plantactinospora* from the *Micromonosporaceae* family. This strain was selected, among few others, for a genome shotgun sequencing project using the next-generation sequencing technology on Illumina Miseq platform. Preliminary bioinformatics analysis of these shotgun sequences has provided a snapshot into its secondary metabolite biosynthetic potential (unpublished data). Assembly of a complete genome sequence was not possible due to the low sequencing coverage or read depth generated from the sequencing project. Therefore, the complete biosynthetic capability of KBS50 could not be ascertained.

Having been identified with promising biosynthetic capability, as well as being a potentially novel species of a rare actinomycete from a family of a well-known producer of natural product compounds were the main motivations for selecting strain KBS50 from the SBC's microbial culture collection to critically investigate its biosynthetic potential.

1.4.   Research aim and objectives

This study aims to evaluate the antibiotics and secondary metabolites biosynthetic capabilities of *Plantactinospora* sp. KBS50 as a potential source of novel bioactive compounds. To achieve the aim of this research project, this work was divided into 3 main components with the following main objectives:

a) To identify, characterize and evaluate the antimicrobial activity of KBS50.
b) To assess the secondary metabolites biosynthetic capabilities of KBS50 through genome sequencing and bioinformatics analysis.
c) To enhance the antimicrobial activity and secondary metabolites detection from KBS50 using the OSMAC fermentation approach.

1.5.   Significance of the study

In general, the findings from this research project revealed that rare actinomycetes from the marine-associated environment in Sarawak are a valuable source of bioactive natural product compounds with potent antimicrobial properties. This potential was demonstrated from the study of *Plantactinospora* sp. KBS50, which showed a great potential as a producer of potent antimicrobial compounds and a plethora of other potentially bioactive secondary metabolites compounds. The genome sequencing and analysis provides an overview of the genetic and enzymatic mechanism underlying the biosynthetic pathway for these secondary metabolites. The availability of this genetic information will further facilitate the discovery of new compounds from KBS50 through the genetic engineering and manipulation of the biosynthetic genes. In addition, the results from the OSMAC fermentation provides the basis for optimisation of cultivation conditions to further enhance the production of secondary metabolites by KBS50. This research work will, therefore, pave the way for the isolation, identification and characterization of potentially novel bioactive natural product compounds from *Plantactinospora* sp. KBS50 which can be developed further as new drug candidates.

1.6. Outline of the thesis

This thesis is comprised of 5 chapters as listed below:

Chapter 1:   Introduction and literature review.

Chapter 2:   Identification, characterization and the evaluation of antimicrobial activity of *Plantactinospora* sp. KBS50.

Chapter 3:   Genome sequencing and analysis for the identification of secondary metabolite biosynthetic gene clusters from *Plantactinospora* sp. KBS50

Chapter 4:   Enhancement of antimicrobial activity and secondary metabolites detection from *Plantactinospora* sp. KBS50 using the OSMAC fermentation approach.

Chapter 5:   General summary and conclusions.

Chapter 1 provides a general introductory background and a comprehensive review of relevant literature related to the natural product research and strategies for bioactive compounds discoveries from actinomycetes. The aim, objectives and the significance of this research project are also stated in this chapter.

Chapter 2 reports on the identification, characterization and the preliminary evaluation of the antimicrobial activities of KBS50. The identity was established using the 16S rRNA gene sequence analysis, while its morphological characteristics were evaluated and compared with other related species. This chapter also reports on the production of secondary metabolites, preliminary antimicrobial screening and HPLC analysis for secondary metabolites profiling from KBS50.

Chapter 3 reports on the genome sequencing and analysis of secondary metabolites biosynthetic capabilities of *Plantactinospora* sp. KBS50 using bioinformatics tools. The complete genome was sequenced using the Pacific Bioscience's SMRT sequencing technology and assembled into a single circular chromosome. This study also reports on the genome annotation results using the RAST server, the identification of secondary metabolites BGCs in the genome sequence using the antiSMASH pipeline, as well as the comparative analysis of biosynthetic genes between KBS50 and other related species.

Chapter 4 describes the application of OSMAC fermentation approach to further enhance the antibiotics and secondary metabolites detection from KBS50. The effect of various types of elicitor elements and cultivation conditions that could affect the antimicrobial activities and the secondary metabolites production from KBS50 are discussed in this chapter. This study also highlights the isolation of two bioactive compounds with potent antibacterial activity using the bioassay-guided fractionation.

Chapter 5 summarises the salient findings and conclusions made from this research project. Future directions and recommendation for further study are also discussed in this concluding chapter.

Identification, Characterization and the Evaluation of Antimicrobial Activity of *Plantactinospora* sp. KBS50

2.1. Introduction

In the present study, an effort was made to explore the potential of an actinomycete strain KBS50, as a new source of bioactive secondary metabolites which could be further developed as a drug candidate. The strain was isolated from a beach sediment sample collected from the Santubong area in Sarawak, Malaysia. Currently, there is a lack of data on natural product discovery from local isolates, especially actinomycetes isolated from the marine environment in Sarawak. At the same time, there is an increasing need to explore new areas for the isolation of novel source of bioactive secondary metabolite compounds, owing to the decline in the discovery of new compounds as well as a surge in the incident involving antibiotic-resistant pathogens (Bérdy 2012). This research gap forms the basis of the present study, in which a rare actinomycete from Sarawak was selected for a comprehensive assessment of its capability to produce bioactive secondary metabolite compounds, especially with antimicrobial activity.

Specifically, the study in this chapter highlighted the effort made to identify, characterize and to evaluate the antimicrobial properties of actinomycete strain KBS50. The identification and classification of the strain among its closest relatives were established using a near-complete 16S rRNA gene sequence that provided a taxonomical and ecological perspective of this actinomycete. Morphological and physiological characteristics of the strain were studied to determine its unique features as compared to its closest relatives. Lastly, its antimicrobial potential against representative test microorganisms was evaluated. The results presented in this chapter provided an insight into the capabilities of a rare actinomycete strain derived from the marine-associated environment in Sarawak as a potential source of novel natural product compounds.

In this chapter, the objectives of the study were as follows:

i) To identify the actinomycete strain KBS50 using 16S rRNA gene sequence.

ii) To characterize its morphological and physiological features.

iii) To evaluate the antimicrobial activity of actinomycete strain KBS50.

2.2. Materials and Methods

2.2.1. Strain cultivation and maintenance

The actinomycete strain KBS50 was maintained on International Streptomyces Project (ISP) medium 2 agar (ISP2, per liter: Yeast extract, 4.0 g, malt extract, 10.0 g, glucose, 4.0 g, agar 15.0 g, pH 7.2 $\pm$ 0.2), while the stock cultures are stored at -80°C freezer in cryovials containing 1 ml of 20% nutrient glycerol.

2.2.2. Morphological and physiological characterization

Morphological characteristics including colony colour, mycelia and spore formation were recorded on ISP2, ISP3, ISP4 and ISP5 agar media according to the method of Shirling and Gottlieb (1966). Microscopic observation of the colony and mycelia was made by light microscope (CX-31, Olympus) directly from agar plate using a 50X long-distance objective lens. Pictures were captured using a Moticam 3 microscope camera through the Motic Image Plus v2.0 software (Motic).

The ability of strain KBS50 to grow at different concentrations of sodium chloride (0, 1, 2, 3, 4, 5, 6, 7, 10, 12, 15, & 20% w/v), different temperature range (4, 10, 20, 25, 28, 30, 37, & 40°C) and pH (4, 5, 6, 7, 8, 9, 10, 11, 12, & 13) was tested on ISP2 Agar. The growth were observed and recorded after 7, 14, 21 and 28 days of incubation. Gram staining was carried out using standard Gram stain method (Bartholomew & Finkelstein 1958).

2.2.3. DNA extraction and purification

The genomic DNA (gDNA) was extracted and purified using the CTAB method adapted from Moore et al. (2004). Strain KBS50 was cultivated in 20 ml ISP2 broth in a 125 ml Erlenmeyer flask at 28°C for 3 days with constant shaking at 200 rpm. Cells were collected from 10 ml of culture broth by centrifugation at 4000 rpm (18 cm radius of rotor, Centrifuge 5810 R, Eppendorf) for 10 minutes. The harvested cells were washed twice with sterile reverse osmosis (RO) water and resuspended in 2375 µl Tris-EDTA buffer. The cells were subjected to enzymatic digestion with the addition of 100 µl of 50 mg/ml lysozyme (final concentration of 2 mg/ml) and mixed thoroughly by inverting the tube several times. Then, 25 µl of 10 mg/ml RNAse was added to digest RNA (final concentration of 0.1 mg/ml). The tube was inverted several times to mix and then incubated at 37°C for 60 minutes. Subsequently, 350 µl of 10% sodium dodecyl sulphate was added to the mixture, followed by 150 µl of 20 mg/ml proteinase K. The mixture was incubated at 55°C for 90 minutes. Then, 600 µl of 5 M NaCl was added and the mixture was incubated at 65°C for 2 minutes. Following incubation, 400 µl of CTAB (pre-warmed at 65°C) was added and the mixture was further incubated at 65°C for another 10 minutes.

The gDNA was extracted using an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The tube was centrifuged at 10,000 g (10 cm radius of rotor, Mikro 22R, Hettich) for 5 minutes and the top layer was transferred to a new tube and extracted using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v). The tube was centrifuged at 14,000 g for 5 minutes. The top layer was transferred to a new tube for another extraction using an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The tube was centrifuged at 10,000 g for 5 minutes and the top layer was collected to a new tube. An equal volume of isopropanol was added, mixed gently and left at room temperature for 5 minutes to precipitate the gDNA. The tube was centrifuged at 14,000 g for 15 minutes to pellet the gDNA. The supernatant was discarded and the gDNA pellet was washed twice with 500 µl of 70% ethanol. The ethanol was discarded and the pellet was dried for 5 minutes in a laminar flow cabinet. Finally, the gDNA was resuspended in 200 µl Tris-Cl (pH 8.0) and stored at -20°C. The concentration was estimated by spectrophotometric measurement at A260 nm, while the quality was estimated by measurement of the A260/A280 and A260/A230 ratios using NanoVue (GE Healthcare). The size and integrity of the gDNA were estimated on 0.8% agarose gel electrophoresis.

2.2.4. Strain identification and phylogenetic analysis using 16S rRNA sequence

The 16S rRNA sequence was amplified using the universal primers pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') (Ludwig et al. 1993) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Wilson et al. 1990) under the following polymerase chain reaction (PCR) mixture: Pfu DNA Polymerase 10X buffer with MgSO$_4$, 3 µl; dNTP mix 10 mM each , 0.6 µl; forward primer 10 µM, 0.6 µl; reverse primer 10 µM, 0.6 µl; Pfu DNA Polymerase 2 u/µl, 0.6 µl; DNA template 0.1 µg/µl, 2 µl; and 22.6 µl sterile water for a total reaction volume of 30 µl. Amplification was performed in a thermal cycler using initial denaturation of 1 minute at 96°C, followed by 30 cycles of 45 seconds denaturation at 96°C, 1 minute primer annealing at 53°C, and 2 minutes extension at 72°C. Final extension was set at 72°C for 7 minutes and the reaction was held at 4°C indefinitely.

PCR product was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) according to the manufacturer's manual. Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an automated DNA sequencer, ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems) using the same primer pair. The raw sequences data obtained (reverse and forward sequence), was processed using Sequence Scanner software v2.0 (Applied Biosystems). The contig assembly and final consensus sequence from both forward and reverse sequences were generated using BioEdit program version 7.2 (Hall 1999). The Basic Local Alignment Search Tool (BLAST$^®$) (http://blast.ncbi.nlm.nih. gov/Blast.cgi) was used to analyse the 16S rRNA gene for sequence similarity in the GenBank database.

The phylogenetic tree was constructed to assess the taxonomic placement of the strain among its closest relatives. Phylogenetic tree was inferred using neighbour-joining method (Saitou & Nei 1987) and evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) with bootstrap analysis (Felsenstein 1985) of 1000 replicates using MEGA version 6 software package (Tamura et al. 2013). Sequence identity matrix was calculated using the BioEdit program version 7.2 (Hall 1999).

2.2.5. Evaluation of extracellular enzyme activity

The ability of the actinomycete strain KBS50 to produce extracellular enzymes was evaluated on agar media. The carboxymethyl cellulose (CMC) agar and Avicel agar was used to evaluate the activity of endo-1,4-ß-D-glucanase and exo-1,4-ß-D-glucanase, respectively, while skim milk agar (SMA) was used to evaluate the proteolytic activity of protease enzyme.

The basal medium for CMC and Avicel agar was prepared according to Kasana et al. (2008) (per liter: NaNO$_3$, 2.0 g, K$_2$HPO$_4$, 1.0 g, MgSO$_4$•7H$_2$O, 0.5 g, KCl, 0.5 g, peptone, 0.2 g, Agar, 17.0 g, pH 7.2 $\pm$ 0.2). One percent (10.0 g/L) of CMC or Avicel was added to the basal medium and then autoclaved at 121°C for 15 minutes. The autoclaved liquid agar was cooled to around 45°C and poured into Petri dishes at 20 ml per plate. Meanwhile, the SMA was prepared using Nutrient Agar (NA) as the basal medium (per litre: Peptone, 5.0 g, NaCl, 5.0 g, beef extract, 1.5 g, yeast extract, 1.5 g, agar, 15.0 g, pH 7.2 $\pm$ 0.2). The NA was prepared in a 900 ml RO water while the skim milk solution (20.0 g skim milk powder) was prepared in 100 ml RO water. Both solutions were autoclaved separately at 121°C for 15 minutes and then cooled to around 45°C before they were mixed and poured into Petri dishes at 20 ml per plate.

The actinomycete strain KBS50 was spot inoculated onto CMC, Avicel or SMA test agar and incubated at 28°C for up to 14-d. Glucanase activity on CMC and Avicel agar plates were detected according to the method of Sazci et al. (1986) and Brigitte and Aloisio (2014) with slight modification. The plates were flooded with 10 ml Congo red solution (1% Congo red in sterile RO water) and shaken at 50 rpm for 15 minutes. Subsequently, the Congo red solution was discarded and the plates were washed with 10 ml of 1M NaCl solution, shaken at 50 rpm for 15 minutes before it was discarded. Glucanase activity was determined from the presence of clear zone around the actinomycete colony following the staining and washing procedure. The production of protease on SMA medium was determined from the formation of a clear zone around the actinomycete colony (Alnahdi 2012; Rydén et al. 1973).

2.2.6. Preliminary screening for antagonistic activity on agar plates

Primary antimicrobial screening was carried out to determine the antagonistic activity of strain KBS50 against representatives of Gram-negative bacteria (*Escherichia coli* NBRC 3301 & *Pseudomonas aeruginosa* NBRC 12689), Gram-positive bacteria (*Staphylococcus aureus* NBRC 12732 & *Bacillus subtilis* NBRC 3134), yeast (*Saccharomyces cerevisiae* ATCC 9763), and fungi (*Aspergillus niger* NBRC 4066, *Ganoderma boninense* and *Rhizoctonia solani*). *G. boninense* and *R. solani* were locally isolated plant pathogenic fungi strains maintained at the Swinburne University of Technology Sarawak Campus.

The antimicrobial activity was evaluated using 4 types of agar medium, namely ISP2, MB (per liter: Soluble starch, 5.0 g, glucose, 5.0 g, meat extract, 1.0 g, yeast extract, 1.0, NZ-Case, 2.0 g, NaCl, 2.0 g, $CaCl_2$-$2H_2O$, 2.0 g, agar, 15.0 g, pH 7.2 $\pm$ 0.2), FM1 (per liter: Corn starch, 20.0 g, glucose, 10.0 g, NZ-Amine A, 5.0 g, yeast extract, 5.0 g, $CaCO_3$, 1.0 g, agar, 15.0 g, pH 7.2 $\pm$ 0.2), and FM8 (per liter: Sago starch, 5.0, peptone NZ Soy, 4.0, wheat flour, 5.0, glucose, 3.0, yeast extract, 3.0, $MgSO_4$•$7H_2O$, 0.5, KH_2PO_4, 0.25, K_2HPO_4, 0.25, agar, 15.0, pH 7.2 $\pm$ 0.2).

The antagonistic activity of strain KBS50 against the test microorganisms was evaluated using modified perpendicular streak method (Gebreyohannes et al. 2013; Velho-Pereira & Kamat 2011). The perpendicular streak agar plates were prepared from cell suspension of a 7-day old actinomycete culture grown on ISP2 agar which was scrapped and suspended in 5 ml sterile RO water. Fifty µl of the homogenised cells suspension was inoculated onto agar media and spread at the designated segment. The plates were then incubated at 28°C for 7 days for the actinomycete to develop and produce bioactive compounds that will diffuse into the agar medium.

Standardised inoculums of bacteria and yeast were prepared according to Andrews (2001) from overnight cultures of the test strains in Luria Broth (LB) and Sabouraud Dextrose Broth (SDB), for bacteria and yeast at 37°C and 30°C, respectively. A sterile cotton swab was dipped into the suspension of the standardised test strain (1.0 x 10^6 CFU/ml) and the excess liquid was removed by gently rotating the swab against the inner side of the tube wall. Each test strain was streaked as a straight line perpendicular to the 7-day old

actinomycete culture, leaving a gap of 5 mm between the test strain and the actinomycete culture. Agar plates without actinomycete culture were streaked similarly as a growth control. The test was carried out in three replicates. Test plates were incubated at 37°C for 24 hours and 30°C for 48 hours for bacteria and yeast, respectively. The inhibition of test strains along the streak line (measured in mm) was used as an indication of the susceptibility of the test strains towards the bioactive metabolites produced by strain KBS50 on the agar media.

For antagonistic properties against fungi, mycelial plugs of 7-day old fungi test strains grown on Potato Dextrose Agar (PDA) at 28°C were made using 6 mm sterile straw and inoculated onto the perpendicular streak agar plates at 25 mm distance from the 7-day old actinomycete culture. Control plates without actinomycete culture were inoculated similarly. Inhibition of fungi mycelia growth on the test plates was recorded after 7 days of incubation at 28°C. The measurement was taken on radial growth of fungal mycelium in the direction towards actinomycete culture. Percentage inhibition of radial growth (PIRG) over control was calculated as follows:

$$PIRG = [C - T]/C \times 100$$

Where;

C – Radial growth of fungi test strain on control plate (in mm).

T – Radial growth of fungi test strain on test plate (in mm).

2.2.7. Production of secondary metabolite in liquid culture

The production of secondary metabolites in liquid culture was induced using fermentation broths ISP2, MB, FM1 and FM8 prepared as described previously without the addition of agar. A preculture was prepared by inoculating 50 ml ISP2 broth in a 125 ml Erlenmeyer flask with a 7-day old actinomycete culture scrapped from ISP2 agar surface. The preculture was grown in an incubator shaker at 28°C for 7 days with constant shaking at 200 rpm. Each fermentation broth was inoculated with the preculture using 5% inoculum size. The cultures were then fermented at 28°C with constant shaking at 200 rpm for 7 days. Secondary metabolites were extracted from the spent broth using an equal volume of ethyl acetate, twice. The resulted organic extracts were dried *in vacuo* and

stored at 4°C until further analysis. Meanwhile, the cell-free culture broths (CFB) were collected by removing the cells by centrifugation at 4000 rpm for 10 minutes, and filter-sterilized through 0.2 μm filter.

2.2.8. Secondary screening of CFBs and crude extracts

The CFBs and crude extracts were tested for antimicrobial activity against *E.* coli NBRC 3301, *P. aeruginosa* NBRC 12689, *S. aureus* NBRC 12732, *B. subtilis* NBRC 3134, *S. cerevisiae* ATCC 9763, and *A. niger* NBRC 4066 using agar-well diffusion assay (AwDA). Sterilised Muller-Hinton and Sabouraud Dextrose liquid agar cooled to approximately 45°C were inoculated with the test strains for a final concentration of 10^6 cells/ml and 10^5 cells/ml for bacteria and yeast/fungi, respectively. Ten ml of the inoculated liquid agar was then poured into 90 mm diameter Petri dishes, and the wells on the solidified agar were prepared using sterilised straw (6 mm diameter). The dried crude extracts were reconstituted in 100% dimethyl sulfoxide (DMSO) to prepare crude extract stock solution of 100 μg/ml concentration which was then further diluted to 2.5 mg/ml using sterile RO water. Forty μl of each the CFBs and crude extracts were loaded into the agar wells for antimicrobial testing. The antibiotics chloramphenicol (100 ppm for Gram-positive bacteria, and 400 ppm for Gram-negative bacteria) and nystatin (100 ppm for yeast/fungi) were used as positive controls, while the CFBs and crude extracts of non-inoculated media, and 2.5% DMSO solution were used as the negative control. The experiment was carried out in triplicate. The assay plates for bacteria were incubated at 37°C for 24 hours, while the assay plates for yeast/fungi were incubated at 30°C for 48 hours. The diameter of inhibition zone was measured (in mm) to determine the antimicrobial activity.

2.2.9. Determination of the MIC of ISP2 crude extract

The MIC of the ISP2 crude extract against *B. subtilis* and *S. aureus* was determined using broth microdilution method adapted from Andrews (2001). The wells in 96-well plate were seeded with 75 μl of standardised test strain of 2.0 X 10^6 cells/ml. Crude extract solution of 100 mg/ml prepared in 100% DMSO was diluted to 2 mg/ml using Mueller-Hinton broth (MHB), with subsequent two-fold dilutions for 10 different concentrations. From each dilution point, 75 μl was added into the inoculated wells. Chloramphenicol as

a positive control was prepared similarly. Wells containing 150 µl test strain without the addition of extract or antibiotic was prepared as the growth control. The experiment was carried out in triplicate. Initial optical density (OD) was measured using 600 nm wavelength. The plates were then incubated at 37°C for 24 hours before the final OD reading was measured at 600 nm. The MIC was measured as the least concentration of crude extract or antibiotic in which no visible growth was detected.

2.2.10. Secondary metabolites profiling of crude extracts using HPLC

Ten ml of dried crude extracts were dissolved in 1 ml of 100% HPLC-grade methanol, filtered through 0.2 µm Regenerated Cellulose filter into HPLC vials and subjected to HPLC analysis on a reverse-phase Analytical HPLC Agilent 1100 system equipped with diode array detector (DAD) (G1315C DAD SL, Agilent). The DAD was set to measure UV spectrum at 200, 210, 230, 254, 273, 330, 400 and 600 nm. Extracts of the non-inoculated media were also analysed using HPLC for background data. Aliquots of 20 µl of each sample were injected into the HPLC column (4.6 x 150 mm, 5 µm, ZORBAX Eclipse XDB-C18, Agilent) held at a temperature of 50°C, and eluted at a flow rate of 1.5 ml/minute for 15 minutes run-time per sample. Solvents and conditions used were as follows: 0-10 minute, 10% Acetonitrile and 90% water; 10-15 minute, 100% Acetonitrile. The HPLC data was used for qualitative analysis of secondary metabolite production in the four type of fermentation broths.

2.2.11. Statistical analysis

The data collected were analysed using one-way analysis of variance (ANOVA) to compare the level of significance of antimicrobial activities between the samples using SPSS version 15 (SPSS Inc.).

2.3. Results

2.3.1. Morphological and physiological characteristics of strain KBS50

Strain KBS50 is a Gram-positive actinomycete that forms extensively branched substrate mycelia on which the spores of approximately 0.2-0.4 µm in diameter appeared to be borne singly (Figure 2.1). On all agar medium tested, the strain formed circular, slightly convex colonies with no formation of aerial mycelia detected. Good growth was observed on ISP2, ISP3 and ISP4, while poor growth was observed on ISP5 medium. The colour of the colonies was orange-yellow on ISP2, ISP3 and ISP4 to slightly pale orange-yellow on ISP5 medium (Figure 2.2). No soluble pigments were observed on any of the media tested.
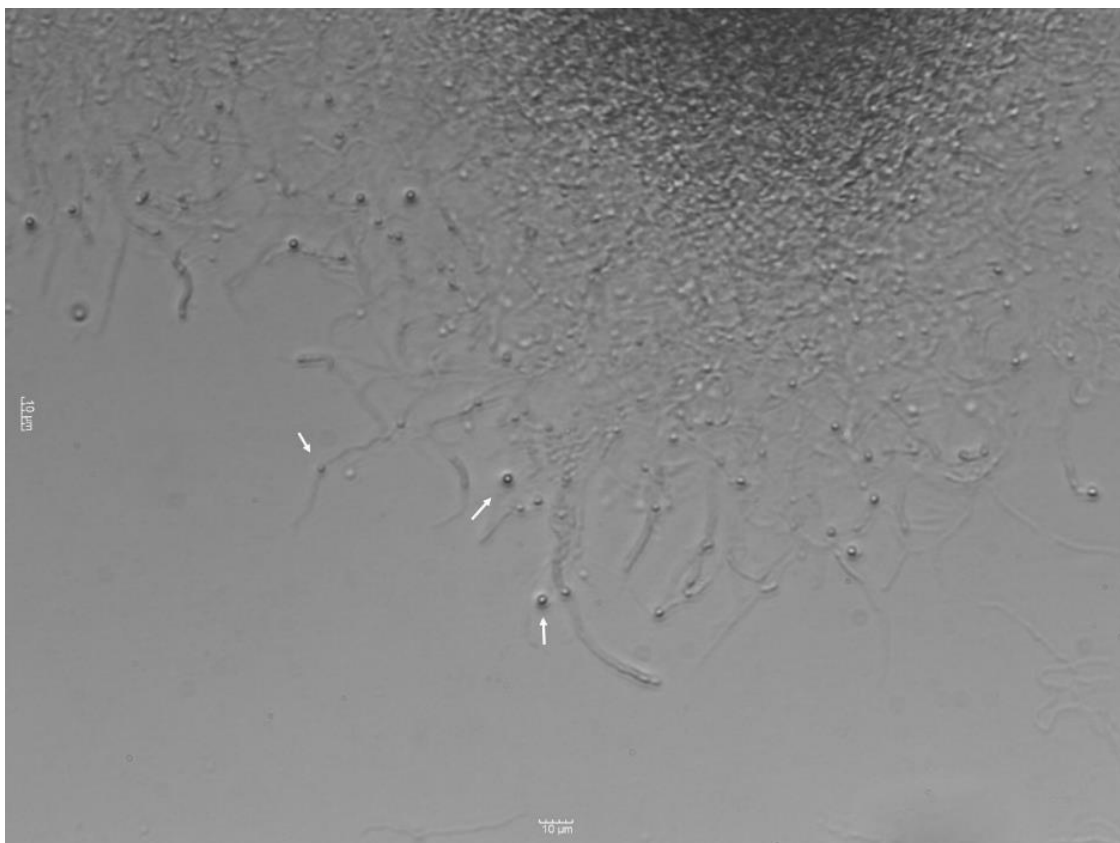
Figure 2.1: Substrate mycelia of strain KBS50 growing on ISP2 agar. Arrows indicate the spores structure formed on the substrate mycelia. The picture was taken from a 14-day old culture incubated at 28°C using light microscope under 50X objective lens. Scale bar = 10 µm.
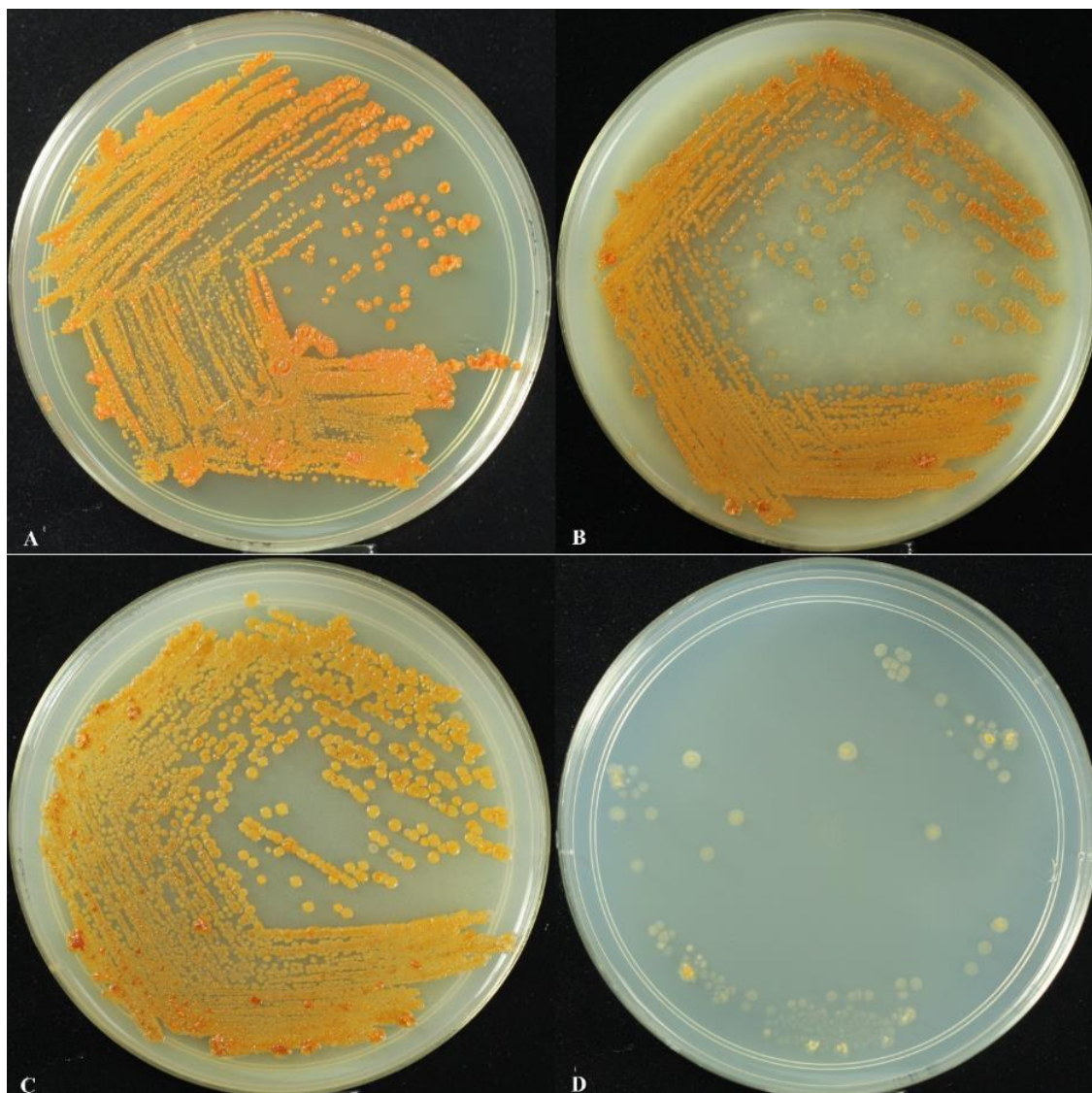
Figure 2.2: Colony morphology of 14-day old KBS50 culture on ISP media incubated at 28°C. A = ISP2, B= ISP3, C = ISP4, D = ISP5.

Strain KBS50 grew well on ISP2 agar with up to 6% NaCl concentration. On ISP2 agar with 7% NaCl, the colonies grew poorly but growth was still evident from the presence of spores borne on the poorly developed substrate mycelia as observed under a light microscope after 21 days of incubation. No growth was observed at 10% and a higher concentration of NaCl tested even after 21 days of incubation.

The incubation temperature in which the actinomycete strain KBS50 was able to grow was in the range of 20-37°C, with very good growth was observed between 25–30°C. Poor growth was observed at 20°C while it was unable to grow at 4°C, 10°C and 40°C. Meanwhile, strain KBS50 showed good growth on ISP2 agar with pH range between pH 6 – 11. The minimum pH tolerated was pH 5 and the maximum was pH 12, on which strain KBS50 grew poorly. No growth was observed on agar with pH 4 and pH 13. The morphological and physiological characteristics of strain KBS50 are summarised in Table 2.1 with a comparison to other species of *Plantactinospora*.

Table 2.1: Morphological and physiological characteristic of strain KBS50 with a comparison to other *Plantactinospora* species.

Characteristics	KBS50	*P. mayteni*	*P. siamensis*	*P. endophytica*	*P. veratri*	*P. sonchi*
Isolation source	Beach sediment	Plant tissue (root)	peat swamp forest soil	Plant tissue (leaves)	Plant tissue (root)	Plant tissue (leaves)
Substrate mycelium	Well developed, extensively branched	Extensively branched	Well developed, extensively branched	Well developed	Well developed	Well developed, extensively branched
Aerial mycelium	None	Sparse, white colour	None	None	None	None
Sporulation	Single, from substrate mycelia	Single, from substrate mycelia	Single, from substrate mycelia	Single, from substrate mycelia	Single, from substrate mycelia	Single, from substrate mycelia
Colony morphology	Circular, convex	Raised, folded	ND	ND	ND	Raised, folded
Colony colour	Orange-yellow (ISP2, ISP3 and ISP4), Pale orange-yellow (ISP5)	Orange-yellow to black (ISP2), pale orange-yellow to black (ISP3), black (ISP4), yellowish (ISP5)	Strong reddish-orange (ISP2)	Orange-red to brown (ISP2, ISP3, ISP4 and ISP5)	Vivid yellow (ISP2, ISP4 and ISP5), brilliant greenish-yellow to vivid orange-yellow (ISP3)	Light orange-yellow (ISP2), brilliant yellow (ISP3), strong reddish-orange (ISP4)
Soluble pigmentation	None	None	Pale yellow (ISP2)	None	Dark orange-yellow pigment on ISP7.	None
Tolerance to NaCl (% w/v)	0-7	0-3	0-2 (data from Zhu et al. 2012)	0-3	0-1	0-1
Growth temperature (°C)	20-37	4-45 (data from Xing et al. 2015)	10-40 (data from Xing et al. 2015)	10-45	15-45	15-40
pH tolerance	5-12	6-8	5-8 (data from Xing et al. 2015)	5-10	6-10	6-10

Data are from Qin et al. (2009b), Thawai et al. (2010), Zhu et al. (2012), Xing et al. (2015), and Ma et al. (2015) for *P. mayteni, P. siamensis*, *P. endophytica*, *P. veratri*, and *P. sonchi*, respectively, unless stated otherwise. ND = Data not available.

2.3.2. Extraction and purification of Genomic DNA

High quality gDNA was extracted and purified from the vegetative cells of strain KBS50. The gDNA was of high molecular weight and good integrity as visualised using the agarose gel electrophoresis (Figure 2.3). The A260/A280 ratio of 1.969 and the A230/A260 ratio of 0.78 indicate good quality that is virtually free from contaminating protein, RNA or polysaccharides, based on the ideal ratios stated by Moore et al. (2004). The concentration as estimated from the A260 reading was 0.576 µg/µl with a total yield of 115 µg gDNA. For PCR application, the gDNA suspension was adjusted to approximately 0.1 µg/µl final concentration.
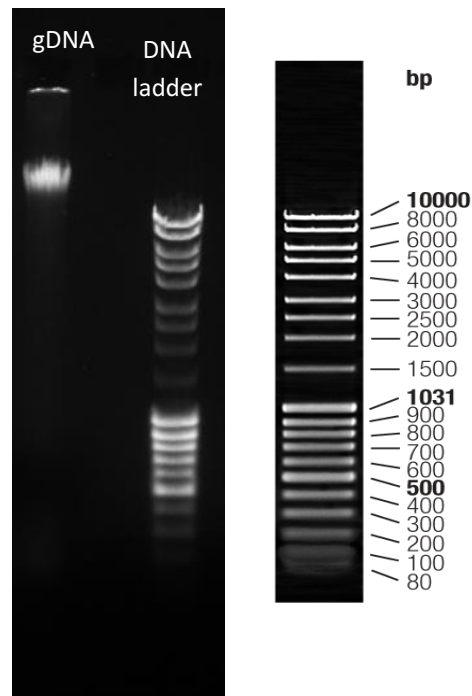
Figure 2.3: Agarose gel electrophoresis of the gDNA sample isolated from strain KBS50. DNA marker used was a Thermo Scientific MassRuler DNA Ladder Mix.

2.3.3. Strain identification and phylogenetic analysis

Strain KBS50 was identified through the amplification of 16S rRNA sequence using primer 27F and 1492R and sequenced using the same primer pair. The resulted 1326 bp-long 16S rRNA sequence was analysed using Nucleotide BLAST® (blastn) tool against "16S ribosomal RNA sequences (Bacteria and Archaea)" on the GenBank database and showed to have highest sequence similarity with *Plantactinospora* species. Based on the BLAST results, four species of *Plantactinospora*, namely *P. mayteni*, *P. siamensis*, *P. endophytica*, and *P. veratri* produced the top four significant alignments of 98% identity with KBS50's 16S rRNA sequence. Other sequences among the top 100 significant alignments results were comprised of other members of *Micromonosporaceae* such as *Verrucosispora*, *Polymorphospora*, *Micromonospora* and *Salinispora* with sequence identity between 96-97%. The "16S ribosomal RNA sequences (Bacteria and Archaea)" database used for BLAST analysis was last updated as of 10 January 2017.

Based on the current publications in the International Journal of Systematic and Evolutionary Microbiology, there were two additional species of *Plantactinospora* that has been published, i.e. *P. sonchi* (Ma et al. 2016) and *P. soyae* (Guo et al. 2016). However, their 16S rRNA sequences have not been added to the 16S ribosomal RNA sequences database used for the BLAST analysis. Sequences of all the published *Plantactinospora* species and other sequences producing significant alignments with KBS50 from the BLAST® search were retrieved from GenBank database for the construction of the phylogenetic tree. Actinomycete strain KBS50 formed a distinct monophyletic clade together with other members of *Plantactinospora* genus. The node showing the relatedness of strain KBS50 with members of *Plantactinospora* species was supported by a bootstrap value of 72% in the neighbor-joining tree (Figure 2.4). The clustering of strain KBS50 with *Plantactinospora* species was in agreement with their high sequence identity of 97-98%, while other members of *Micromonosporaceae* with 96-97% sequence identity were clustering out from the clade. Sequence identity matrix calculated using the BioEdit program version 7.2 (Hall 1999), indicate the highest identity with *P. mayteni* (97.9%), followed by *P. siamensis* (97.7%), *P. endophytica* (97.5%), *P. veratri* (97.5%), *P. sonchi* (97.2%), and *P. soyae* (96.9%). The 16S rRNA gene sequence of strain KBS50 was deposited into GenBank nucleotide database under the accession number KY348801.
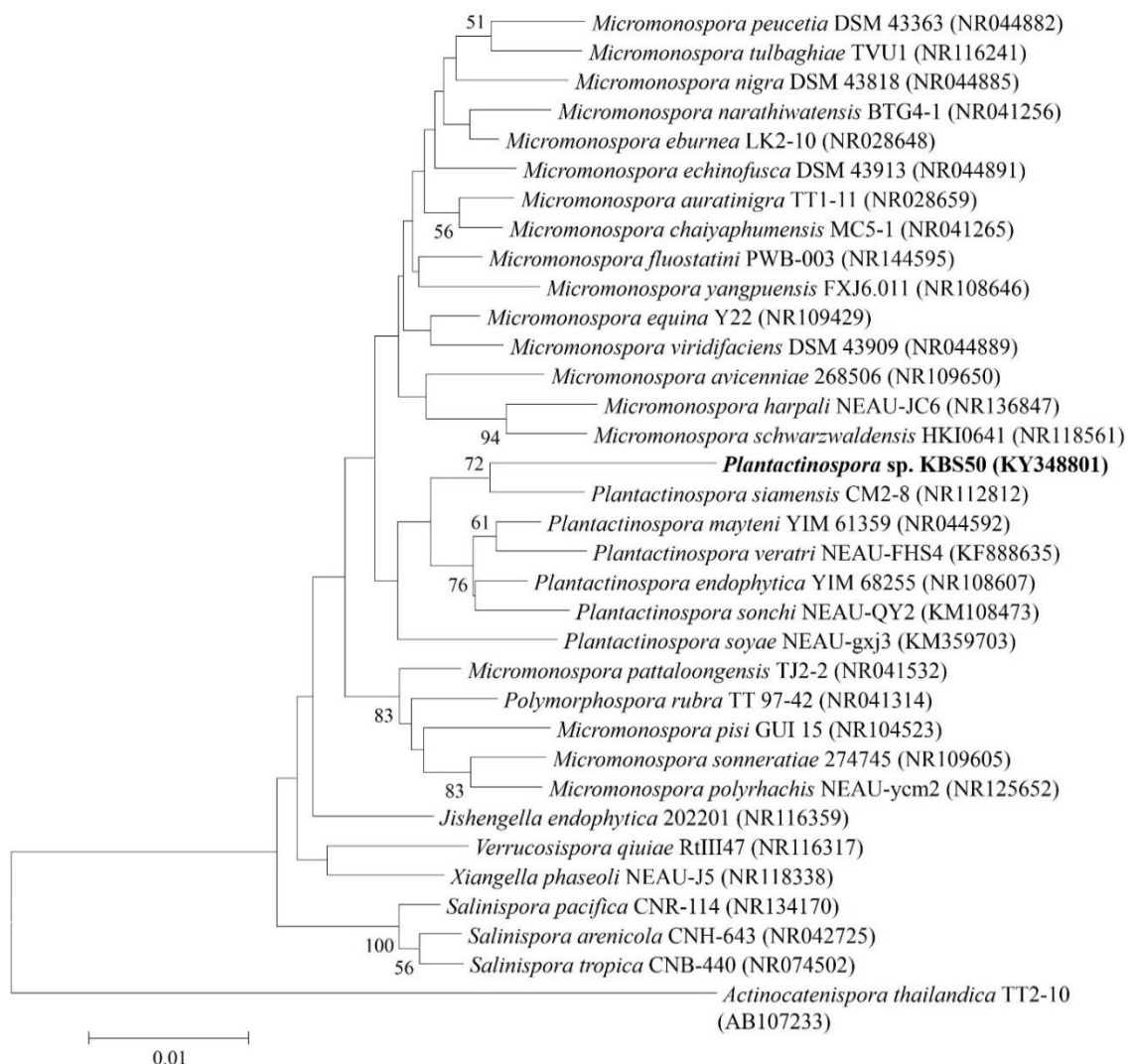
Figure 2.4: The neighbor-joining tree based on 16S rRNA gene sequence showing the relationship between strain KBS50 and other species of *Plantactinospora*. Bootstrap values (≥50%) based on 1000 replications are indicated at the branch point. *Actinocatenispora thailandica* TT2-10 sequence (accession no. AB107233) served as the outgroup. Bar = 0.01 nucleotide substitutions per site.

2.3.4. Extracellular enzyme activity

Actinomycete strain KBS50 was tested positive for proteolytic activity as demonstrated from the formation of clearance zone on SMA agar (Figure 2.5). The diameter of the clear zone was recorded at 23.67 ± 0.88 mm on the 7-day and increased to 44.00 ± 1.00 mm on the 14-day, demonstrates strong production of the protease enzyme. On the other hand, no glucanase activity was detected on CMC or Avicel agar even after 14 days of incubation.
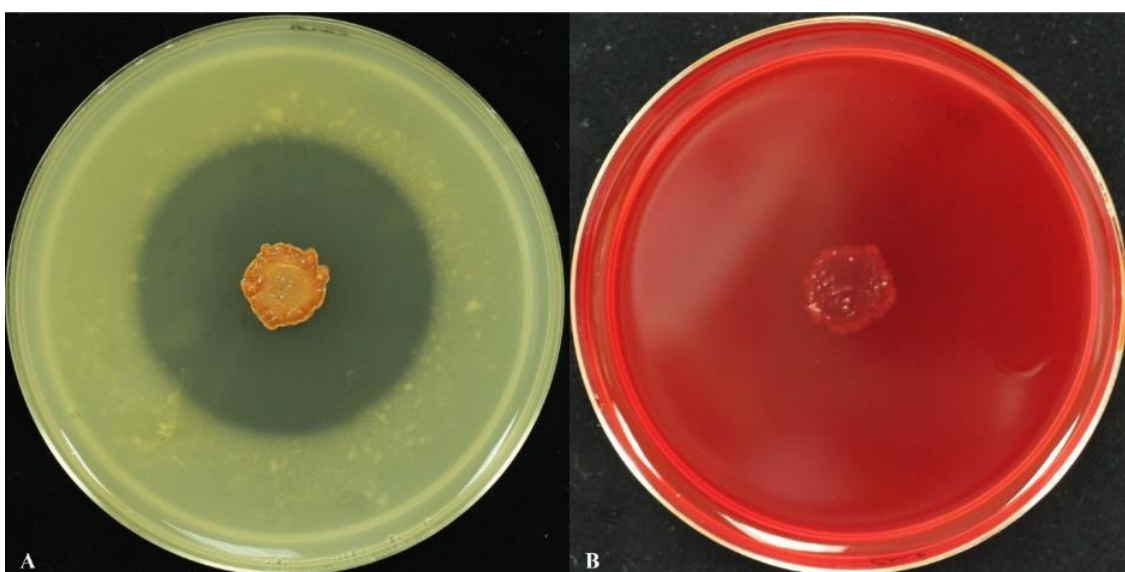
Figure 2.5: Extracellular enzyme activity of strain KBS50. The strain was positive for proteolytic activity based on the formation of clear area around the actinomycete colony on SMA agar (A). No glucanase activity was detected on CMC agar stained with 1% Congo red (B). The pictures were taken on the 14-day of incubation at 28°C.

2.3.5. Antagonistic activities on agar plates

The antagonistic activity of actinomycete strain KBS50 was screened using perpendicular streak method on 4 different types of agar media. Strain KBS50 showed a narrow spectrum antagonistic activity against representatives of Gram-positive bacteria, *B. subtilis* and *S. aureus* (Figure 2.6). The antagonistic activity was observed on all four type of agar media tested (Table 2.2). However, there was a significant difference in inhibition on these four type of agar as determined by one-way ANOVA: *S. aureus* ($F(3,8) = 206.400$, $p = 0.000$), *B. subtilis* ($F(3,8) = 408.733$, $p = 0.000$). The inhibition of both test strains (*S. aureus* and *B. subtilis*) was significantly stronger on MB agar compared to ISP2, FM1 and FM8 ($p < 0.05$). No inhibition was detected against any of the Gram-negative bacteria or yeast tested in the perpendicular streak assay.
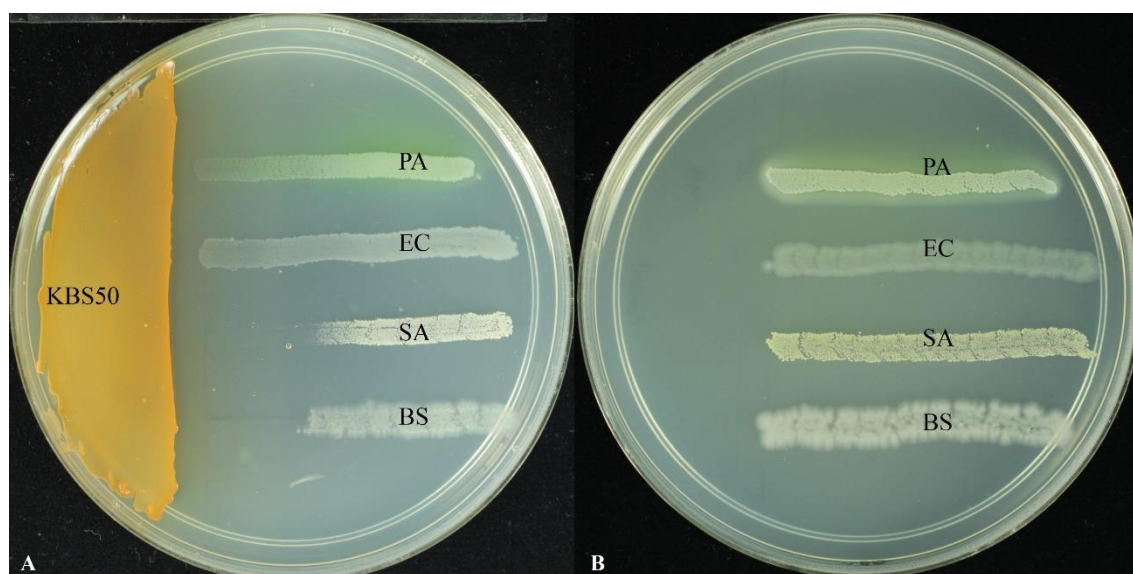
Figure 2.6: Antagonistic activity of strain KBS50 against *S. aureus* and *B. subtilis* on the perpendicular streak assay agar. Inhibition of test strain was observed along the streak lines perpendicular to the actinomycete culture on MB agar plate (A). The control plate without strain KBS50 showed no inhibition of the test strains (B). Similar antagonistic activity was also observed on ISP2, FM1 and FM8 agar media (pictures not shown). Test strains: *P. aeruginosa* (PA), *E. coli* (EC), *S. aureus* (SA) and *B. subtilis* (BS).

Table 2.2: Inhibition of *B. subtilis* and *S. aureus* on the perpendicular streak agar assay.

Media	Inhibition of bacteria test strain (mm) $\pm$ SEM	
	B. subtilis	*S. aureus*
ISP2	15.67 ± 0.67	17.00 ± 0.00
MB	23.67 ± 0.88	21.33 ± 0.33
FM1	18.67 ± 0.67	15.00 ± 0.58
FM8	2.00 ± 0.00	3.67 ± 0.33

Antagonistic activity against fungi test strain (*A. niger*, *G. boninense* and *R. solani*) was also observed in the perpendicular streak assay (Table 2.3). Inhibition of fungi was measured based on the percentage inhibition of radial growth of fungal mycelium in the direction towards actinomycete culture. It was considered as no inhibition if the fungal mycelium overgrowth the actinomycete culture. Inhibition of fungal mycelium by strain KBS50 was also showed to be dependent on the type of agar media. There was a significant difference in inhibition on the four type of agar as determined by one-way ANOVA: *A. niger* ($F(3,8) = 1663.631$, $p = 0.000$), *G. boninense* ($F(3,8) = 19.298$, $p = 0.001$), *R. solani* ($F(3,8) = 83.264$, $p = 0.000$). For *A. niger*, significantly strong inhibition was recorded on MB agar ($72.59 \pm 0.74\%$) as compared to FM1 ($46.67 \pm 1.28\%$, $p = 0.000$) and FM8 ($46.67 \pm 0.00\%$, $p = 0.000$), while there was no inhibition recorded on ISP2 agar. For *G. boninense*, significantly strong inhibition was also recorded on MB agar ($53.33 \pm 1.92\%$) as compared to ISP2, FM1 and FM8 ($p < 0.05$). Meanwhile, inhibition of *G. boninense* on ISP2, FM1 and FM8 agar showed no significant difference ($p > 0.05$). The inhibitory effect of KBS50 on the radial growth of fungal mycelium toward the actinomycete culture on MB agar plates is illustrated in Figure 2.7.

Table 2.3: Inhibition of fungal mycelia on the perpendicular streak agar assay.

Media	Percentage inhibition of radial growth $\pm$ SEM		
	A. niger	*G. boninense*	*R. solani*
ISP2	-	37.75 ± 0.25	-
MB	72.59 ± 0.74	53.33 ± 1.92	42.22 ± 4.63
FM1	46.67 ± 1.28	38.87 ± 2.04	-
FM8	46.67 ± 0.00	36.92 ± 2.15	-
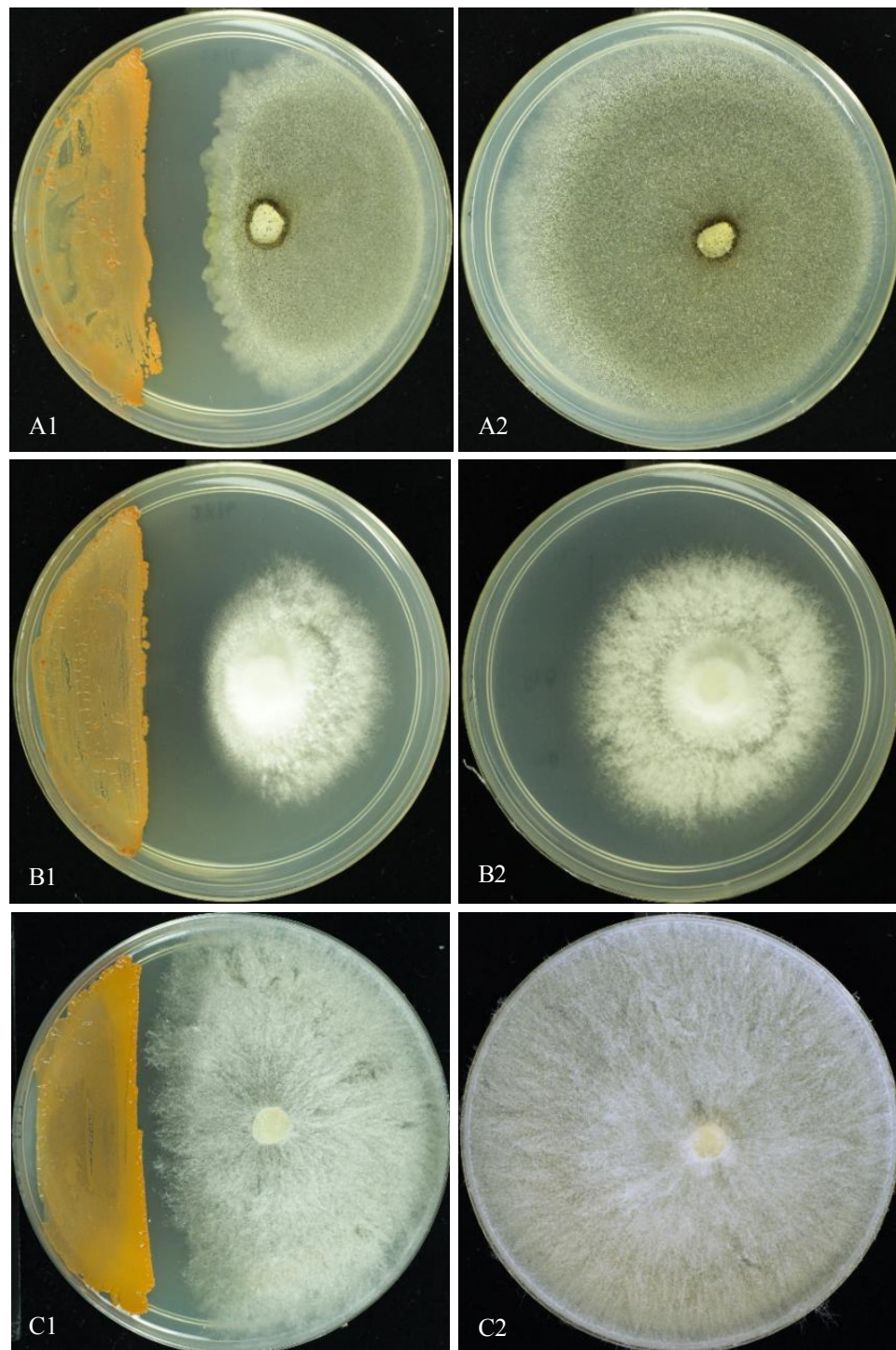
Figure 2.7: Antagonistic activity of strain KBS50 against plant pathogenic fungi on the perpendicular streak assay agar. The inhibition of radial growth of *A. niger* (A1), *G. boninense* (B1) and *R. solani* (C1) as compared to their growth on the control plates (A2, B2, and C2, respectively) on MB agar medium are shown. The picture was taken after 7 days of incubation at 28°C.

2.3.6. Antimicrobial activity in secondary screening

The CFB of actinomycete strain KBS50 exhibited antimicrobial activity against the Gram-positive bacteria (Table 2.4). There was a significant difference in the antimicrobial activity among the CFB as determined by one-way ANOVA: *B. subtilis* ($F(3,8) = 332.000$, $p = 0.000$), *S. aureus* ($F(3,8) = 507.167$, $p = 0.000$). The CFB of ISP2 was found to have the strongest antimicrobial activity against *B. subtilis* (16.33 ± 0.33 mm, $p < 0.05$). Meanwhile, the CFB of ISP2 and FM8 showed no significant difference in inhibition of *S. aureus* ($p = 0.067$). No antimicrobial activity was recorded from the CFB of MB medium.

Table 2.4: Antimicrobial activity of CFBs in AwDA.

CFB	Inhibition diameter (mm) $\pm$ SEM	
	B. subtilis	*S. aureus*
ISP2	16.33 ± 0.33	11.33 ± 0.33
MB	10.00 ± 0.00	0.00 ± 0.00
FM1	11.00 ± 0.00	10.00 ± 0.00
FM8	10.00 ± 0.00	10.00 ± 0.33
Positive control (CP100)	17.67 ± 0.33	16.00 ± 0.58

The crude extracts of actinomycete strain KBS50 exhibited similar antimicrobial activity as the CFB, which only inhibited the growth of Gram-positive bacteria (Table 2.5). There was a significant difference in the antimicrobial activity among the crude extracts as determined by one-way ANOVA: *B. subtilis* ($F(3,8) = 41.867$, $p = 0.000$), *S. aureus* ($F(3,8) = 984.000$, $p = 0.000$). ISP2 crude extract was found to have the strongest antimicrobial activity against *B. subtilis* (17.67 ± 0.33 mm, $p < 0.05$) and *S. aureus* (12.33 ± 0.33 mm, $p < 0.05$).

Table 2.5: Antimicrobial activity of crude extracts in AwDA.

Crude extract	Inhibition diameter (mm) $\pm$ SEM	
	B. subtilis	*S. aureus*
ISP2	17.67 ± 0.33	12.33 ± 0.33
MB	12.00 ± 0.00	9.00 ± 0.00
FM1	15.67 ± 0.67	8.00 ± 0.00
FM8	14.00 ± 0.00	0.00 ± 0.00
Positive control (CP100)	17.67 ± 0.33	16.00 ± 0.58

2.3.7. The MIC of ISP2 crude extract against *S. aureus* and *B. subtilis*

Based on the AwDA result, crude extract from ISP2 broth was identified to have the highest antimicrobial activity against *S. aureus* and *B. subtilis*, and therefore its MIC against both test strains was determined using the broth microdilution method. The MIC of the ISP2 crude extract was 5.21 ± 1.30 µg/ml against *B. subtilis* and 15.63 ± 0.00 µg/ml against *S. aureus*. As a comparison, the MIC of chloramphenicol as determined from this experiment was 3.91 ± 0.00 µg/ml for *B. subtilis* and 5.21 ± 1.30 µg/ml for *S. aureus*.

2.3.8. HPLC analysis for secondary metabolites profiling

To evaluate the production of secondary metabolite in the four type of fermentation broths, the crude extracts were analysed using HPLC. Peaks that were present in the crude extract, as well as the blank sample (similar retention time and UV profile), were considered as medium components. Comparative analysis of the HPLC chromatograms revealed different chemical profiles between ISP2, MB, FM1 and FM8 crude extracts (Figure 2.8). The highest number of unique peaks with relatively higher abundance was detected in the ISP2 crude extract. The five compounds were listed in Table 2.6 and their UV profiles were shown in Figure 2.9.

Table 2.6: List of peaks detected in ISP2 crude extract and their signal strength.

Peak	Retention time (minute)	Signal area [mAU*s]	Peak height [mAU]	Presence in other crude extract	Maximum UV signal (nm)
1	3.795	558.846	111.266	MB (<20 mAU) FM1 (68.213 mAU)	200
2	5.455	237.367	34.565	MB (<20 mAU) FM1 (20.907 mAU) FM8 (<20 mAU)	200
3	6.055	777.654	155.073	FM1 (21.762 mAU) FM8 (<20 mAU)	273
4	7.687	6173.086	1214.9924	MB (<20 mAU) FM1 (<20 mAU) FM8 (<20 mAU)	273
5	7.817	4103.286	815.271	MB (<20 mAU) FM1 (<20 mAU) FM8 (<20 mAU)	273
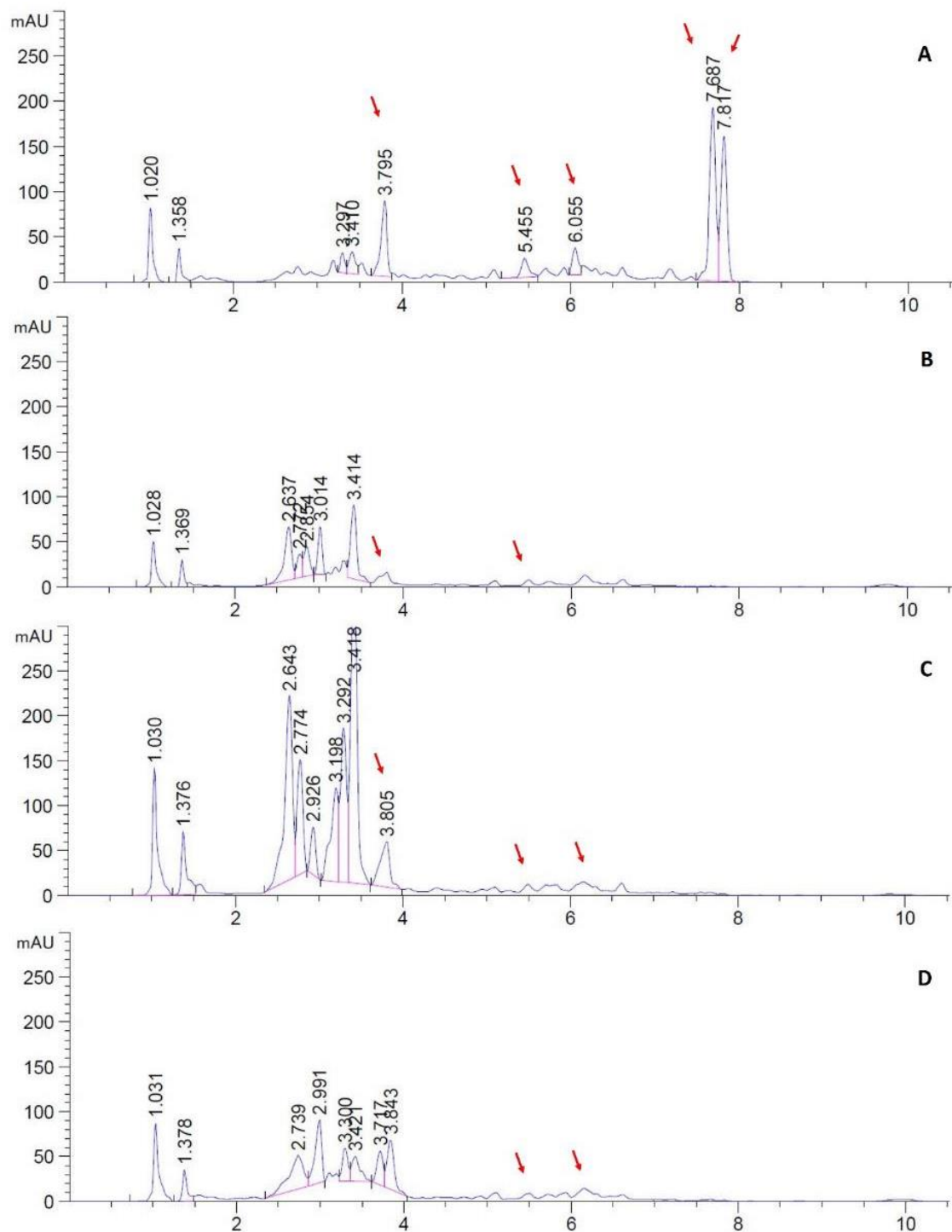
Figure 2.8: Comparative secondary metabolites production by KBS50 in four types of fermentation broths using HPLC analysis. Arrows indicate unique peaks that were present in the HPLC chromatogram of ISP2 (A), MB (B), FM1 (C) and FM8 (D) at UV detection of 210 nm wavelength. The retention times of peaks with higher than 20 mAU are shown.
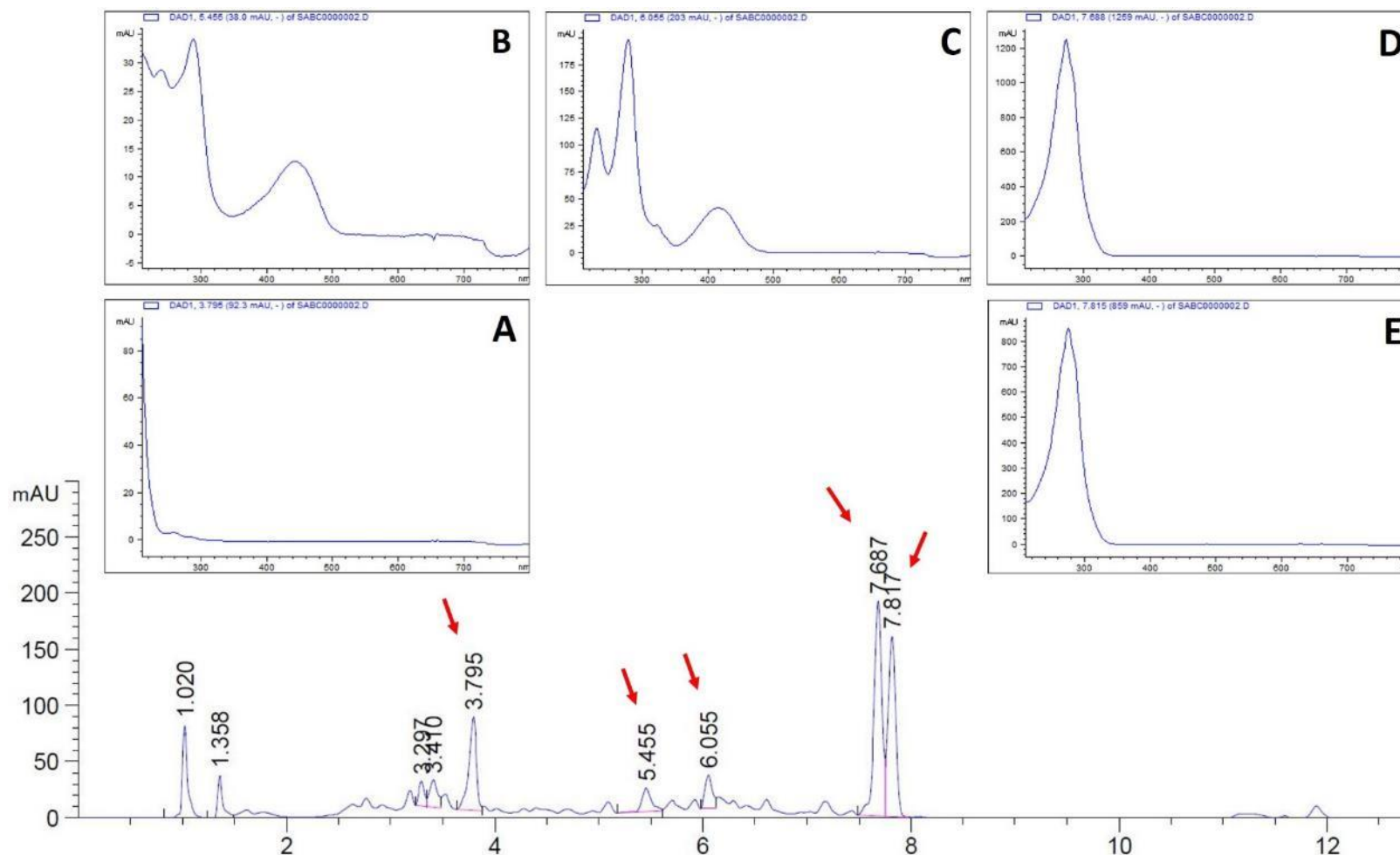
Figure 2.9: HPLC chromatogram of ethyl acetate crude extract from ISP2 fermentation broth at the UV detection of 210 nm wavelength. The UV profiles of unique peaks detected at the retention time of 3.795 (A), 5.455 (B), 6.055 (C), 7.687 (D) and 7.817 (E) are shown. Peaks D and E shared almost identical UV profile although the peaks were well separated at different retention time.

2.4.   Discussion

In this study, the identity of a rare actinomycete, strain KBS50, was established using 16S rRNA gene sequence. The resulted near complete 16s rRNA gene sequence of 1326 bp long was within the recommended length for species description (Stackebrandt et al. 2002). The use of 16S rRNA gene as a genetic marker for identification and to study the evolutionary relationship among bacteria are a common practice in bacterial taxonomy because the genes are universal, highly conserved and stable markers that are less affected by horizontal gene transfer (Schleifer 2009; Větrovský & Baldrian 2013). A bacteria isolate having less than 97% similarity in 16S rRNA gene sequence from its closest phylogenetic relatives can be established as a new taxon or species (Janda & Abbott 2002; Stackebrandt & Goebel 1994). Similarly, if the isolate share similar to or greater than 97% similarity in the 16S rRNA gene sequence, then the isolate may or may not be classified as new species, and in this case, DNA-DNA hybridization should be performed to resolve the issue (Stackebrandt & Goebel 1994). However, Stackebrandt and Ebers (2006) has revised the recommendation and increased the cut-off point to 98.7-99% sequence similarity for a mandatory DNA-DNA hybridization experiment to be carried out to confirm a new species. Based on this cut-off point, actinomycete strain KBS50 was considered as a new species of *Plantactinospora*.

The *Plactactinospora* genus was first coined by Qin et al. (2009b) to describe "a spore-forming actinomycete isolated from plant tissues" with the first type strain, *P. mayteni* YIM 61359T. Following that, five new type species were added to this genus i.e. *P. siamensis* (Thawai et al. 2010), *P. endophytica* (Zhu et al. 2012), *P. veratri* (Xing et al. 2015), *P. sonchi* (Ma et al. 2016), and *P. soyae* (Guo et al. 2016). *P. siamensis* was originally described as *Actinaurispora siamensis* (Thawai et al. 2010) before it was amended by Zhu et al. (2012), and it was the only type species isolated from peat swamp forest soil. Other type species were isolated from plant tissues such as roots and leaves. Strain KBS50 was isolated from a marine-associated environment, an ecologically different habitat, but phylogenetically closely related to these terrestrial actinomycetes. One may argue that the actinomycete, isolated from beach sediment, could have been originated from a terrestrial environment which ends up being wash-off to the coastal area. Nonetheless, its ability to tolerate up to 7% NaCl concentration was higher than any other species of *Plantactinospora*.

Interestingly, the genus *Plantactinospora* is only distantly related to *Salinispora*, a genus of true marine actinomycetes isolated from deep-sea sediment (Maldonado et al. 2005). Strain KBS50 shared between 96.9-97.0% of 16S rRNA gene sequence similarity with *Salinispora* species. Tolerance to the high concentration of NaCl might have enabled strain KBS50 to survive the highly saline environment, a trait which may have been acquired during its transition from terrestrial to the marine environment. Even for *Salinispora* species, there was evidence suggesting that these marine actinomycetes were evolved from a terrestrial environment (Penn & Jensen 2012). Nevertheless, strain KBS50 does not require NaCl or seawater for growth, unlike its true marine relatives (Maldonado et al. 2005). With the addition of a marine-derived strain KBS50 as a new member of *Plantactinospora*, this genus is not restricted to the endophytic or terrestrial actinomycetes. It is expected that more new species of this genus will be discovered from diverse ecological habitats including the marine environment, considering that this genus is only distantly related with the marine actinomycetes, *Salinispora*.

Apart from its high tolerance to NaCl, other physiological characteristics of strain KBS50 that clearly distinguish it from other *Plantactinospora* species include its ability to tolerate higher pH, and its inability to grow at an incubation temperature of 40°C and above. These physiological differences may have resulted from the strain adaptation to its new environment (Retchless & Lawrence 2012). Meanwhile, the basic morphological characteristics of strain KBS50 on agar media were somewhat similar to other *Plantactinospora* species and also shared with many members of *Micromonosporaceae*. In this study, features such as tolerance to a various range of pH, temperature and salinity were determined as part of the strain characterization, but are also useful to understand the suitable growth parameters for its cultivation. All these factors can be taken into consideration when designing an optimum growth condition for this strain, or they can be manipulated to create a unique or stressful environment for the actinomycetes to grow in order to study their secondary metabolites production under diverse cultivation conditions.

On the basis of 16S rRNA gene sequence analysis and the differences in physiological characteristics, it was proposed that strain KBS50 represents new species in the *Plantactinospora* genus. To ascertain its taxonomic position within the genus, detailed study of the phenotypic and chemotaxonomic properties need to be carried out as required

for the full description of new bacterial species (Stackebrandt et al. 2002). However, a full taxonomical aspect of strain KBS50 was not within the scope of this project. For the rest of this thesis, actinomycete strain KBS50 is designated as *Plantactinospora* sp. KBS50 with the GenBank accession number, KY348801, for the 16S rRNA gene sequence deposited into the database.

The *Plantactinospora* genus is a relatively new group of rare actinomycetes from the *Micromonosporaceae* family, and to the best of our knowledge, their biological activity especially antimicrobial potential was not well studied. Most of the species in this genus were isolated from plant tissues such as roots and leaves, indicating their endophytic nature and ecological association with plants. The fact that many endophytic actinomycetes are often associated with having antimicrobial and antifungal activity (Golinska et al. 2015; Strobel & Daisy 2003; Taechowisan et al. 2003) is an indication of potential aspect that has yet to be explored on these endophytic *Plantactinospora* species. Meanwhile, the origin of strain KBS50 as a marine-associated actinomycete is also an interesting factor for the screening of its antimicrobial activities. The marine environment is considered underexplored habitat for natural product screening, but over the years, there are several notable discoveries made from marine actinomycetes especially from the *Salinispora* species (Matsuda et al. 2009; Udwary et al. 2007). Strain KBS50's close association with these marine actinomycetes indicates its potential as a producer of bioactive secondary metabolites that requires further exploration.

To determine the antimicrobial potential of strain KBS50, preliminary and secondary screening was carried out against the selected test microorganism. It has been reported that media composition can affect the production of secondary metabolite compounds from actinomycetes (Bode et al. 2002). Therefore, four different types of media were employed in the antimicrobial screening to determine its antagonistic activity on agar plates as well as for the production of secondary metabolites in broths. The purpose was to increase the chances of discovering antimicrobial activity which might be left undetected if only a single type of medium were to be used. The antimicrobial screening showed that the activity was indeed affected by the cultivation media, although the differences were only in the inhibition level against the same test strains. HPLC analysis of the crude extracts provided evident on the diversity and relative abundance of secondary metabolites that were produced in the fermentation broths. Based on the HPLC

analysis and secondary screening results, ISP2 broth appeared to be the best medium among the four type of media tested for the production of bioactive secondary metabolite compound by the actinomycete strain KBS50.

The preliminary screening showed that *Plantactinospora* sp. KBS50 exhibited antagonistic activity against the Gram-positive bacteria and the fungi test strains. However, secondary screening of the CFB and crude extracts showed antimicrobial activity against the Gram-positive bacteria only. It was possible that the antagonistic activity against these fungi strains was contributed by the proteolytic activity of protease enzyme on the agar media. Screening of CFB from liquid culture did not result in the inhibition of *A. niger*, suggesting that the antifungal compound or the protease enzyme was preferentially produced on solid agar media instead of liquid culture. Other potential reason could be the presence of a very low amount of the antifungal compound that was below its effective inhibitory concentration. It is also possible that the antagonistic activity resulted from the direct interaction between the actinomycete and fungal strain on agar media. If this was the case, strain KBS50 might have the potential application as a biocontrol agent for plant pathogenic fungus, such as *G. boninense* owing to its strong antagonistic activity. *G. boninense* is the causative agent for stem rot disease in oil palm that is adversely affecting oil palm plantation industry in the South East Asian countries (Rees et al. 2009). It is certainly of interest to screen other endophytic species of *Plantactinospora* as well for their biocontrol potential against *G. boninense* and other plant pathogenic fungi.

The qualitative HPLC analysis of the ethyl acetate extractable secondary metabolites from the four types of fermentation media strongly indicates the influence of media composition towards the production of secondary metabolites. It is well known that rich-nutrient conditions may suppress secondary metabolites production, while nutrient-poor conditions trigger secondary metabolites production in actinomycetes (Sanchez & Demain 2002). Among the four fermentation media used in the initial screening, higher production of secondary metabolites was detected from the ISP2 medium, although being relatively the simplest medium with regard to the nutrient compositions, as compared to the MB, FM1 and FM8. It appears that the more complex carbon or nitrogen sources, such as the soluble starch, sago starch, wheat flour, meat extract, and peptone that are present in these media negatively affecting secondary metabolites synthesis by KBS50.

Based on the HPLC analysis and secondary screening results, ISP2 broth appeared to be the best medium among the four types of media tested for the production of bioactive secondary metabolite compound by the actinomycete strain KBS50.

2.5.   Summary

The study in this chapter reports on the identification, characterization, and the preliminary investigation of the antimicrobial potential of a rare actinomycete, strain KBS50. The strain was isolated from a beach sediment sample collected from the Santubong area in Sarawak, Malaysia and was considered as a marine-associated actinomycete. It was identified as a novel species of *Plantactinospora*, a relatively new genus of actinomycetes within the *Micromonosporaceae* family. Major physiological differences between strain KBS50 and other species of *Plantactinospora* includes its ability to tolerate up to 7% (w/v) NaCl and grow on agar medium with pH 12, while it was unable to grow at an incubation temperature of 40°C and above. Higher tolerance to NaCl was expected as its isolation source was collected from a saline environment. Based on the molecular identification and its unique physiological features, strain KBS50 was proposed as a new species, in which it was designated as *Plantactinospora* sp. KBS50. This study, however, did not encompass full taxonomic aspects of strain KBS50, and therefore, a detailed study of its phenotypes and chemotaxonomic properties will still be required to ascertain its taxonomic position within the genus according to the current standards in bacterial systematics. Antimicrobial screening carried out in this chapter provided an overview of the antibacterial and antifungal potential of strain KBS50. Strong antagonistic activity against *S. aureus*, *B. subtilis*, *A. niger*, *G. boninense* and *R. solani* was recorded, although the secondary screening of CFB and crude extracts only detected antimicrobial activity against the Gram-positive bacteria. These screenings indicate the potential influence of nutrient composition as well as other cultivation parameters on the biological activity of strain KBS50. Based on the antimicrobial results highlighted in this chapter, *Plantactinospora* sp. KBS50 was found to be an interesting producer of bioactive secondary metabolite compounds, especially against the Gram-positive bacteria. Further investigation of its secondary metabolite's biosynthetic capabilities from a genomic perspective is presented in Chapter 3, a study that takes advantages of the current technological advancement in microbial genome sequencing and the latest bioinformatics tools for genome mining.

Genome Sequencing and Analysis of Secondary Metabolite Biosynthetic Capabilities of *Plantactinospora* sp. KBS50

3.1. Introduction

In the present study, the actinomycete strain *Plantactinospora* sp. KBS50 has been identified as a potential producer of bioactive secondary metabolite compounds, exhibiting the antimicrobial activities against fungi and bacteria test strains as reported in Chapter 2. Being a rare actinomycete from the marine-associated environment and its unique phylogenetic placement, the strain's genome could hold a significant genetic capability to synthesise novel secondary metabolite compounds. For the study in this chapter, genome mining strategy was employed to unveil this potential and to identify the genes that might be involved in its secondary metabolism. This study provided the first genome sequence data of an actinomycete from the *Plantactinospora* genus and, to the best of our knowledge, is the first reported study that explores the genetic capability of a rare actinomycete isolated from the marine-associated environment in Sarawak for natural product screening.

For this study, a complete genome sequence of strain KBS50 was generated using the Pacific Bioscience's SMRT sequencing technology for the *de novo* genome assembly. The genome sequence was annotated using automated annotation pipeline and subsequently analysed using bioinformatics tool for the identification of secondary metabolites BGCs.

In this chapter, the objectives of the study were as follows:

i) To sequence and characterize the complete genome of *Plantactinospora* sp. KBS50.

ii) To identify the secondary metabolites BGCs in the genome sequence of *Plantactinospora* sp. KBS50.

3.2. Materials and Methods

3.2.1. Strain cultivation and genomic DNA isolation

The cultivation of strain KBS50 and the total gDNA isolation for whole genome sequencing was as described in Chapter 2 (Section 2.2.3). Hundred µl of the gDNA was mixed with 25 µl DNAstable® Plus (Biomatrica, USA) and shipped at room temperature for genome sequencing.

3.2.2. Genome sequencing

Genome sequencing was performed on a Pacific Biosciences (PacBio) RS II instrument at the University Malaysia Sabah, as an outsourced service. Briefly, the gDNA preserved in DNAstable® Plus was purified using AMPure® PB (Pacific Biosciences, USA), then analysed for quality control using NanoDrop (Thermo Scientific, USA) and Qubit™ (ThermoFisher Scientific, USA), while the integrity and fragments size was evaluated on a field-inversion gel. SMRTbell™ library was constructed with approximately 5 µg of gDNA using a 20 kb template preparation method, followed by 15 kb size selection using the BluePippin™ Size Selection (Sage Science). No DNA shearing was performed during the library preparation. The SMRTbell™ template library was loaded into a single SMRT Cell and sequenced using P6-C4 chemistry with 4 hours movie time. Raw sequence data generated from the sequencing facility was analysed in-house.

3.2.3. *De novo* genome sequence assembly

The *de novo* assembly of PacBio sequence reads was carried out using the hierarchical genome assembly process (HGAP) pipeline under the 'RS_HGAP_Assembly.3' protocol from the SMRT® Analysis Software v2.3.0 (Pacific Biosciences), including consensus polishing with quiver (Chin et al. 2013). The predicted genome size and the minimum seed read length in the protocol parameter were set to 6.7 Mbp and 20kbp, respectively. The *de novo* assembly resulted in 2 polished contigs of 6.7 Mbp and 1439 bp long. The shorter contig (1439 bp) was identified as the 'Internal Control Complex Sequence' that was part of PacBio sequencing control, therefore omitted from further analysis. The

longest contig was assigned as a reference sequence for remapping of the reads using the 'RS_Resequencing.1' protocol.

The circular topology of the genome sequence was determined via a dot plot analysis using Gepard v1.40 (Krumsiek et al. 2007) and double-checked for the self-similar end for manual genome closure. Dot plot analysis and manual examination of the sequence confirmed the presence of overlapping ends with self-identity of 98%. The repeat sequence at 3'-end was trimmed off, followed by a couple of remapping using 'RS_Resequencing.1' protocol to drive down the number of corrections remaining by resubmitting the resulted consensus sequence as a reference sequence.

The putative origin of replication (OriC) region was predicted using Ori-Finder web server (Gao & Zhang 2008) with a default parameter. The predicted OriC sequence was confirmed using BLAST search on Dori-C database (Gao & Zhang 2007). A sequence containing 2 kbp downstream and 2 kbp upstream of the putative OriC region was then analysed with BLAST® tool on NCBI GenBank protein database using a translated nucleotide query (blastx) against 'Bacteria and Archaea" genetic code for identification of chromosomal replication initiator protein, Bac_DnaA gene sequence. The open reading frame of this gene was double-checked using ORFfinder tool (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Blast and ORFfinder result indicates that the gene was located on the reverse strand of the genome sequence. The entire sequence was reverse complemented using BioEdit (Hall 1999), then the sequence upstream of the Bac_DnaA gene was moved to the downstream position. The permuted sequence started with Bac_DnaA gene in a forward orientation was finally polished using 'RS_Resequencing.1' protocol twice.

3.2.4. Genome annotation

Genome annotation was performed with the RAST annotation server available at http://rast.nmpdr.org/rast.cgi which was specifically developed for annotation of prokaryotic genomes (Aziz et al. 2008; Overbeek et al. 2014). RAST provided high-quality annotations that identify and assign biological function and information to the genome sequence (Ekblom & Wolf 2014; Koonin & Galperin 2003). The genome

sequence including its general features was visualised as a graphical map using DNAPlotter (Carver et al. 2009).

3.2.5. Identification of secondary metabolite biosynthetic gene clusters

Identification of secondary metabolite BGCs was carried out using the "antibiotics & Secondary Metabolite Analysis Shell" (antiSMASH) version 3.0 available as a web server (http://antismash.secondarymetabolites.org/). The RAST-annotated genome sequence (GenBank file format) was uploaded onto the antiSMASH web server with the following input parameters enabled: Gene Cluster Blast analysis, Known Gene Cluster Blast analysis, Subcluster Blast analysis, smCOG analysis and Active site finder. A series of automated analysis pipeline was performed on the genome sequence including gene cluster identification using HMMer3 tool, NRPS/PKS domain analysis and annotation, core chemical structures prediction, gene cluster comparative analysis using ClusterBlast and secondary metabolism protein family analysis (Blin et al. 2013; Medema et al. 2011; Weber et al. 2015).

Comparative analysis of secondary metabolite BGCs between *Plantactinospora* sp. KBS50 and other related species were carried out. These genome sequences included *Salinispora arenicola* CNS-205 (NC_009953), *Salinispora tropica* CNB-440 (NC_009380), and *Micromonospora narathiwatensis* BTG4-1 (LT594324). The genome sequence of *Streptomyces coelicolor* A3(2) (NC_003888) was included in the comparison as the strain is a well-known producer of many secondary metabolite compounds (Bentley et al. 2002). At the time of analysis, no genome sequence of other *Plantactinospora* species was available in public databases for the comparative analysis. The complete genome sequences were downloaded from NCBI GenBank database, annotated using RAST and analysed using the antiSMASH, under the same parameters as with the *Plantactinospora* sp. KBS50.

3.3. Results

3.3.1. Isolation of genomic DNA

Using the CTAB method, high-quality gDNA was extracted and purified from the vegetative cells of strain KBS50, with the A260/A280 ratio of 1.969, A230/A260 ratio of 0.78, and showed minimal degradation on agarose gel electrophoresis (Chapter 2, Section 2.3.2). For genome sequencing application, 25 µl of DNAstable® Plus was mixed with 100 µl gDNA containing approximately 46 µg gDNA, to preserve and stabilise the gDNA for shipment at room temperature.

3.3.2. Genome sequencing and assembly

The gDNA was put through a stringent quality control to ensure the input-DNA quality required for PacBio SMRT sequencing was met. Size-selection of the SMRTbell library using BluePippin system effectively removed shorter fragments from the SMRTbell template library, favouring fragments of 15 kbp and longer, which had resulted in an overall long sequencing subreads. The single SMRT Cell produced 594,209,170 nucleotide bases generated through 61,115 reads, with the mean subread length of 9,722 bp and N50 value of 15,807. These subreads were *de novo* assembled using HGAP protocol with consensus polishing using Quiver and multiple resequencing using the consensus sequence as a reference sequence. The graphical summary for the *de novo* assembly report is shown in Figure 3.1.

The genome of *Plantactinospora* sp. KBS50 was determined as a circular chromosome based on the presence of overlapping regions at both ends of the assembled contig (Figure 3.2). Using Ori-Finder (Gao & Zhang 2008), the putative OriC regions in the genome sequence which contained three DnaA boxes was predicted (Figure 3.3). BLAST search on Dori-C database indicates that the sequence shared between 90-93% identities with OriC regions of other actinomycetes from the *Micromonosporaceae* family, including *Actinoplanes*, *Micromonospora* and *Salinispora*. The Z-curves showing GC, AT, RY and MK disparity curves, the location of the DnaA genes and DnaA box clusters is shown in Figure 3.4. Using the putative location of the OriC region, DnaA gene sequence was identified using BLAST analysis. The identified gene showed high sequence similarity of

99% with the chromosomal replication initiator protein DnaA gene from other closely related actinomycetes including the members of *Micromonospora*, *Salinispora* and *Verrucosispora*. The open reading frame of this gene was double-checked using ORFfinder tool to determine the first codon.
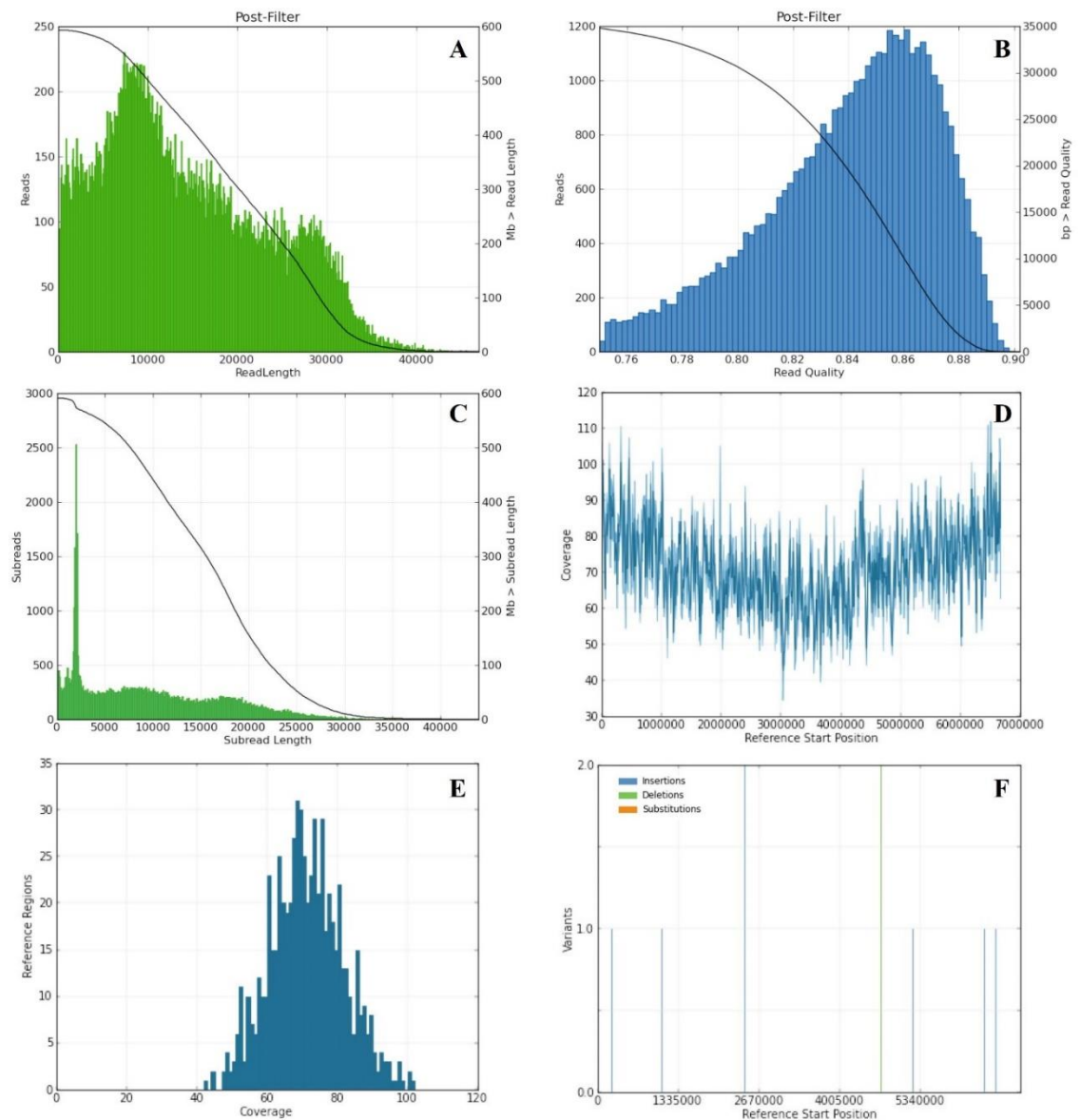
Figure 3.1: A graphical summary of the PacBio RS II sequence data from a 15 kbp size-selected KBS50 library, analysed with SMRT Analysis v2.3. The graphs show the polymerase read length (A), polymerase read quality (B), subread filtering (C), the coverage across the reference sequence (D), depth of coverage (E), and the variants across the reference sequence (F).
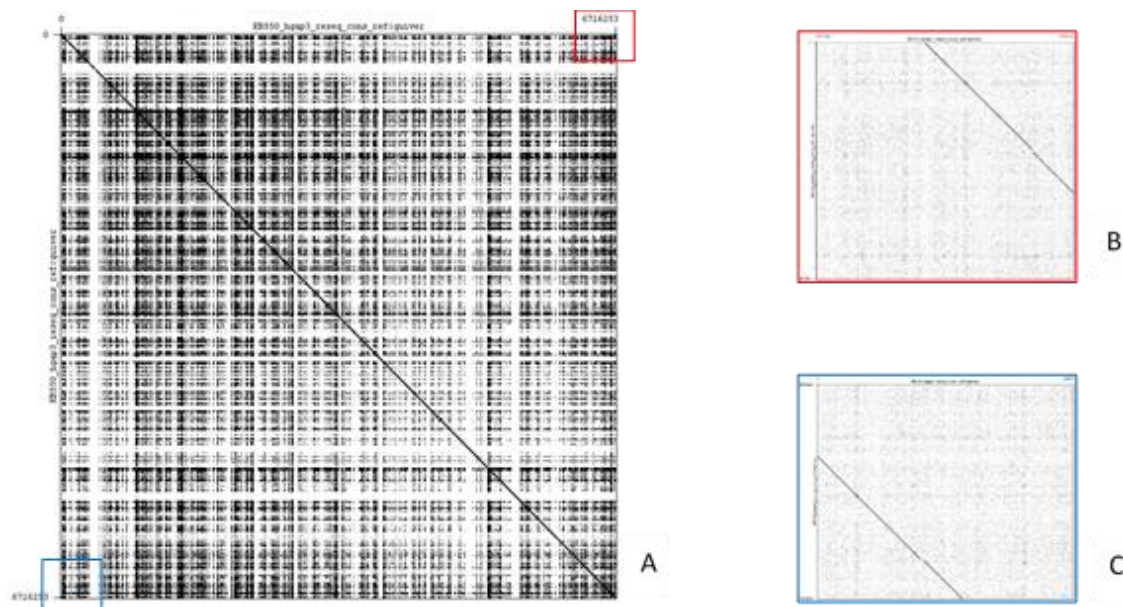
Figure 3.2: Dot plots (A) of a complete genome sequence of KBS50 generated using Gepard v1.40. The diagonal lines (B & C) indicate the presence of a repeat at the beginning and end of the sequence.

5'-

cctTTATCCACAgccTGTGGATAGcgaccggatgatcacggtgag**TTATCCACA**ccggtggacaacggcctgtgg **-3'**

Figure 3.3: DNA sequence of putative OriC region detected using Ori-Finder. The DnaA boxes are bold and capitalised.
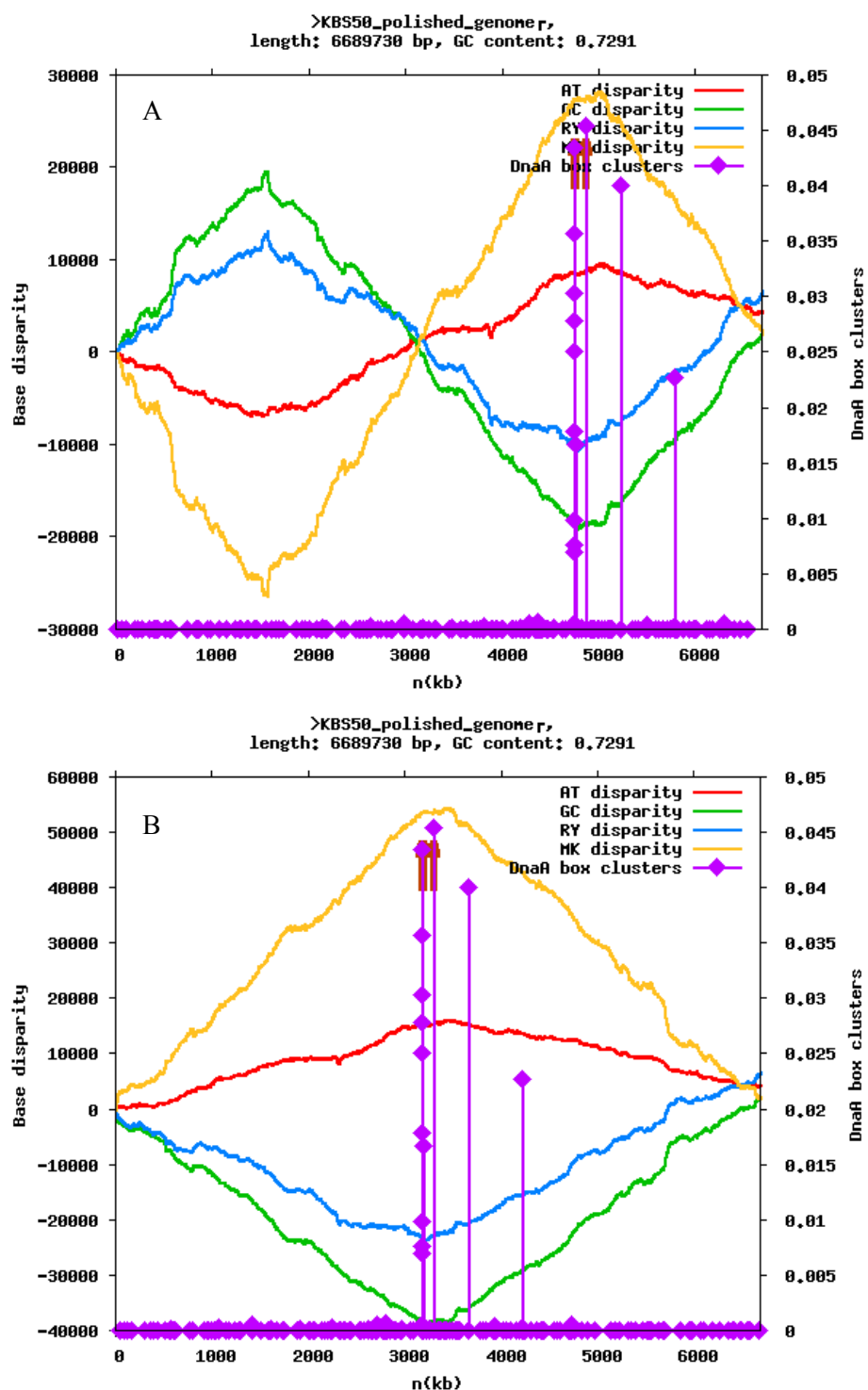
Figure 3.4: The putative origin of replication (OriC) region for the complete genome of KBS50 predicted using Ori-Finder web server. The integrated plots show the Z-curves (AT, GC, RY and MK disparity curves) for the original sequence (A), and for the rotated sequence begins and ends in the maximum of the GC disparity curve (B). The short vertical red line indicates the indicator gene (such as DnaA) location and purple peaks with the diamonds indicate the DnaA box clusters.

3.3.3. Genes annotation and the general features of KBS50's genome

The genome of *Plantactinospora* sp. KBS50 was assembled as a single, circular chromosome of 6,689,726 nucleotides, with an estimated sequencing coverage of 88.8 X. The genome sequence was started with the Bac_DnaA gene on a positive strand, located at the position 1 to 1005. The G+C content of the genome is 72.9% and is comparable to other *Plantactinospora* species (Guo et al. 2016; Ma et al. 2016; Qin et al. 2009b; Xing et al. 2015; Zhu et al. 2012). The genome contains three rRNA operons (5S-16S-23S), and 47 tRNAs that represent all 20 amino acids including one that represents the twenty-first amino acid, selenocysteine. There are 5,820 coding sequences (CDS) predicted with a total length of 5,870,697 nucleotide bases, which represent 87.8% of the total genome sequence length. Out of these, 4,093 CDS (70.3%) were functionally annotated while the rest were annotated as hypothetical proteins. The largest CDS comprises of 12,663 bp and the average length of CDS is 1,008 bp. The number of coding sequences in the genome is slightly biased towards the positive strand (52.8%), a trend shared with many other microbial genomes (Bentley et al. 2002). The genome also contains a considerable number of regulatory genes such as transcriptional regulators, histidine kinase, and RNA polymerase sigma factor, which amount to 6.6% of the total genome. Interestingly, the genome also contains 12 genes encoding for the "clustered regularly interspaced short palindromic repeats" (CRISPR) or CRISPR-associated protein. Approximately 50% of sequenced bacterial genomes contain the CRISPR (Grissa et al. 2007) that provides protection against viruses (Sorek et al. 2013). The genome features of *Plantactinospora* sp. KBS50 is summarised in Table 3.1 while the graphical representation of the genome is shown in Figure 3.5.

RAST annotation pipeline identified protein-encoding genes and categorises them according to subsystems category, which are groups of functionally related proteins (Overbeek et al. 2005). Based on this annotation pipeline, 35% of the CDS in KBS50 genome were connected to various subsystems in the database, while another 65% were not in the subsystems. These genes were distributed in 413 different subsystems categories. The highest number of genes (526) are connected to carbohydrate metabolism, where 141 genes are involved with monosaccharides metabolism and utilisation, while 5 genes are involved in polysaccharides (glycogen) metabolism. Other subsystems features in which KBS50's genes are connected to include the metabolism of aromatic compounds

(65 genes), nitrogen metabolism (10 genes), stress response (130 genes), iron acquisition and metabolism (15 genes), and protein metabolism (332 genes), among others. The subsystems category distribution and features counts of KBS50 genes based on RAST analysis are presented in Figure 3.6.

Table 3.1: Genome assembly statistics for *Plantactinospora* sp. KBS50.

Parameter	Genome feature
Sequencing data	PacBio RSII SMRT sequencing, P6-C4 chemistry
Number of reads	61,115
Number of bases	594,209,170
Mean subread length	9,722
N50 value	15,087
Average reference coverage	88.8 X
Assembly type/ process	De novo/ HGAP assembly
GenBank BioProject number	PRJNA396887
GenBank BioSample number	SAMN07440307
Genome sequence accession number	CP022961
Genome size	6,689,726
G+C content	72.9%
Chromosome	Single, circular
tRNA	47
rRNA	9 (3 operons)
Number of CDS	5,820
Coding capacity	87.8%
CDS from (+) strand	3,084
CDS from (-) strand	2,736
Longest CDS length	12,663 bp
Average CDS length	1,008 bp
Genes for regulatory function	439 (6.6% of total genome)
Functionally annotated proteins (%)	4,093 (70.3%)
Hypothetical proteins (%)	1,727 (29.7%)
Genes with FIGfam protein family (%)	3544 (60.9%)
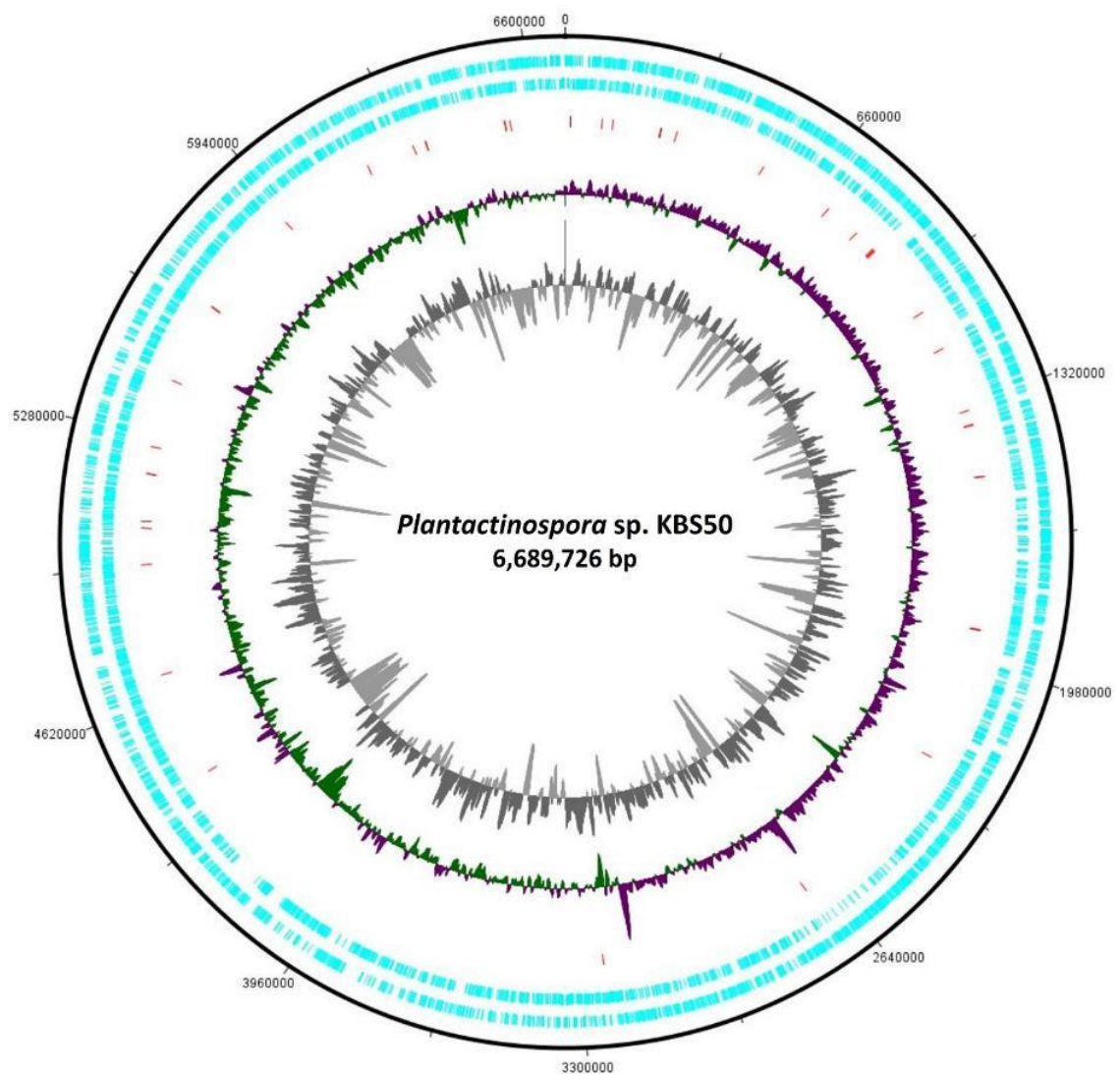
Figure 3.5: A graphical representation of circular genome sequence of *Plantactinospora* sp. KBS50 oriented to the chromosomal replication initiator DnaA gene in a forward direction. From centre to outside ring: GC content, GC skew, tRNA, genes on the reverse strand, genes on the forward strand. The graphical map was generated using DNAPlotter (Carver et al. 2009).
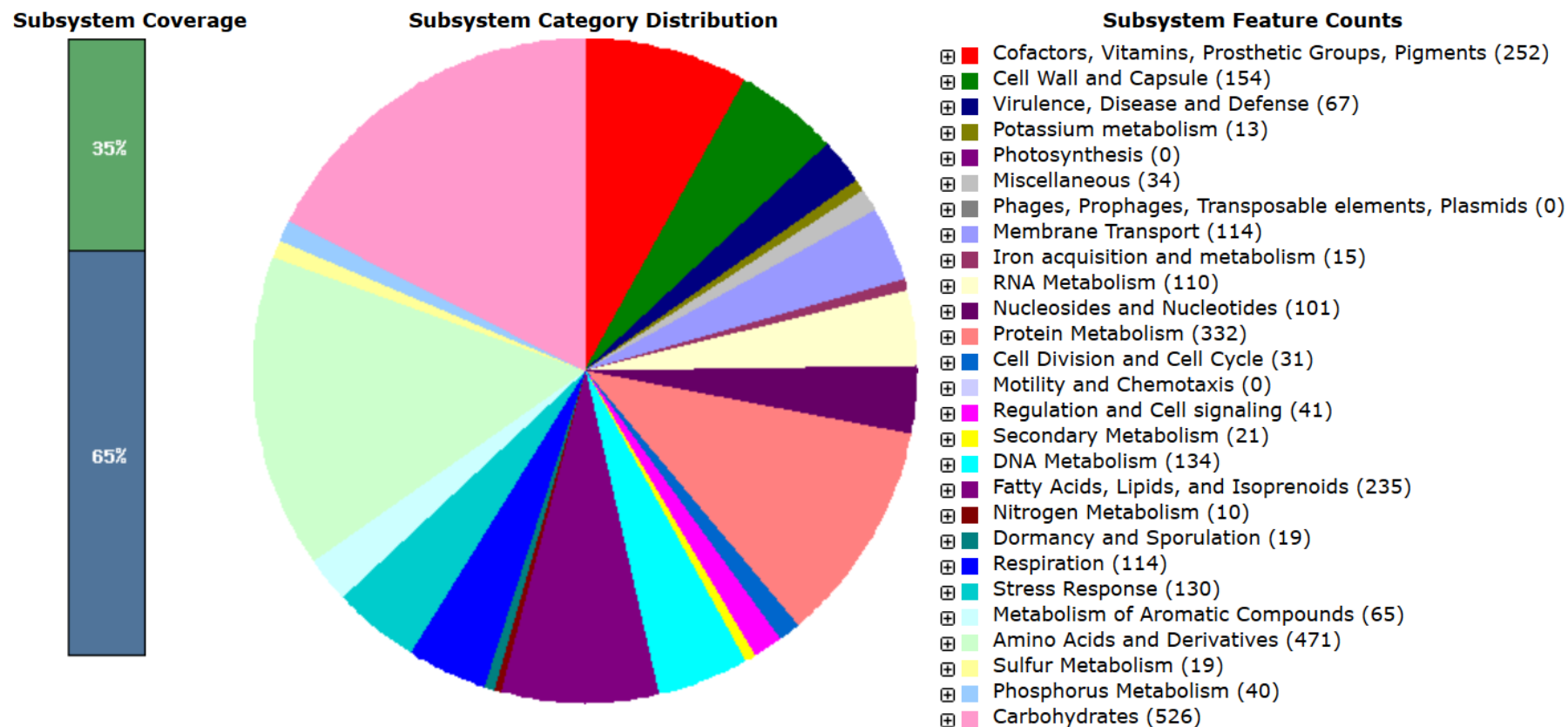
Figure 3.6: Overview of *Plantactinospora* sp. KBS50's genes based on RAST annotation pipeline. The pie chart generated by the RAST server shows the distribution and the number of genes connected to various subsystems categories.

3.3.4. Genes for protein and carbohydrate metabolism and utilisation

The genome of *Plantactinospora* sp. KBS50 revealed a wide spectrum of enzymes for protein metabolism. There are 44 genes identified for protein degradation. These genes encode enzymes for proteolysis, metallocarboxypeptidase, proteasome, and aminopeptidase. The most abundant class of protease encoded by the genome are the serine proteases, which include protease II (oligopeptidase B), ATP-dependent protease La (endopeptidase La) and ATP-dependent protease Clp (endopeptidase Clp). Other class of proteases identified include zinc (metallo) and cysteine proteases. BLAST search of the protease II protein sequence (CDS no. 348) showed the highest identity to oligopeptidase B found in *Micromonospora* species, such as *M. zamorensis* (79%), *M. viridifaciens* (79%), and *M. chokoriensis* (79%). The protein sequence also showed 77% identity with protease enzyme found in the marine actinomycetes, *S. tropica* and *S. pacifica*. The BLAST search of the endopeptidase La protein sequence (CDS no. 313) showed the highest identity to the endopeptidase La found in *M. rhizosphaerae* (91%).

The genome of KBS50 contains the highest number of genes for carbohydrate metabolism, with a total of 526 genes listed under the subsystems category. There are 6 identified genes for hydrolysis or with binding function towards cellulose, including the endo-1,4-ß-glucanase (cellulase A, E1, and E4) and exoglucanase B (Exocellobiohydrolase B). A total of 32 genes identified for chitin and N-acetylglucosamine utilisation, 66 genes for disaccharides/ oligosaccharides utilisation, and 5 genes for polysaccharides (glycogen) metabolism. Meanwhile, 141 genes are identified for monosaccharides metabolism and utilisation, where 47 genes accounted for xylose, 15 genes for D-ribose, 13 genes for L-arabinose, and 47 genes for D-galacturonate/ glucuronate utilisation. The high number of genes dedicated for carbohydrate metabolism indicate the ability of KBS50 to utilise a wide variety of carbon sources as energy.

3.3.5. Biosynthetic gene clusters for secondary metabolism

Genome analysis using antiSMASH indicated the presence of 60 secondary metabolite BGCs predicted to be involved in the production of polyketides, nonribosomal peptides, terpenes, siderophores, and lantipeptides, among others. From the 60 BGCs detected, 37 are putative clusters including fatty acid and saccharide, while the major type of BGCs identified includes 7 NRPS, 7 PKS (Type 1, 2 & 3, abbreviated T1pks, T2pks and T3pks) and 2 T1pks/NRPS hybrid (Figure 3.7). Almost all of the identified BGCs have low gene similarity to any known cluster, where 34 clusters have between 4-46% gene similarities to known gene clusters while 23 clusters have no homologous known gene clusters (Table 3.2). Meanwhile, cluster 47 (Cf_putative) and cluster 6 (Terpene) showed high gene similarity to the sioxanthin BGC at 75% and 100%, respectively, while Cluster 56 (T3pks) has 71% gene similarity to the alkyl-O-dihydrogeranyl-methoxyhydroquinones BGC. A large number of unique BGCs in the genome with low gene similarity to any known gene cluster indicates the potential novelty of their metabolic products.
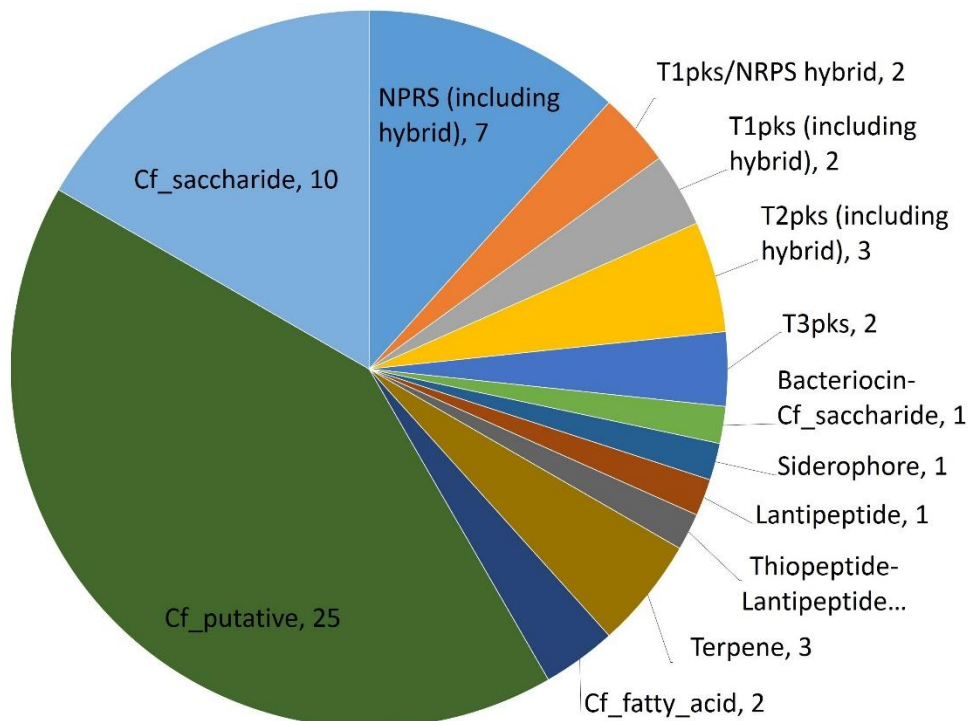
Figure 3.7: Overview of the type of secondary metabolite BGCs in the genome sequence of *Plantactinospora* sp. KBS50 identified using the antiSMASH platform.

Table 3.2: List of secondary metabolite BGCs identified from the genome sequence of *Plantactinospora* sp. KBS50 using the antiSMASH platform.

Cluster	Type of BGC	Most similar known cluster (gene similarity)
Cluster 1	Cf_putative	Herboxidiene (5%)
Cluster 2	Cf_putative	Fengycin (20%)
Cluster 3	Cf_putative	-
Cluster 4	Cf_putative	-
Cluster 5	Cf_putative	-
Cluster 6	Terpene	Sioxanthin (100%)
Cluster 7	Cf_putative	-
Cluster 8	Cf_putative	-
Cluster 9	Cf_putative	Methylenomycin (14%)
Cluster 10	Cf_putative	Fluostatin (6%)
Cluster 11	Cf_putative	-
Cluster 12	Cf_putative	-
Cluster 13	Cf_putative	Rabelomycin (10%)
Cluster 14	Cf_saccharide	Bottromycin_A2 (15%)
Cluster 15	Cf_putative	-
Cluster 16	Cf_putative	Enduracidin (8%)
Cluster 17	Cf_putative	-
Cluster 18	Nrps-Otherks-Lantipeptide-T1pks	Retimycin (10%)
Cluster 19	T1pks	Azinomycin_B (4%)
Cluster 20	Cf_putative	Kinamycin (8%)
Cluster 21	Cf_saccharide	-
Cluster 22	Cf_saccharide	-
Cluster 23	T2pks-Oligosaccharide-Otherks-Cf_saccharide	Mithramycin (37%)
Cluster 24	Thiopeptide-Lantipeptide	Streptomycin (12%)
Cluster 25	Nrps-Cf_fatty_acid	Oxazolomycin (18%)
Cluster 26	Cf_saccharide	-
Cluster 27	T3pks	Furaquinocin_A (43%)
Cluster 28	Cf_putative	Asukamycin (4%)
Cluster 29	Cf_putative	-
Cluster 30	Cf_saccharide	-

Table 3.2: Continue…

Cluster	Type of BGC	Most similar known cluster (gene similarity)
Cluster 31	Cf_putative	Novobiocin (15%)
Cluster 32	Terpene	Hopene_biosynthetic_gene_cluster (46%)
Cluster 33	T2pks	Pradimicin_biosynthetic_gene_cluster (25%)
Cluster 34	Lantipeptide	-
Cluster 35	Cf_saccharide	Cyclomarin (8%)
Cluster 36	Cf_saccharide	-
Cluster 37	NRPS	Clarexpoxcin (10%)
Cluster 38	Lantipeptide- NRPS	Bacillibactin (46%)
Cluster 39	NRPS	Bacillibactin (46%)
Cluster 40	NRPS	Cyclomarin (17%)
Cluster 41	NRPS -Bacteriocin	-
Cluster 42	Blactam-T1pks	Maklamicin (30%)
Cluster 43	NRPS-T1pks-Lantipeptide	Maklamicin (28%)
Cluster 44	Cf_putative	-
Cluster 45	Cf_putative	Lymphostin (30%)
Cluster 46	Terpene	-
Cluster 47	Cf_putative	Sioxanthin (75%)
Cluster 48	Cf_fatty_acid	Chlorizidine_A (7%)
Cluster 49	Cf_fatty_acid	-
Cluster 50	Cf_saccharide	-
Cluster 51	NRPS	Daptomycin (4%)
Cluster 52	Cf_putative	-
Cluster 53	Cf_putative	Tetarimycin (5%)
Cluster 54	T2pks	Xantholipin (18%)
Cluster 55	Cf_saccharide	Phosphonoglycan (5%)
Cluster 56	T3pks	Alkyl-O-Dihydrogeranyl-Methoxyhydroquinones (71%)
Cluster 57	Cf_putative	Fosfazinomycin (14%)
Cluster 58	Siderophore	-
Cluster 59	Bacteriocin-Cf_saccharide	Carbapenem_MM_4550 (6%)
Cluster 60	Cf_saccharide	Rifamycin (5%)

Cf = putative cluster identified with the ClusterFinder algorithm in the antiSMASH platform. Otherks = Other type of PKS cluster.

3.3.5.1. PKS and NRPS gene clusters

A total of 16 gene clusters encoding for polyketides, nonribosomally synthesised peptides as well as hybrids clusters containing either T1pks, NRPS or both, were identified from the genome sequence of KBS50. Based on the co-linearity rules of the modular biosynthetic pathway, the antiSMASH analysis pipeline was able to predict the core chemical structures of putative polyketides and peptides product of the modular T1pks and NRPS clusters (Medema et al. 2011; Weber et al. 2015). Additional tailoring reaction and cyclisation to produce the final product were not taken into account for the structure prediction. A total of 11 core structures of the putative metabolic products were predicted from the T1pks and NRPS clusters including hybrids cluster containing either T1pks or NRPS or both. The genetic map of the BGCs identified by antiSMASH with their predicted core structure and the homologous known gene clusters are shown in Figure 3.8-3.18.
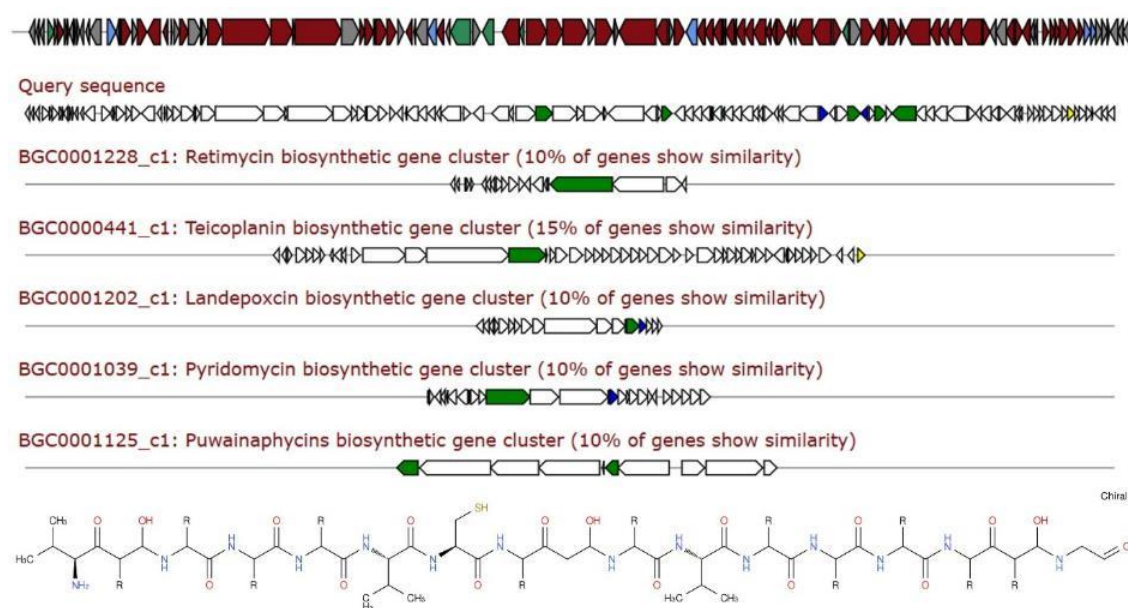
Figure 3.8: Cluster 18, a hybrid genes cluster comprising of NRPS, T1pks, lantipeptides, and other type of PKS genes. The genes showed 10% similarity to retimycin gene clusters.
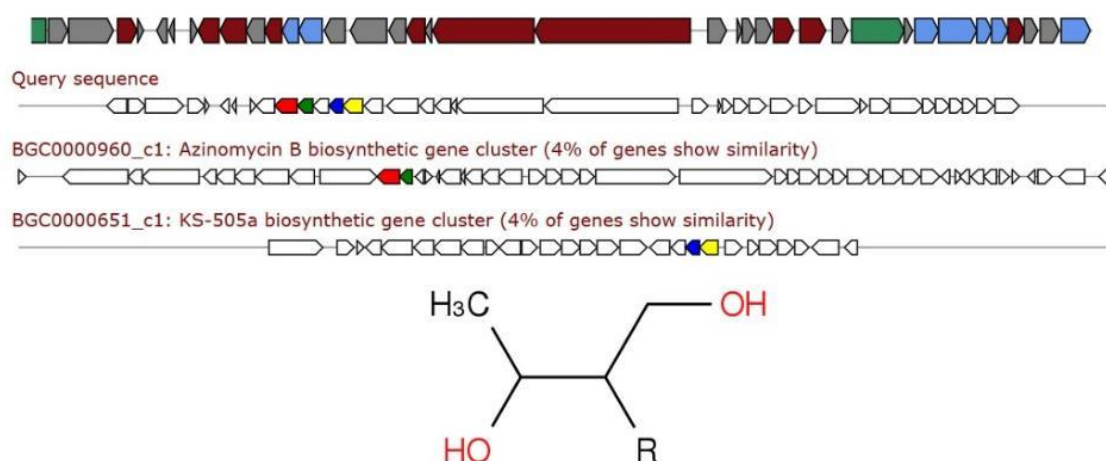
Figure 3.9: Cluster 19, a T1pks gene cluster with 4% similarity to the azinomycin B gene clusters.
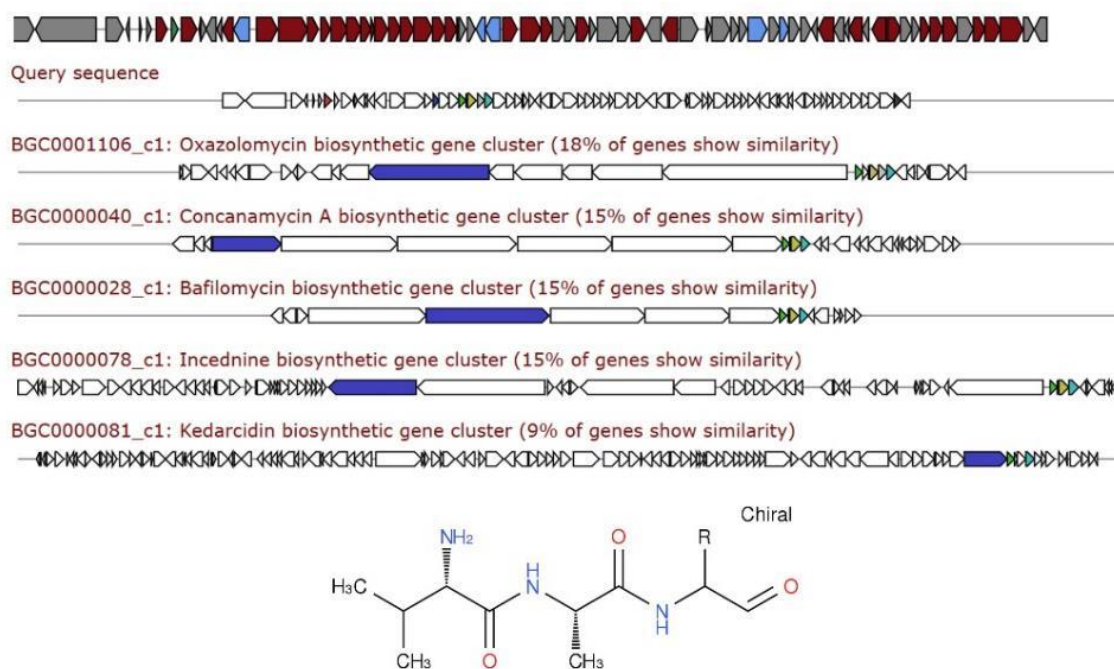
Figure 3.10: Cluster 25, a hybrid gene cluster of NRPS and fatty acid genes with 18% similarity to oxazolomycin gene cluster.

Figure 3.11: Cluster 37, an NRPS gene cluster with 10% similarity to clarexpoxcin gene cluster.

Figure 3.12: Cluster 38, a hybrid gene cluster of NRPS and lantipeptides genes. The genes showed 46% similarity with bacillibactin gene cluster.
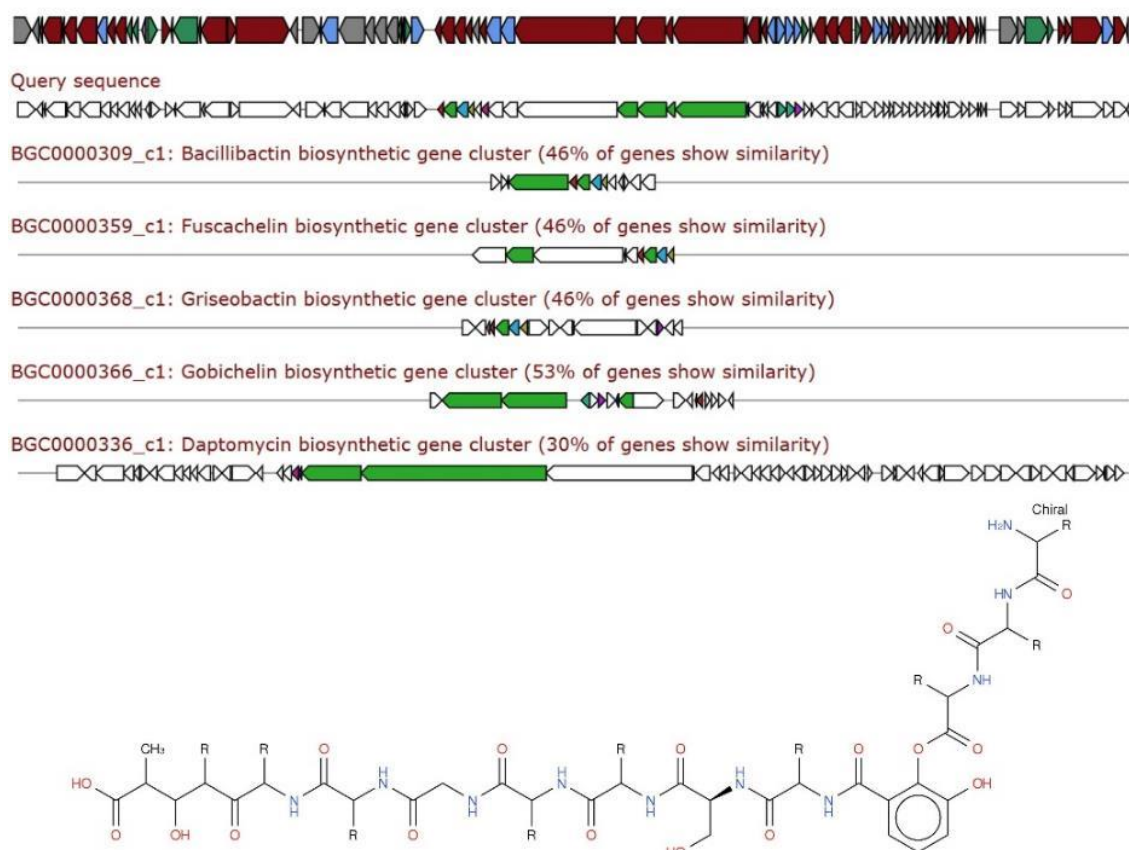
Figure 3.13: Cluster 39, an NRPS gene cluster with 46% similarity to the bacillibactin gene cluster.
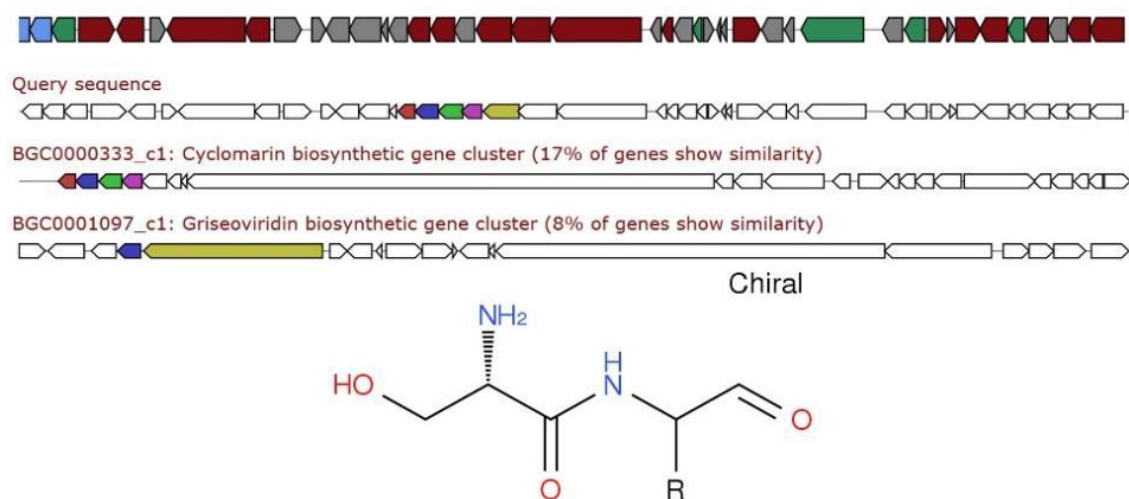
Figure 3.14: Cluster 40, an NRPS genes with 17% similarity to cyclomarin gene cluster.
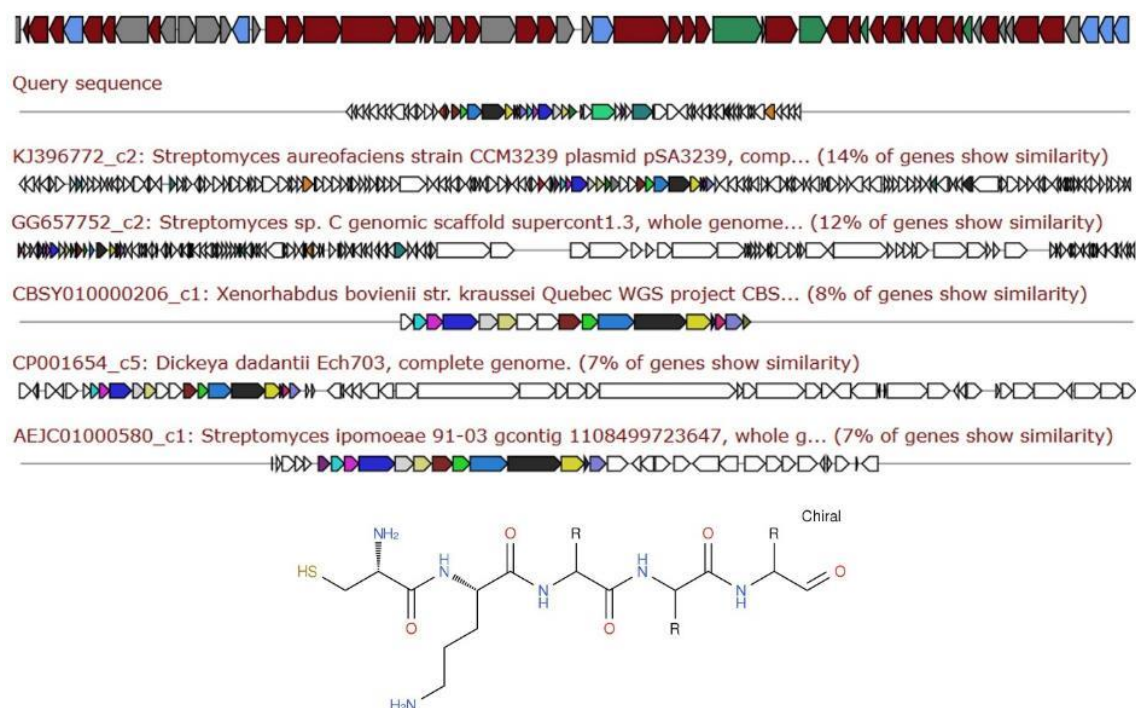
Figure 3.15: Cluster 41, a hybrid of NRPS and bacteriocin genes with 14% similarity to the gene cluster found in *Streptomyces aureofaciens*. This cluster showed no similarity to any known gene cluster.
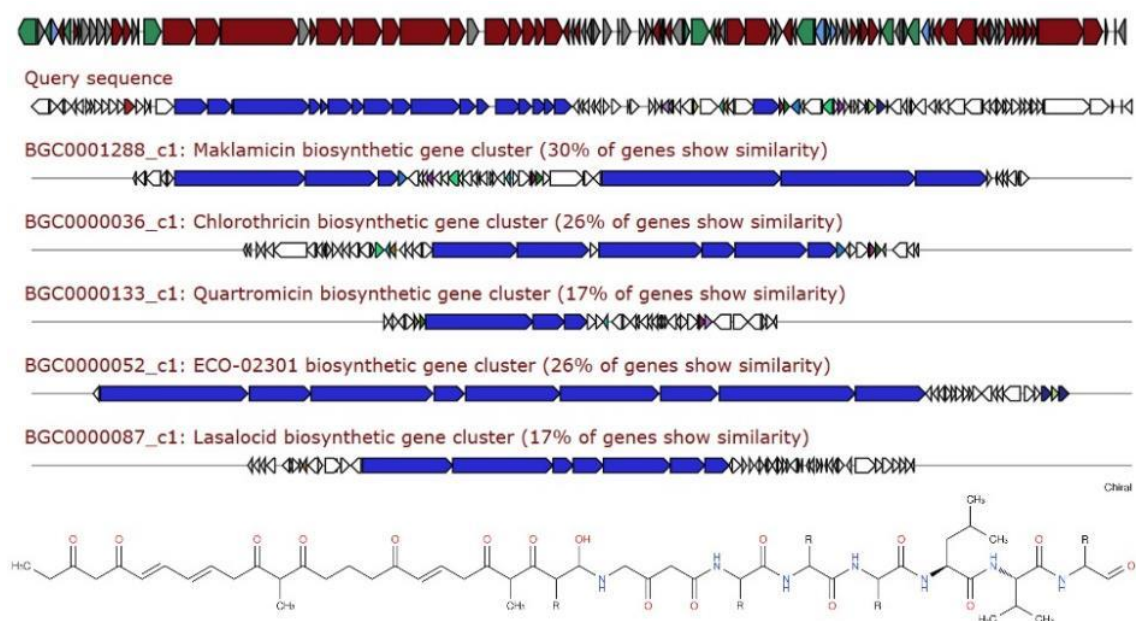
Figure 3.16: Cluster 42, a hybrid of T1pks and ß-lactam genes with 30% similarity to the maklamicin gene cluster.
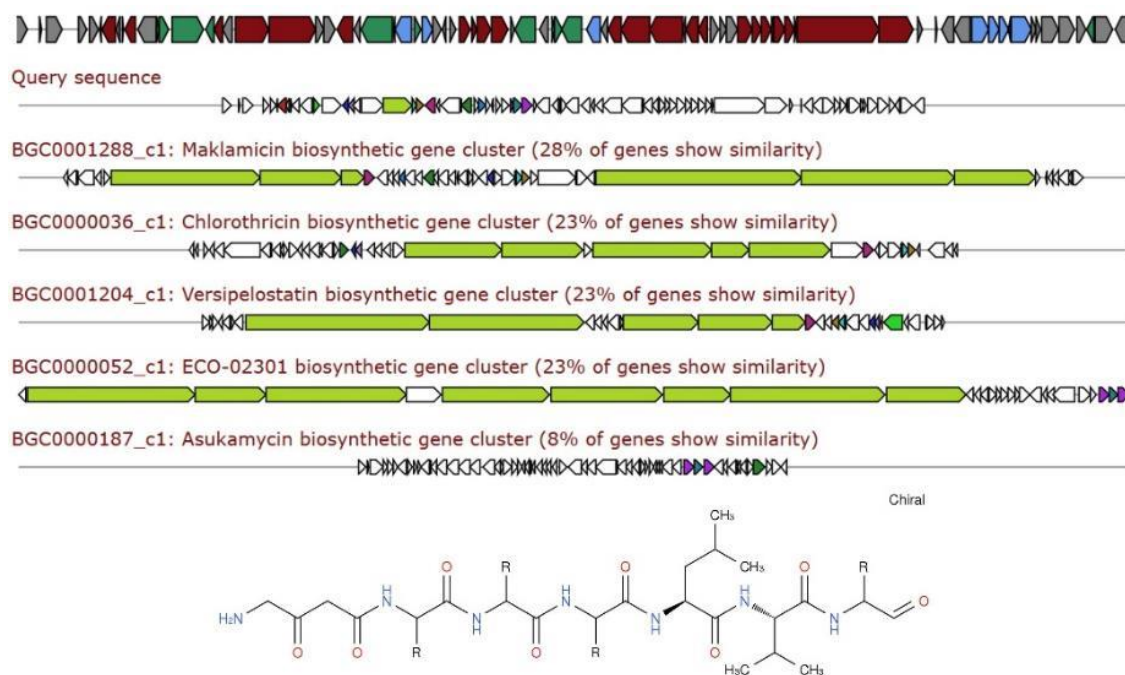
Figure 3.17: Cluster 43, a hybrid of NRPS, T1pks and lantipeptides genes with 28% similarity to the maklamicin gene cluster.
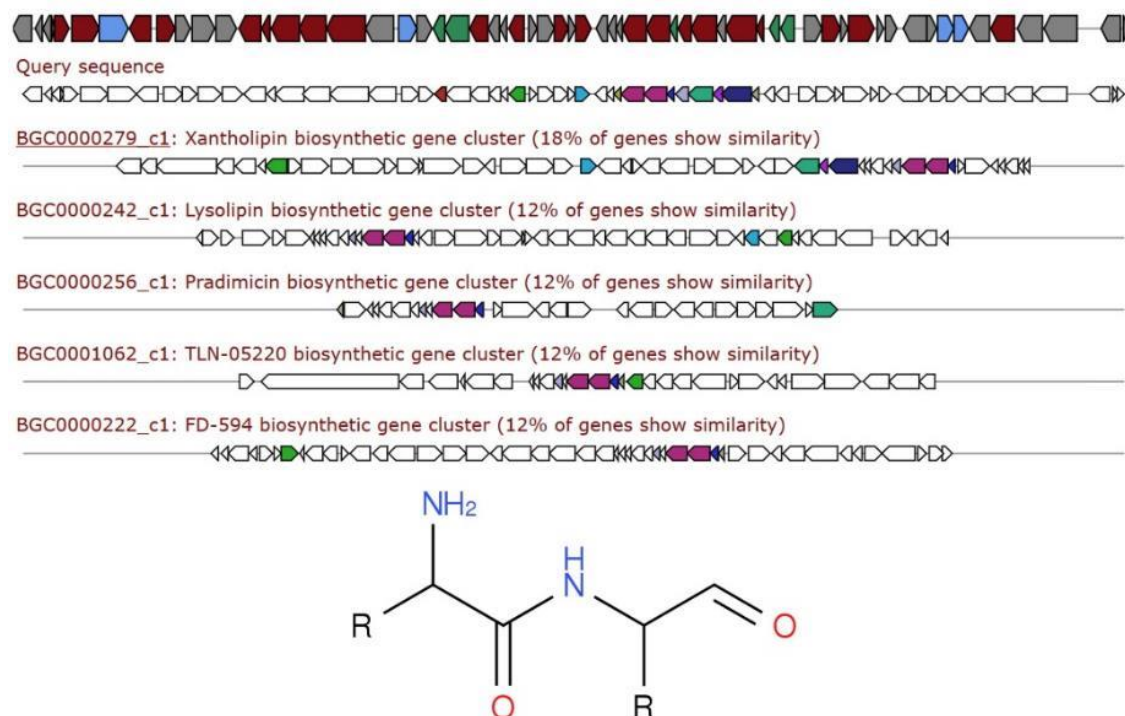
Figure 3.18: Cluster 51, an NRPS gene cluster with 18% similarity to the xantholipin gene cluster.

3.3.5.2. Biosynthetic gene clusters for other secondary metabolites

In addition to PKS, NRPS and PKS/NRPS-hybrids BGCs, the genome of strain KBS50 also contains several gene clusters for bacteriocin, lantipeptide, terpene and siderophores biosynthesis, which adds to the diversity of secondary metabolites that this strain could potentially produce. A total of 7 gene clusters including terpenes (Cluster 6, 32, 46), lantipeptides (Cluster 24, 34), siderophores (Cluster 58) and bacteriocin (Cluster 59) were identified.

Cluster 6 (terpene) showed remarkably high gene similarity (100%) to the sioxanthin BGC. The genetic map of this cluster also showed highly identical gene cluster organisation with the sioxanthin gene cluster (Figure 3.19). Sioxanthin is a novel carotenoid compound first characterized from *S. tropica* CNB-440 that is responsible for the orange pigmentation observed in *Salinispora*, and its biosynthetic pathway also present in other genera such as *Micromonospora* and *Verrucosispora* (Richter et al. 2015). Cluster 32 is another terpene gene cluster with 46% similarity to the hopene gene cluster (Figure 3.20), while cluster 46, another terpene, showed no gene similarity to any known gene cluster although it showed 45% similarity to the uncharacterized gene clusters found in *Micromonospora* species (Figure 3.21).
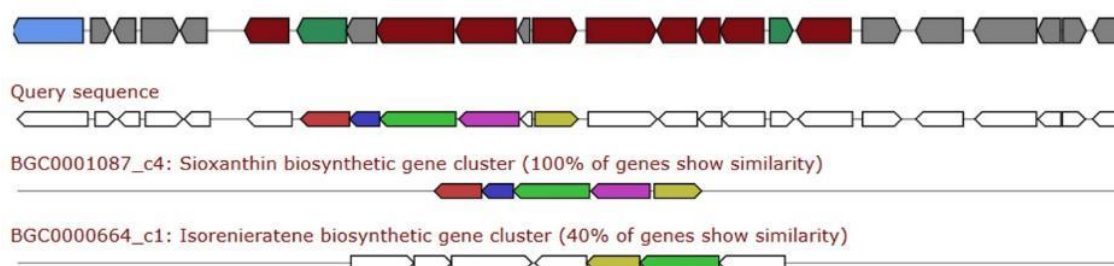
Figure 3.19: Genetic map of cluster 6 (terpene) and its homologous known gene clusters. The gene cluster organisation highly resembled the sioxanthin BGC.
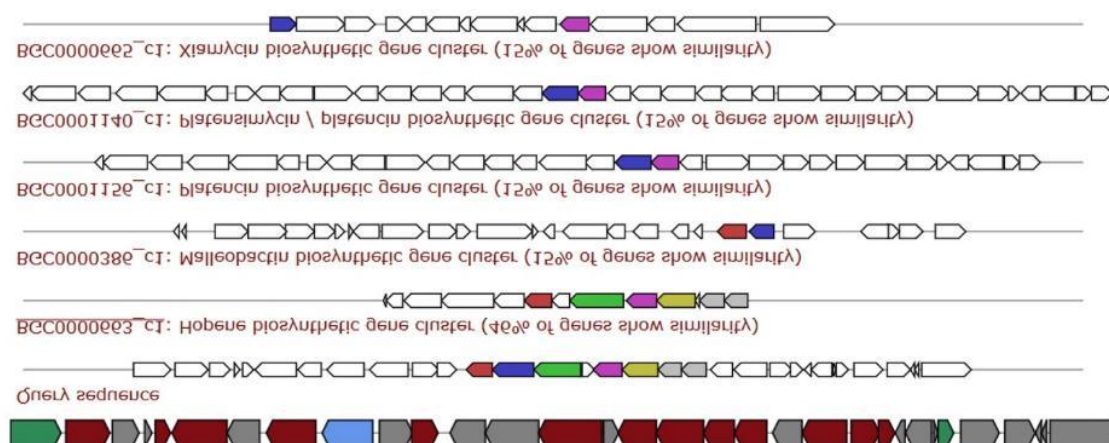
Figure 3.20: Genetic map of cluster 32 (terpene) shows 46% gene similarity and almost similar gene cluster organisation to the hopene BGC.
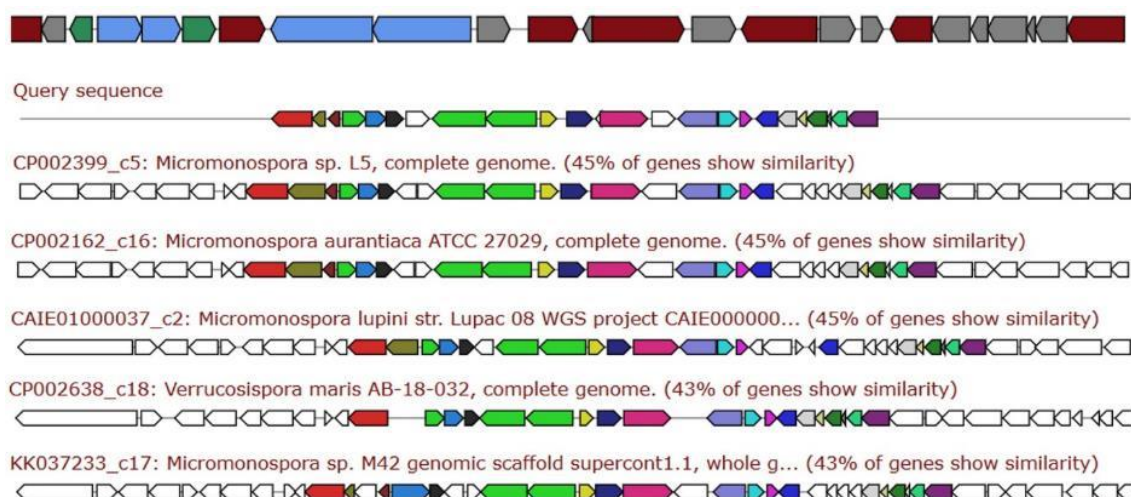
Figure 3.21: Genetic map of cluster 46 (terpene) shows 45% gene similarity to an uncharacterized gene cluster found in *Micromonospora* sp. Cluster 46 has no homologous known gene clusters.

Cluster 59 was identified as bacteriocins-saccharide hybrid gene cluster with 6% similarity to the carbapenem gene cluster. Bacteriocins are ribosomally synthesised peptides or proteins compound produces by both Gram-negative and Gram-positive bacteria that may exhibit narrow or broad spectrum antibacterial activity (Cotter et al. 2005; Yang et al. 2014). Lantipeptides and thiopeptides are another types of ribosomally

synthesised peptides that may exhibit antimicrobial properties. One lantipeptide gene cluster (Cluster 34) was identified with no homologous known gene cluster. Another 4 clusters containing the lantipeptides genes are Cluster 18 and 43 (NRPS-T1PKS-lantipeptide hybrid), cluster 38 (NRPS-lantipeptide hybrid), and cluster 24 (thiopeptide-lantipeptide hybrid). The genetic maps of these ribosomally synthesised peptides (except for the NRPS/T1pks hybrids) are shown in Figure 3.22.
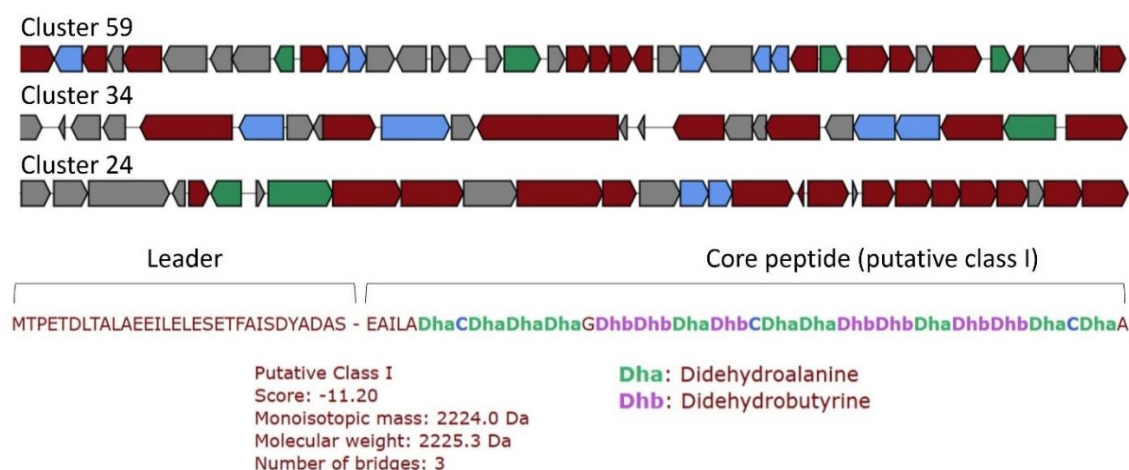
Figure 3.22: Genetic map of cluster 59 (bacteriocin), 34 (lantipeptide), and 24 (thiopeptide/lantipeptide). Predicted structure for cluster 24 including the leader, core peptide and the molecular weight is shown below the genetic map.

Siderophores are chelating agents produced by bacteria and fungi which primarily used for scavenging Fe^{3+} ion from the environment (Chu et al. 2010; Johnstone & Nolan 2015; Neilands 1995). One siderophore gene cluster (Cluster 58) was identified with no homologous known gene cluster, although it showed 12% similarity to the uncharacterized gene clusters found in *Micromonospora* and *Streptomyces* species (Figure 3.23). The presence of TetR family transcriptional regulator in this gene cluster suggests that the synthesis of the siderophores may be regulated by the protein. Meanwhile, the presence of genes related to those coding for IucA/IucC protein family suggests that this gene cluster may synthesise aerobactin-like siderophores for iron transport system (de Lorenzo & Neilands 1986).
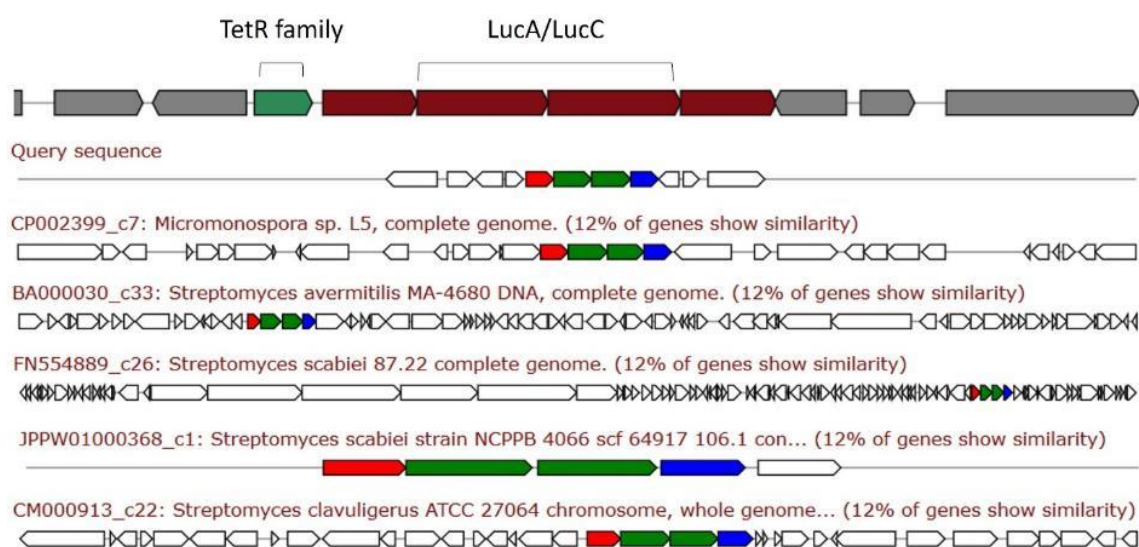
Figure 3.23: Genetic map of cluster 58 (siderophore) which may involve with the biosynthesis of aerobactin-type siderophores. The cluster contains genes encoding for TetR family transcriptional regulator and the IucA/IucC protein family.

3.3.6. Comparative secondary metabolite biosynthetic gene clusters analysis

The genome size of strain KBS50 (6.689 Mb) is larger than other species from the *Micromonosporaceae* family being compared, although only slightly bigger than *M. narathiwatensis* (6.612 Mb). However, the linear chromosome of *S. coelicolor* is almost 2 Mb larger than KBS50 genome (Table 3.3). Having a larger genome also contributed to the overall higher number of BGCs in *S. coelicolor*, at 96 total clusters. KBS50 has a total of 60 BGCs compared to 62 in *S. arenicola*, 51 in *S. tropica* and 61 in *M. narathiwatensis*. Based on the analysis in this study, the secondary metabolites BGCs in KBS50 genome spans across approximately 35% of the total genome, as compared to *S. arenicola* (40%), *S. tropica* (36%), *M. narathiwatensis* (27%), and *S. coelicolor* (28%). This was most likely an overestimate because the gene clusters identified through antiSMASH also includes adjacent genes which might not be associated with the gene cluster, as well as many putative gene clusters identified using the ClusterFinder algorithm (Medema et al. 2011; Weber et al. 2015).

The variety of secondary metabolite biosynthetic pathway in strain KBS50 is comparable to other species being compared, with the exception of the *Salinispora* species which have an additional type of gene clusters including ladderane, indole, amglyccycl, and butyrolactone (Table 3.3). Ectoine BGC is found only in *S. coelicolor*. Among the four species of *Micromonosporaceae*, KBS50 has the highest number of NRPS (7), T2pks (3) and T3pks (2) gene clusters, whereas *S. arenicola* has the highest number of T1pks (7). Other than cluster 6 (terpene) and cluster 47 (Cf_putative) which showed highly similar genes and cluster organization to the sioxanthin BGCs found in *S. tropica*, other gene clusters found in KBS50 genome have low similarity to any known gene clusters, as well as lack of similarity to any gene clusters found in the species of which it was compared with. However, a number of gene clusters found in KBS50 share high gene similarity and cluster organization to uncharacterized gene clusters found in other species, such as *Micromonospora* (cluster 56, T3pks, 80% similarity), *Kibdelosporangium* (cluster 54, T2pks, 79% similarity), and *Streptomyces* (cluster 23, T2pks, 53% similarity). Lack of homologous gene clusters indicates the uniqueness of secondary metabolite BGCs found in KBS50 and their putative metabolites.

Table 3.3: Secondary metabolite BGCs in strain *Plantactinospora* sp. KBS50 genome in comparison to other species.

Organism	*Plantactinospora* sp. KBS50	*Salinispora arenicola*[1]	*Salinispora tropica*	*Micromonospora narathiwatensis*[2]	*Streptomyces coelicolor*
Chromosome organization	Circular	Circular	Circular	Circular	Linear
Genome size	6.689 Mb	5.786 Mp	5.183 bp	6.612 Mb	8.667 Mb
T1pks (including hybrids)[3]	2	7	3	-	4
T2pks	3	1	2	1	2
T3pks	2	1	1	1	1
NRPS (including hybrids)[4]	7	4	3	3	3
PKS-NRPS hybrid	2	2	3	5	1
Bacteriocin	1	-	1	1	2
Lantipeptide/ thiopeptide	2	2	1	2	3
Terpene	3	3	1	5	4
Siderophore	1	1	1	1	3
Ladderane	-	1	-	-	-
Indole	-	1	-	-	1
Amglyccycl	-	1	1	-	-
Butyrolactone	-	1	1	-	-
Ectoine	-	-	-	-	1
Others[5]	37	37	33	42	71
TOTAL	**60**	**62**	**51**	**61**	**96**

[1]One gene cluster of T1pks in *S. arenicola* contains lantipeptides-bacteriocin genes. [2]Five gene clusters of PKS-NRPS hybrid in *M. narathiwatensis* contain the T1pks genes. [3]T1pks-hybrid clusters contain T1pks and other non-NRPS genes. [4]NRPS-hybrid clusters contain NRPS and other non-PKS genes. [5]Other type of BGCs identified with the ClusterFinder algorithm: Cf_putative, Cf_saccharide, Cf_fatty acids.

3.4. Discussion

Genome mining approach to natural product discovery incorporates the genome sequencing and bioinformatics analysis to identify gene clusters that involve in the biosynthesis of secondary metabolite compounds (Choi et al. 2015; Ziemert et al. 2016; Zotchev et al. 2012), allowing an estimates of the full metabolic capability of the actinomycete strain. The objectives of this study were to sequence and to characterize the complete genome of strain KBS50, and to identify the secondary metabolite BGCs in the genome sequence using bioinformatics tool. In Chapter 2, strain KBS50 has been identified as a new species of *Plantactinospora*, while the antimicrobial screening showed its ability to produce antimicrobial compounds against fungi and bacteria test strains. *Plantactinospora* sp. KBS50 is a unique addition to the rare actinomycete genus by being the first species isolated from the marine-associated environment in Sarawak to date. Therefore, this study presented the first complete genome sequence of a *Plantactinospora* species that provided insights into the genome structure and the capability of this species to synthesise diverse type of secondary metabolite compounds.

In this study, the complete genome of KBS50 was successfully sequenced and assembled as a single contig that represents the finished genome sequence. This was possible with the application of PacBio SMRT sequencing technology that is capable of producing long read length and very high consensus accuracy (Koren & Phillippy 2015; Rhoads & Au 2015). DNA integrity and size-selection of the prepared SMRTbell library were the contributing factors in generating longest sequencing reads possible. Due to the preferential loading of shorter molecules in the zero-mode waveguide of the SMRTCell, size-selection using the BluePippin system was necessary for preferential selection of longer molecules during library preparation, which results in an overall longer reads and higher N50 value (Rhoads & Au 2015). The N50 refers to 50% of the entire assembly is contained in contigs or scaffolds equal to or longer than the N50 value (Salzberg et al. 2012). Sequencing coverage for strain KBS50 genome was estimated at 88.8X which is lower than the recommended 100X coverage for generating a finished bacterial genome (Koren & Phillippy 2015). However, with the improvement in sequencing chemistry, high consensus accuracy can be obtained for the finished genome (Koren & Phillippy 2015), as in the case of KBS50 genome assembly where the consensus accuracy is over 99.999%. High quality and complete genome sequence generated using PacBio SMRT sequencing

technology is essential for downstream analysis, especially so for actinomycetes genome with high GC content that can be difficult to resolve using other sequencing technology (Shin et al. 2013).

The circular topology of the KBS50 chromosome is in agreement with other reported genomes from the *Micromonoporaceae* family (Trujillo et al. 2014; Udwary et al. 2007). The genome size is moderately larger than other actinomycetes such as *Salinispora* (Udwary et al. 2007), but smaller compared with *Streptomyces* (Bentley et al. 2002) and *Nonomuraea* (D'Argenio et al. 2016). Larger genome usually translates to higher coding capacity and this is true for KBS50 which has higher number of CDS compared to the smaller-sized genomes of *S. tropica* (Udwary et al. 2007) and *E. coli* (Riley et al. 2006), but lower compared to larger-sized genome such as the *N. gerenzanensis* (D'Argenio et al. 2016). The GC skew often used as an indicator to determine replication origin as well as to distinguish between the leading strand and the lagging strand (Sernova & Gelfand 2008). In the case of KBS50 genome, the leading strand has positive GC skew right after the origin of replication, and this changes around halfway in the genome where there is excess of C, over G (Figure 3.5). The identification of replication origin is essential to better understand the distribution of genes in the genome sequence (Gao & Zhang 2008).

RAST annotation pipeline provides high-quality genome annotation that automatically identifies coding sequences and assigns their respective functions. Annotations were made by comparison with other protein families in the database (Aziz et al. 2008; Overbeek et al. 2014). Apart from being completely automated annotation process, the result obtained using RAST is estimated to match the quality of the best manual genome annotation (Overbeek et al. 2014). KBS50 genome was annotated with a wide array of genes for carbohydrate, amino acids and protein metabolism, making it highly capable of utilising a different type of carbon and nitrogen sources as energy. The genome also revealed a high number of genes encoding enzymes for protein degradation. High level of protease activity detected on SMA medium (tested in Chapter 2) indicates that the protease enzyme was produced and excreted to the extracellular environment. Protease activity could be linked to antagonistic activity against fungi test strains by inhibiting the growth of fungal mycelia (Illakkiam et al. 2013; Liu & Yang 2013). Meanwhile, there was no glucanase activity detected on CMC and Avicell agar, although genome analysis indicates the presence of genes for cellulose hydrolysis. The absence of glucanase activity

could be attributed to weak enzyme activity resulted from the secretion of very small amount of the enzyme into the media if excreted at all (Begum & Absar 2009). Compared to an endophytic actinomycete, *Micromonospora lupine* which was reported to have 46 genes for cellulose degradation and high level of *in vitro* cellulase activity (Trujillo et al. 2014), the small number of cellulase-related genes present in KBS50 genome suggests that it may be incapable of hydrolyzing cellulose completely.

As a novel strain from a rare group of actinomycetes, the genome of KBS50 contains a repertoire for diverse types of secondary metabolites BGCs (Table 3.2 and Table 3.3). Notably, it has the highest number of BGCs for polyketides and nonribosomal peptides biosynthesis as compared to *S. tropica* and *S. arenicola*, the marine actinomycetes which are highly regarded as a prolific producer of bioactive compounds (Fenical & Jensen 2006). The presence of a diverse type of BGCs showed that it is genetically capable of producing a considerable number of secondary metabolite compounds high in structural diversity and possibly diverse biological activity. The metabolites particularly the one resulted from PKS and NRPS biosynthetic pathways may exhibit a wide range of biological activity including antimicrobial and anticancer (Cane & Walsh 1999).

Based on the initial antimicrobial screening results for KBS50, the antimicrobial properties particularly the narrow spectrum antibacterial activity against *S. aureus* and *B. subtilis* might be attributed to the presence of bacteriocins, thiopeptides, or lantibiotics compounds. Diversity in the lantipeptides biosynthetic pathway leads to their high structural diversity and bioactivity (Knerr & van der Donk 2012). Also known as lantibiotics, this type of compounds is produced by Gram-positive bacteria and may exhibit antibacterial properties primarily against Gram-positive bacteria (Brotz and Sahl 2000). Thiopeptides is another class of ribosomally synthesised peptides that are highly active against the Gram-positive bacteria (Just-Baringo et al. 2014). Another possible compound produced by KBS50 which may exhibit antimicrobial activity is terpene. In general, terpenes are diverse class of natural product, mostly produced by plants and fungi (Yamada et al. 2015). Terpenes have diverse functions in it host producer including antagonistic agents for a defence mechanism, among others (Gershenzon & Dudareva 2007). Having a diverse type of BGCs, it is possible that the secondary metabolites produced by KBS50 may possess other biological activity beyond antimicrobial properties, which was not evaluated in the present screening.

The advent of the next generation sequencing technology and comprehensive bioinformatics analysis tools are two key components that facilitate genome mining for natural product discovery from microorganisms. The current limitations, however, may perhaps lay upon the efficiencies of the bioinformatics tools to detect and identify secondary metabolites gene clusters from genome sequence data, as these tools depend heavily on pre-existing information on the specific type of secondary metabolites biosynthetic pathways that have been manually curated (Ziemert et al. 2016). As more data on the lesser known type of secondary metabolites become available in the future, we can expect that additional types of secondary metabolite genes may be identified from the genome sequence. Therefore, the number and type of BGCs in KBS50 genome may be more than what has been reported in the present study.

One of the limitations in antiSMASH is that it may not distinguish different type of gene clusters that happen to be close to each other, resulting in hybrid clusters (Medema et al. 2011). Therefore, the actual number of BGCs in the genome could be higher because two different gene clusters might have been merged and identified as a single hybrid by the antiSMASH. The actual number of genes especially the accessory genes within the identified cluster may also be overestimated due to the nature of BGCs detection by the antiSMASH (Medema et al. 2011). Manual examination of the gene cluster of interest may be necessary to define the actual gene cluster's boundary. Core chemical structure of a fully functional gene cluster can be predicted using antiSMASH (Zotchev et al. 2012), providing a clue as to whether the identified gene cluster consists of the necessary genes to synthesise the compound. The prediction was based on the substrate specificity of the acyltransferase (AT) and adenylation (A) domains of PKS and NRPS, respectively (Medema et al. 2011). Based on these predictions, 11 core structures of PKS and NRPS gene clusters were presented (Figure 3.8 to 3.18), which provides an overview of the diverse structural variations between the secondary metabolite products of these gene clusters. Some degree of similarity between the predicted core structures can be observed especially for adjacent clusters where the gene boundaries were not well separated (Figure 3.12 and 3.13). Although structural prediction offers some idea of structural diversity and functionality of the BGCs, it is still far from being able to predict the structure of the end product due to many possible tailoring reactions and cyclisation that may take place post-PKS/NRPS reactions (Weber et al. 2015).

With the large amount of secondary metabolite BGCs, one might expect to detect a number of these metabolites from the strain's fermentation product. However, HPLC analysis of crude extracts from the fermentation of KBS50 in four different broths detected a small number of compounds (Figure 2.8). This result indicates that many of the BGCs might not be expressed or poorly expressed when the strain was fermented in those media. Other researchers have shown similar findings for actinomycetes rich in secondary metabolite BGCs (Bentley et al. 2002; Ikeda et al. 2003; Oliynyk et al. 2007; Udwary et al. 2007). These circumstances may pose a challenge to the discovery of secondary metabolite compounds from KBS50, as many of the gene clusters may be silent that requires activation in order to isolate its metabolites. Production of very small amount of metabolite in fermentation would make it difficult to detect let alone to isolate in sufficient amount for further analysis. Even if the metabolite might possess potent antimicrobial properties, its presence in very low concentration in an extract may result in undetected biological activity (Rutledge & Challis 2015).

To overcome the limitation of low gene expression or silent genes, strategies such as fermentation using the OSMAC approach, genetic manipulation of the gene cluster of interest, ribosome engineering or heterologous expression can be used to induce the secondary metabolites biosynthesis from KBS50 (Choi et al. 2015; Zerikly & Challis 2009). The genetic map of the BGCs from KBS50 indicate that most of the cluster are conceivably regulated by regulatory proteins such as transcriptional regulators. Hence, it might be possible to manipulate these regulatory genes in order to increase the expression level or to activate the BGCs of interest (Reen et al. 2015). Meanwhile, the more traditional strategy using OSMAC approach has been shown to be quite successful for the discovery of many secondary metabolite compounds from a single strain (Bode et al. 2002). Fermentation of KBS50 in a small number of media as demonstrated in Chapter 2 showed that its secondary metabolites production could be influenced by the different type of cultivation media. By increasing the level of expression or activating silent BGCs in strain KBS50 using the OSMAC approach, one might possibly detect broader antimicrobial spectrum or different type of biological activity from the crude extracts.

3.5.   Summary

In this study, a complete genome of the actinomycete strain, *Plantactinospora* sp. KBS50 was sequenced and analysed to evaluate its genetic capability in synthesising various type of secondary metabolite compounds. The high-quality genome was assembled from the PacBio SMRT sequencing data that represent the finished genome. The circular chromosome of KBS50 comprised of 6,689,726 nucleotides. Genome annotation performed using RAST server identified 56 RNAs and 5,820 coding sequences with the coding capacity of 87.8%. Strain KBS50 is highly capable of utilising various source of carbon and nitrogen for energy based on a large number of genes dedicated for carbohydrates, proteins, and amino acids metabolism. The presence of many genes that encode for proteolytic enzymes, which was supported by strong *in vitro* protease activity makes the actinomycete a promising source of proteases. The strain is also an interesting candidate for biocontrol agent against plant pathogenic fungi. *Plantactinospora* sp. KBS50 genome contains a highly diverse type of secondary metabolite BGCs, making it an interesting source of natural product compounds such as polyketides, nonribosomal peptides, lantibiotics, terpenes, bacteriocins, siderophores and siderophores. These compound are estimated to exhibit a wide range of biological activities including antimicrobial, anticancer and iron transport function. The fact that strain KBS50 exhibits strong antibacterial activity against Gram-positive bacteria showed that the antibacterial compound is readily produced in liquid culture, allowing for its isolation and purification. The antibacterial activity by strain KBS50 might have been contributed by more than one type of compounds, due to the presence of many gene clusters for biosynthesis of various compounds with antimicrobial properties. The genome sequencing and analysis of *Plantactinospora* sp. KBS50 provides valuable genetic information that reveals its ability to synthesise diverse type of secondary metabolite compound. The large number of unique secondary metabolite BGCs strongly indicate that strain KBS50 is a promising source for discovery of novel bioactive natural product compounds.

Enhancement of Antimicrobial Activity and Secondary Metabolites Detection from *Plantactinospora* sp. KBS50 using the OSMAC fermentation approach

4.1. Introduction

Actinomycetes are an excellent producer of secondary metabolite compounds with various biological activity and this capability is further supported by the genome sequencing and analysis which reveals that many actinomycetes' genome contains various gene clusters for secondary metabolites biosynthesis (Perić-Concha & Long 2003). Similarly, the genome analysis of *Plantactinospora* sp. KBS50 revealed that the actinomycete strain possesses many BGCs for the biosynthesis of various type of secondary metabolite compounds including polyketides, nonribosomal peptides, bacteriocins, lantipeptides, thiopeptides and terpenes. Many of these compounds could potentially exhibit narrow spectrum or broad spectrum antimicrobial activity. However, the preliminary screening, as presented in Chapter 2, showed that KBS50 only demonstrated a narrow spectrum antibacterial activity against the representatives of Gram-positive bacteria. In addition, the HPLC analysis revealed a relatively small number of secondary metabolite compounds present in the crude extracts. It was possible that some of the secondary metabolite compounds were produced in a very small quantity which limits the detection of their antimicrobial activity during the bioassay screening (Rutledge & Challis 2015). Since nutrient composition can affect secondary metabolites production (Bode et al. 2002), it was anticipated that the cultivation media used for the fermentation could have limited the yield or the number of compounds produced by this actinomycete. Under these 'standard' cultivation conditions, the secondary metabolite biosynthetic genes may remain silent or poorly expressed (Rutledge & Challis 2015; Zerikly & Challis 2009).

Having the genetic capacity to synthesise various type of antimicrobial compounds, it was further hypothesised that the antimicrobial activity and secondary metabolites detection

from KBS50 could be enhanced using the OSMAC fermentation approach. Hence, the study in this chapter was designed to evaluate the antibiotics production by KBS50 using the fermentation media supplemented with selected chemical and biological elicitors, as well as cultivation at different pH value and incubation temperature. The fermentation medium, ISP2, was selected as the basal medium whereby small variation was then made. To evaluate the antibiotics production, the crude extracts from the OSMAC fermentation were screened against test microorganisms to gauge its antimicrobial activity. Statistical analysis was carried out to determine whether there were any significant increases in antibiotics production as the results of the OSMAC fermentation. Comparative HPLC analysis was carried out to monitor the changes of secondary metabolite production in the OSMAC media as compared to the basal medium. The possible number of secondary metabolite compounds with antibacterial activity against the Gram-positive bacteria was estimated using the bioassay-guided fractionation, which eventually leads to the isolation of the bioactive compounds. The potency of these compounds was determined by the evaluation of the MIC and the MBC.

The objectives of the study in this chapter were as follows:

i)   To enhance the antibiotics and secondary metabolites production by KBS50 using the OSMAC fermentation approach.

ii)   To evaluate the effect of the OSMAC fermentation on antibiotics and secondary metabolites production by KBS50, using antimicrobial screening and comparative HPLC analysis.

iii)   To isolate the antimicrobial compounds through bioassay-guided fractionation for the determination of its MIC and MBC.

4.2. Materials and Methods

4.2.1. Fermentation media compositions and preparation

The fermentation broth ISP2 was selected as the basal medium and small variations in its composition were made by the addition of elicitor elements and changes to the cultivation parameters (Table 4.1) All the broth were prepared at final pH of 7.2 (unless stated otherwise) and sterilised by autoclaving at 121°C for 15 minutes.

Table 4.1: Fermentation broths for the OSMAC approach.

No.	Fermentation broth	Medium composition (per L) and preparation
1	ISP2 (basal medium)	Yeast extract, 4.0 g, malt extract, 10.0 g, glucose, 4.0 g.
2	ISP2+NaCl 1%	ISP2 basal medium supplemented with 1% NaCl (w/v). Added before sterilization.
3	ISP2+NaCl 1.5%	ISP2 basal medium supplemented with 1.5% NaCl (w/v). Added before sterilization.
4	ISP2+DMSO 3%	ISP2 basal medium supplemented with 3% DMSO. The stock concentration of DMSO was filter-sterilized through 0.2 μm filter and added into the basal medium after sterilisation.
5	ISP2+Sc 25 μM	ISP2 basal medium supplemented with scandium chloride ($ScCl_3 \cdot 6H_2O$) at 25 μM final concentration. Scandium chloride stock concentration was filter-sterilized through 0.2 μm filter and added into the basal medium after sterilisation.
6	ISP2+Sc 50 μM	ISP2 basal medium supplemented with $ScCl_3 \cdot 6H_2O$ at 50 μM final concentration.
7	ISP2+BS cells[1]	ISP2 basal medium supplemented with 2.5% (v/v) dead cells of *B. subtilis* (stock concentration of 1.0×10^8 cells/ml).
8	ISP2+EC cells[1]	ISP2 basal medium supplemented with 2.5% (v/v) dead cells of *E. coli* (stock concentration of 1.0×10^8 cells/ml).

Table 4.1: Continue…

No.	Fermentation broth	Medium composition (per L) and preparation
9	ISP2+AN filtrate[2]	ISP2 basal medium supplemented with 1.5% (v/v) *A. niger* culture filtrate.
10	ISP2+GB filtrate[2]	ISP2 basal medium supplemented with 1.5% (v/v) *G. boninense* culture filtrate.
11	ISP2+PPB 5 mM	ISP2 basal medium supplemented with potassium phosphate buffer (pH 7.2) at 5 mM final concentration. Added before sterilisation.
12	ISP2+PPB 30 mM	ISP2 basal medium supplemented with potassium phosphate buffer (pH 7.2) at 30 mM final concentration.
13	ISP2 37°C	Fermentation at 37°C in ISP2 broth.
14	ISP2 pH 9	ISP2 broth adjusted to pH 9.
15	½ ISP2	Half-strength ISP2 broth.
16	½ ISP2+GlcNac 25 mM	Half-strength ISP2 basal medium supplemented with N-acetylglucosamine (GlcNac) at 25 mM final concentration. The stock concentration of GlcNac was filter-sterilized through 0.2 μm filter and added into the basal medium after sterilisation.
17	ISP2/GYM	Yeast extract, 4.0 g, malt extract, 10.0 g, glucose, 4.0 g, peptone (NZ-Amine A), 1.0 g, NaCl, 2.0 g, morpholinopropanesulfonic acid (MOPS) 1 M, 50 ml. Adapted from GYM broth (Ochi 1987).

[1] The dead cells of *B. subtilis* and *E. coli* were prepared from overnight cultures according to the method of Luti and Yonis (2013).

[2] The culture filtrates for *A. niger* and *G. boninense* were prepared according to the method of Wang, D et al. (2013).

4.2.2. Fermentation and extraction of secondary metabolites

Pre-culture of KBS50 was prepared by inoculating 50 ml ISP2 broth in 125 ml Erlenmeyer flasks with a 7-day old actinomycete culture, scrapped from the ISP2 agar surface. The preculture was grown in an incubator shaker at 28°C for 7 days with constant shaking at 200 rpm. After 7-day, the pre-culture was inoculated into 50 ml fermentation broths in 125 ml Erlenmeyer flasks, using 5% inoculum size. The inoculated broths were fermented at 28°C (except for the fermentation at 37°C) with constant shaking at 200 rpm for 7 days. Secondary metabolites were extracted from the spent broths using an equal volume of ethyl acetate, twice. The resulted organic extracts were dried *in vacuo* and stored at 4°C until further analysis. Meanwhile, the CFBs were collected by centrifugation at 4,000 rpm for 10 minutes and filter-sterilized through 0.2 μm filter.

4.2.3. Antimicrobial screening of the CFBs from OSMAC fermentation

Antimicrobial activity of the CFBs was evaluated using agar well diffusion assay (AwDA) against *E*. coli NBRC 3301, P. *aeruginosa* NBRC 12689, *S. aureus* NBRC 12732, *B. subtilis* NBRC 3134, *S. cerevisiae* ATCC 9763, and *A. niger* NBRC 4066. The method for the AwDA was as described in Chapter 2 (Section 2.2.8). The data collected were analysed using one-way ANOVA to compare the level of significance of antimicrobial activities between the samples in SPSS version 15. The antimicrobial activity was considered as significant if the *p*-value was lower than 0.05 ($p < 0.05$).

4.2.4. Antimicrobial screening of crude extracts

Antimicrobial screening of the crude extracts was evaluated using the microtiter plate (MTP) assay with 96-well plate format. The test strains were similar to the AwDA for CFB, with the addition of a yeast strain, *Candida albicans*. Standardised cells suspension of bacteria, at 2.0×10^6 cells/ml prepared in MHB medium, and yeast at 2.0×10^5 cells/ml prepared in SDB medium, was prepared from an overnight culture at 37°C and 30°C, respectively. A standardised spore suspension of *A. niger* (2.0×10^5 spores/ml) was prepared from a stock spore suspension kept at 4°C. Twenty ml of the dried crude extracts in 15-ml centrifuge tubes were reconstituted in 10 ml of methanol. Then 300 μl of each extract was transferred into the 96-well plates and air-dried under a fume hood. For the

antimicrobial assay, 5 µl of DMSO was added to dissolve the extract in each well, followed by the addition of 70 µl of sterile broth (MHB for bacteria, SDB for yeast/fungi). Finally, 75 uL of broth containing the standardized suspension of bacteria or yeast/fungi was added to each well for a final volume of 150 µl cells/crude extract mixture, where the concentration of the crude extracts tested was at 4-fold of its original concentration, while the DMSO concentration initially used to dissolve the extract was reduced to 3.33% final concentration.

The antibiotics chloramphenicol (CP100, 100 ppm for Gram-positive bacteria; CP400, 400 ppm for Gram-negative bacteria), nystatin (NYS100, 100 ppm for *S. cerevisiae* and *A. niger*), and miconazole (MCZ100, 100 ppm for *C. albicans*) served as positive controls. Wells containing standardised cells/spores suspension only served as the growth controls. To test the effect of DMSO, wells containing cells/spores suspension with 3.33% DMSO served as the vehicle control. These wells also contain air-dried methanol. The MTP assay for each test strain was carried out in triplicate. Initial optical density (IOD) was measured at 600 nm wavelength using 96-well plate reader. The plates were incubated at 37°C for 24 hours (bacteria) and 30°C for 48 hours (yeast/fungi) before the final OD (FOD) reading was measured at 600 nm. The level of inhibition of tests strains by the crude extracts was measured based on the differences in optical density (OD) reading before and after incubation (FOD – IOD), as compared to the growth control, calculated as follow;

Growth inhibition or reduction (%) = [C − T] / C x 100

Where;
C = FOD – IOD value of the growth control
T = FOD – IOD value of the test sample

The data collected were analysed using one-way ANOVA to compare the level of significance of antimicrobial activities between the crude extracts in SPSS version 15. The antimicrobial activity was considered as significant if the *p*-value was lower than 0.05 ($p < 0.05$).

4.2.5. Comparative secondary metabolites profiling of crude extracts using HPLC

Ten ml of dried crude extracts were dissolved in 1 ml of 100% HPLC-grade methanol, filtered through 0.2 μm regenerated cellulose filter into HPLC vials and subjected to HPLC analysis on a reverse-phase Analytical HPLC Agilent 1100 system. The method for the HPLC profiling was as described in Chapter 2 (Section 2.2.10). The HPLC data was used for qualitative analysis of secondary metabolites present in the crude extracts.

4.2.6. Production of antimicrobial compounds in 2 litre fermentation

Plantactinospora sp. KBS50 was fermented in 2 litre ISP2 broth for the production of secondary metabolite compounds with antibacterial activity against the Gram-positive test strains. Pre-cultures were prepared by scraping 7-day old cultures on ISP2 agar, inoculated into 50 ml ISP2 broth in 125-ml Erlenmeyer flasks, and incubated at 28°C for 7 days, with constant shaking at 200 rpm. The ISP2 fermentation broth was prepared in 4 separate flasks of 2-liter volume, each containing 500 ml of the broth. Each flask was inoculated with 5% pre-culture and fermented at 28°C for 7 days with constant shaking at 200 rpm. Secondary metabolites from the fermentation broth were extracted using an equal volume of ethyl acetate. After the addition of solvent, the mixture was shaken at 200 rpm for an hour. The organic layer was separated using a separatory funnel. The aqueous layer was extracted again twice, using an equal volume of ethyl acetate. The organic extracts from 3 times extraction steps were pooled together and transferred into a round-bottom flask. The extract was concentrated to dryness using rotary evaporator while being heated at 45°C on a water bath. Dried crude extract on the wall of the flask was reconstituted using the minimum amount of 100% HPLC-grade methanol and transferred into several pre-weighted glass vials. The crude extract was dried *in vacuo* and its dried weight was recorded. Antimicrobial activity of the crude extract (2.5 mg/ml) against *B. subtilis* and *S. aureus* was confirmed using the AwDA. The assay was carried out in triplicates.

4.2.7. Bioassay-guided fractionation and purification of the antimicrobial compounds

Approximately 170 mg of the crude extract was reconstituted in 2 ml 100% HPLC-grade methanol and filtered through 0.2 μm regenerated cellulose filter into an HPLC vial. The crude extract was analysed using Agilent 1200 series semi-preparative HPLC instrument equipped with DAD (G1315D, Agilent). The DAD was set to measure UV spectrum at 200, 210, 230, 254, 273, 330, 400 and 600 nm. Aliquots of 50 μl were injected into the HPLC column (9.4 x 250 mm, 5 μm, Agilent Eclipse XDB-C18) held at a temperature of 35°C, and eluted at a flow rate of 4.0 ml/minute for 25 minutes runtime per sample. Solvents and conditions used were as follows: 0-15 minute, 10% acetonitrile and 10% water; 15-25 minute, 100% acetonitrile. The crude extract was subsequently fractionated into 10 fractions using a time-based fraction collection of 2.5 minutes/fraction. Multiple rounds of fractionation were carried out from 100 μl injection volume of the crude extract solution, under the same HPLC conditions. The collected fractions from the same retention time were pooled together and dried *in vacuo*. The antimicrobial activity of these fractions at 1 mg/ml concentration was evaluated against *B. subtilis* and *S. aureus* using the AwDA. The fraction with antimicrobial activity was further fractionated using the semi-preparative HPLC for purification of the single peaks. The purified compounds were analysed on a reverse-phase Analytical HPLC Agilent 1100 system. The antimicrobial activity of the compounds at 1 mg/ml concentration was evaluated using the AwDA against *S. aureus*, as the representative of the Gram-positive test strain.

4.2.8. Determination of the MIC of semi-pure compounds

The MIC of semi-pure compounds against *S. aureus* was determined using the broth microdilution method adapted from Andrews (2001). The wells in 96-well plate were seeded with 75 μl of standardised cells suspension of *S. aureus* (2.0 X 10^6 cells/ml), prepared in double-strength MH broth. The stock solutions of semi-pure compounds (100 mg/ml) were prepared in 100% DMSO, diluted to 1 mg/ml concentration using sterile RO water followed by two-fold dilutions for 12-point concentrations. From each dilution point, 75 μl of the semi-pure compound solution was transferred into the inoculated well, for a final assay volume of 150 μl per well. The antibiotics chloramphenicol served as the positive control. Wells containing standardised cells suspension only served as the growth

controls. Other growth controls include wells containing 0.75% DMSO and 1.5% DMSO. The highest DMSO concentration for compound dilution was 1.5%. The wells containing growth medium only served as the negative control. The MIC assay was carried out in triplicate. Initial optical density (IOD) was measured at 600 nm wavelength using 96-well plate reader. The plates were incubated at 37°C for 24 hours before the final OD (FOD) reading was measured at 600 nm. The level of inhibition of test strain was measured based on the differences in optical density (OD) reading before and after incubation (FOD – IOD). The MIC was determined as the lowest concentration of the compound in which no visible growth was detected (Andrews 2001).

4.2.9. Determination of the MBC of the semi-pure compounds

The MBC of the semi-pure compounds was determined to estimate the bactericidal activity of the compounds. From the broth microdilution plates for the MIC test, 100 µl culture broth from the well of the MIC value and from 5 wells above the MIC value was pipetted out and inoculated onto MH agar plates. This experiment was carried out for all the three replicates. The number of bacterial colonies (CFU) on the MH agar was counted after 24 hours of incubation at 37°C. The MBC is the least concentration of antibacterial compound that can kill over 99.9% of the test strain (Balouiri et al. 2016; French 2006). In this experiment, the concentration of the bioactive compound that resulted in less than five colonies (CFU) on the MH agar plates was set as the MBC (Chawawisit et al. 2015).

4.3. Results

4.3.1. Antimicrobial activity of the CFBs from OSMAC fermentation

The antimicrobial screening of the CFBs using the AwDA recorded broad spectrum antimicrobial activity against the Gram-positive and Gram-negative bacteria, as well as yeast (Table 4.2). The antimicrobial activities were analysed using one-way ANOVA to compare the level of inhibition among the CFBs. The inhibition level was also compared with the basal medium, ISP2, to determine whether there was any significant difference in antimicrobial activity of the CFBs from OSMAC fermentation approach. The inhibition of test strains by the CFBs on AwDA are as shown in Figure 4.1. No inhibition of the test strains was recorded for all CFBs collected from the non-inoculated media (data not shown).

All CFBs tested exhibited antimicrobial activity against *B. subtilis*. Based on the one-way ANOVA, there was a significant difference in the inhibition of *B. subtilis* by the CFBs ($F_{(16,34)} = 67.472$, $p = 0.000$). The strongest inhibition was recorded for the CFB of ISP2 pH 9 medium with the mean diameter of inhibition zone of 16.33±0.33 mm, whereas the weakest inhibition was recorded for the CFB of ISP2 + NaCl 1.5% medium, at 9.67±0.33 mm mean diameter of inhibition zone (Figure 4.2). The antimicrobial activity of the CFB of ISP2 pH 9 and ½ ISP2 medium was significantly stronger than the CFB of ISP2 basal medium ($p < 0.05$). Meanwhile, there was no significant difference in the inhibition of *B. subtilis* resulted from the CFB of ISP2 + AN filtrate, ½ ISP2 + GlcNAc 25 mM, ISP2 + BS cells, ISP2 + PPB 5 mM, ISP2 + EC cells, ISP2 + GB filtrate, and ISP2 + DMSO 3% medium, as compared to the CFB of the basal medium, ISP2 ($p > 0.05$). Other CFBs showed significantly weaker antimicrobial activity against the test strain, as compared to the CFB of ISP2 medium ($p < 0.05$).

S. aureus was inhibited by all of the CFBs tested on the AwDA. Based on the one-way ANOVA, there was a significant difference in the inhibition of *S. aureus* by the CFBs ($F_{(16,34)} = 55.264$, $p = 0.000$). The strongest inhibition was recorded for the CFB of ISP2 pH 9 medium whereas the weakest inhibition was recorded for the CFB of ISP2 37°C, at 16.67 ± 0.33 mm and 9.33 ± 0.33 mm diameter of inhibition zone, respectively (Figure 4.3). No CFBs tested had significantly stronger inhibition as compared to the CFB of ISP2

medium ($p > 0.05$). Meanwhile, the CFB of ISP2 + NaCl 1.5% and other CFBs with 14.33 ± 0.33 mm and smaller mean diameter of inhibition zone, showed significantly weaker antimicrobial activity as compared to the CFB of ISP2 medium ($p < 0.05$).

The CFBs also exhibited antimicrobial activity against *P. aeruginosa*, whereby 5 CFBs were recorded to inhibit the test strain on AwDA (Figure 4.4). These CFBs include ISP2 + NaCl 1%, ISP2 + NaCl 1.5%, ISP2 + PPB 30 mM, ISP2 37°C, and ISP2/GYM. Based on the one-way ANOVA, there was a significant difference in the inhibition of *P. aureus* by the CFBs ($F_{(16,34)} = 1915.625$, $p = 0.000$). The strongest inhibition was recorded from the CFB of ISP2 37°C (12.00 ± 0.00 mm), followed by the CFB of ISP2/GYM (11.33±0.33 mm). The antimicrobial activity between these two CFBs was significantly different ($p < 0.05$). Meanwhile, the one-way ANOVA showed no significant difference in the antimicrobial activity between the CFB of ISP2 + NaCl 1%, ISP2 + NaCl 1.5%, and ISP2 + PPB 30 mM medium. The CFB of the basal medium, ISP2, did not exhibit any antimicrobial activity against *P. aeruginosa*. No antimicrobial activity of the CFBs was recorded against another Gram-negative test strain, *E. coli*.

Only one CFB, which is ISP2 + Sc 50 μM, exhibited antimicrobial activity against the yeast strain, *S. cerevisiae*, as indicated by the inhibition zone around the well, at 10.00 ± 0.00 mm (Figure 4.1). Although no clear zone of inhibition against *A. niger* was recorded, possibly weak inhibitory activity was observed for the CFB of the ISP2/GYM medium. The mycelial growth of the fungal around the well inoculated with the CFB appeared to be stunted, whereby similar observation was recorded from all the 3 replicates (Figure 4.1).

Table 4.2: The inhibition zone recorded on the AwDA from the screening of the CFBs of *Plantactinospora* sp. KBS50.

No.	Sample (CFB)	Inhibition zone (mm) ± SEM					
		B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*	*S. cerevisiae*
1	ISP2	14.67 ± 0.33	15.67 ± 0.33	-	-	-	-
2	ISP2+NaCl 1%	11.00 ± 0.00	14.67 ± 0.33	-	9.67 ± 0.33	-	-
3	ISP2+NaCl 1.5%	9.67 ± 0.33	14.33 ± 0.33	-	10.00 ± 0.00	-	-
4	ISP2+DMSO 3%	15.67 ± 0.33	15.00 ± 0.00	-	-	-	-
5	ISP2+Sc 25 µM	13.00 ± 0.00	16.00 ± 0.00	-	-	-	-
6	ISP2+Sc 50 µM	13.00 ± 0.00	16.00 ± 0.00	-	-	-	10.00 ± 0.00
7	ISP2+BS cells	14.67 ± 0.33	14.00 ± 0.00	-	-	-	-
8	ISP2+EC cells	15.00 ± 0.00	15.33 ± 0.33	-	-	-	-
9	ISP2+AN filtrate	13.67 ± 0.33	12.67 ± 0.33	-	-	-	-
10	ISP2+GB filtrate	15.00 ± 0.00	15.00 ± 0.00	-	-	-	-
11	ISP2+PPB 5 mM	14.67 ± 0.33	16.33 ± 0.33	-	-	-	-
12	ISP2+PPB 30 mM	11.00 ± 0.00	16.00 ± 0.00	-	10.00 ± 0.00	-	-
13	ISP2 37°C	10.67 ± 0.33	9.33 ± 0.33	-	12.00 ± 0.00	-	-
14	ISP2 pH9	16.33 ± 0.33	16.67 ± 0.33	-	-	-	-
15	1/2 ISP2	16.00 ± 0.00	16.00 ± 0.00	-	-	-	-
16	1/2 ISP2+GlcNAc 25 mM	13.67 ± 0.33	13.00 ± 0.00	-	-	-	-
17	ISP2/GYM	13.00 ± 0.00	15.67 ± 0.33	-	11.33 ± 0.33	-	-
18	CP100	21.67 ± 0.33	17.67 ± 0.33	NT	NT	NT	NT
19	CP400	NT	NT	20.00 ± 0.00	20.00 ± 0.00	NT	NT
20	NYS100	NT	NT	NT	NT	25.00 ± 0.00	30.67 ± 0.67

NT = Not tested (NT); '-' = no inhibition zone recorded; CP100 = chloramphenicol, 100 ppm; CP400 = chloramphenicol, 400 ppm; NYS100 = nystatin, 100 ppm.
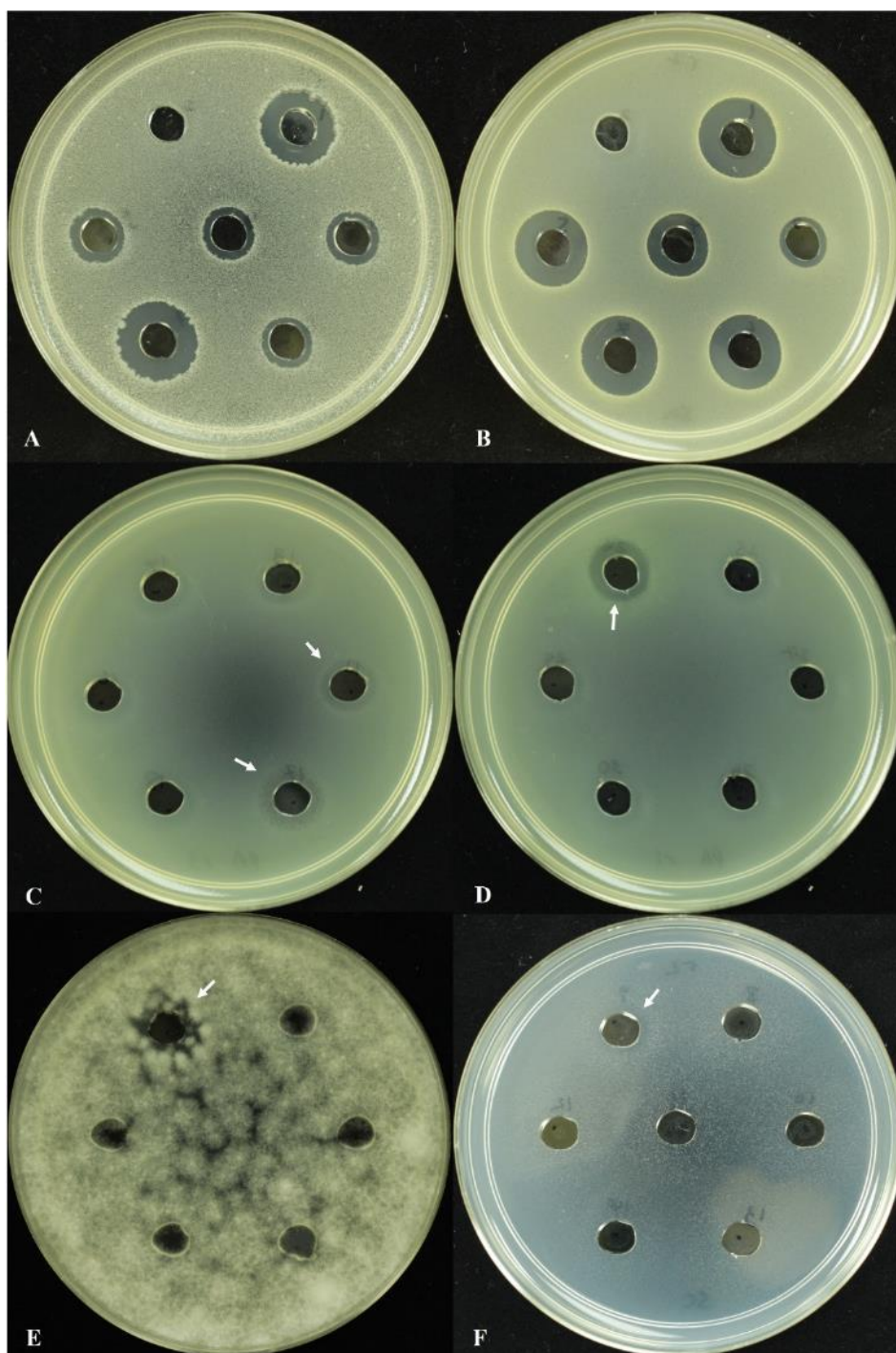
Figure 4.1: The inhibition of test strains by the CFBs of KBS50 in the AwDA. Strong antimicrobial activities were recorded against *B. subtilis* (A) and *S. aureus* (B) as indicated by the clear zone of inhibition. Weak inhibition against *P. aeruginosa* was recorded for a number of CFBs (C & D). Possibly weak antifungal activity against *A. niger* was observed for the CFB of the ISP2/GYM medium, although no clear zone of inhibition was recorded (E). Weak antimicrobial activity against *S. cerevisiae* was recorded, as indicated by the small zone of inhibition on the AwDA (F).
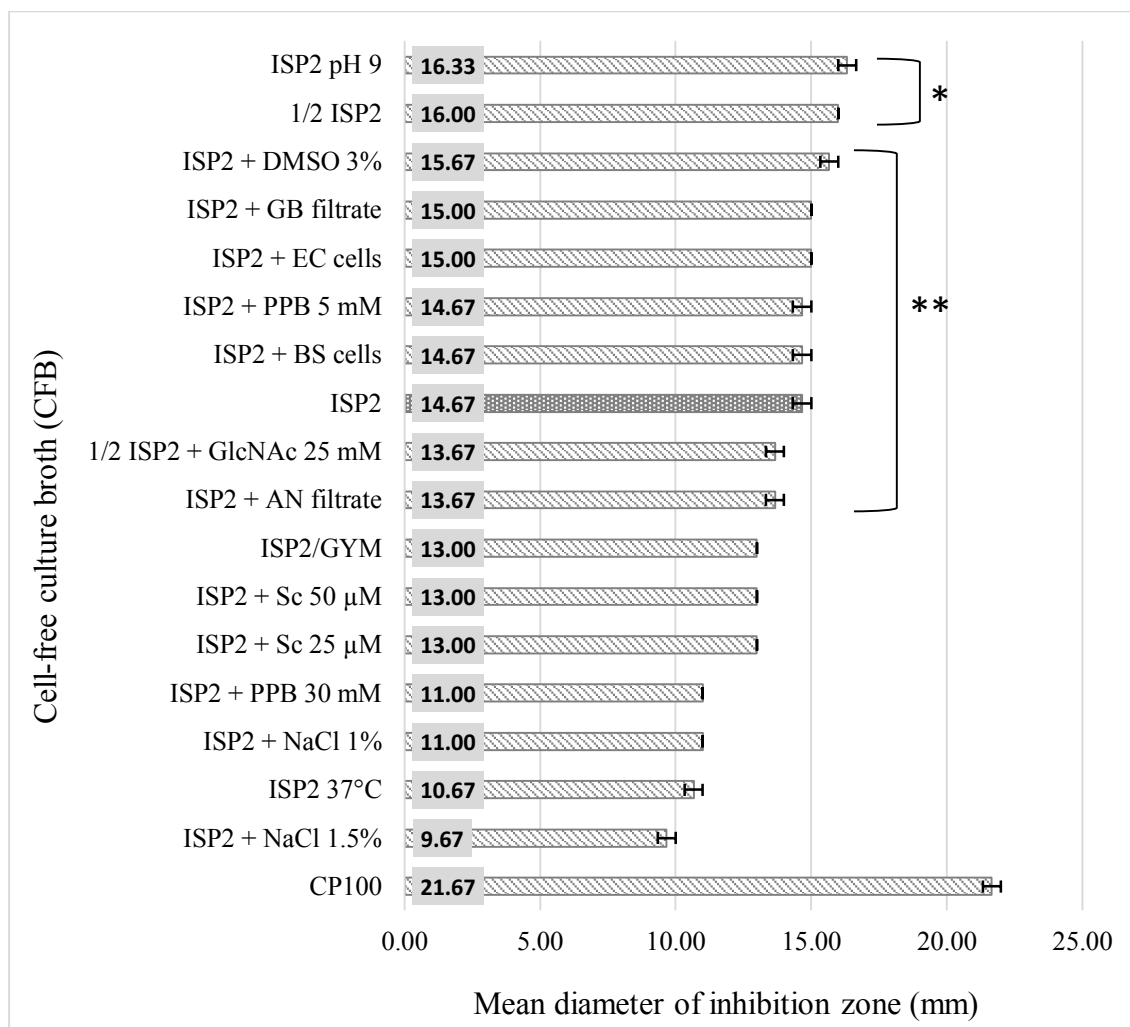
Figure 4.2: Growth inhibition of *B. subtilis* resulted by the CFBs of KBS50 in the AwDA. *Significantly stronger inhibition as compared to the CFB of ISP2 medium ($p < 0.05$). **No significant difference in growth inhibition as compared to the CFB of ISP2 medium ($p > 0.05$).
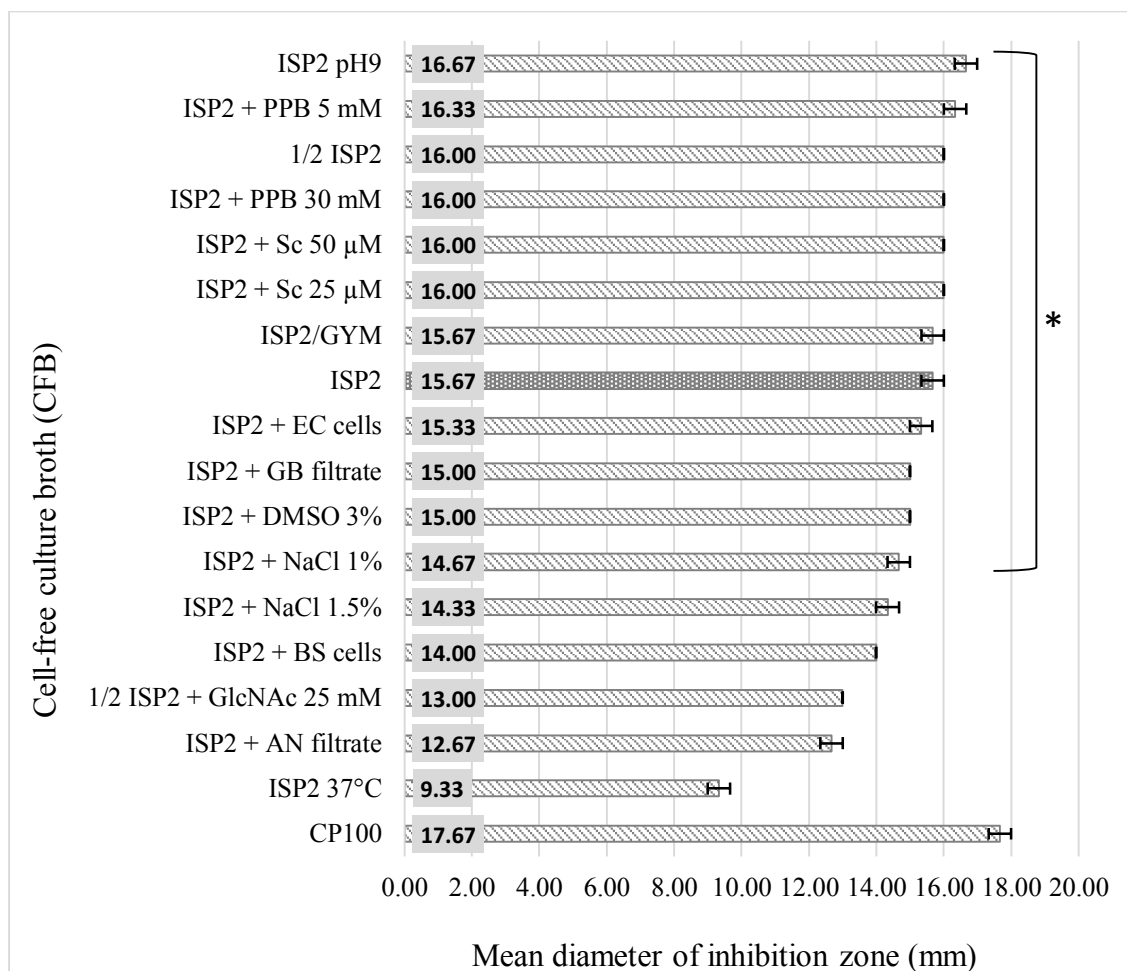
Figure 4.3: Growth inhibition of *S. aureus* resulted by the CFBs of KBS50 in the AwDA. *No significant difference in growth inhibition as compared to the CFB of ISP2 medium ($p > 0.05$).
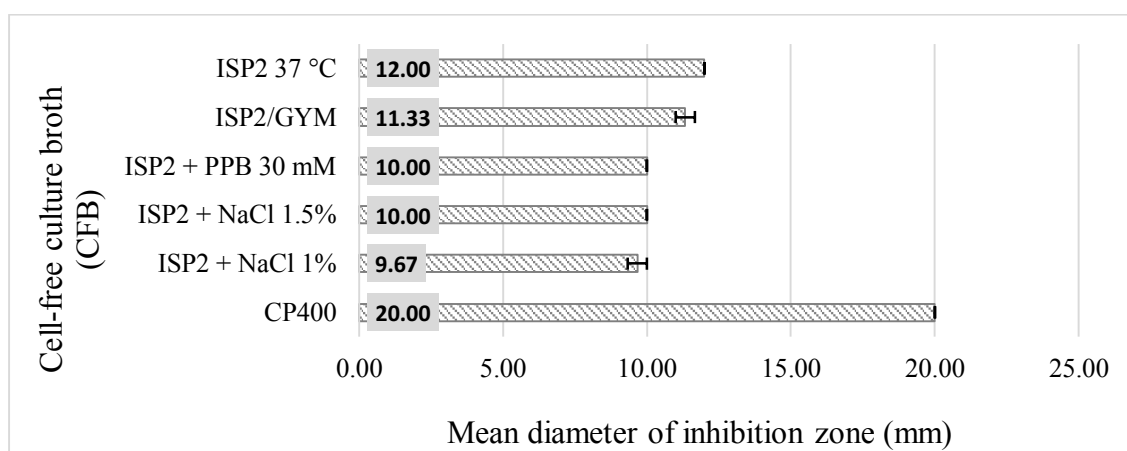
Figure 4.4: Growth inhibition of *P. aeruginosa* resulted by the CFBs of KBS50 in the AwDA. No inhibition was recorded for the CFB of the ISP2 medium.

4.3.2. Antimicrobial activity of the crude extracts

The secondary metabolites from the fermentation broths were extracted using ethyl acetate and the resulted crude extracts were screened for antimicrobial activity using the MTP assay. The minimum growth reduction of 50% and 80% in comparison to the growth control was set as the threshold for the crude extracts to be considered as exhibiting weak or strong antimicrobial activity, respectively. The antimicrobial activities were analysed using one-way ANOVA to compare the level of antimicrobial activity among the crude extracts, as well as to determine whether there was any significant difference in the antimicrobial activity of other crude extracts as compared to the basal medium, ISP2.

Overall, broad spectrum antimicrobial activities were recorded against Gram-positive and Gram-negative bacteria, fungi and yeast test strains (Table 4.3). The crude extract of ISP2 exhibited strong antimicrobial activity against *B. subtilis*, *S. aureus* and *A. niger*, and weak antimicrobial activity against *C. albicans*. Interestingly, the inhibition of *A. niger* by the ISP2 crude extract was recorded on the MTP assay, but not on AwDA as tested previously. This shows that the MTP assay was more sensitive for the detection of antimicrobial activity against the fungal test strain. Fermentation of KBS50 in ISP2 broth supplemented with the elicitors, as well as pH 9, had resulted in an increased in antimicrobial activities, especially against *E. coli*, *P. aeruginosa* and *C. albicans*. However, no antimicrobial activity was recorded against *S. cerevisiae* for all crude extracts tested. The increases in antimicrobial activity indicate that there was an increase in antibiotics production when the actinomycete strain was cultivated in these fermentation media, as compared to the basal medium, ISP2. Screening of crude extracts from the non-inoculated media showed no growth inhibition on all test strains (data not shown). A similar observation was recorded for the DMSO and methanol growth controls, indicating that the growth of these test strains was not affected by the DMSO or dried methanol that was used to reconstitute the crude extracts.

Table 4.3: MTP assay results from the screening of the crude extracts of *Plantactinospora* sp. KBS50.

No.	Extract	Growth inhibition (%) ± SEM						
		B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*	*S. cerevisiae*	*C. albicans*
1	ISP2	96.62 ± 1.44	96.71 ± 0.38	36.03 ± 0.51	29.86 ± 1.55	99.95 ± 0.54	-18.37 ± 13.06	52.54 ± 5.32
2	ISP2+NaCl 1%	93.72 ± 0.21	96.31 ± 0.11	53.13 ± 0.64	47.02 ± 0.96	27.56 ± 1.74	-54.26 ± 13.66	2.99 ± 2.88
3	ISP2+NaCl 1.5%	87.23 ± 0.45	75.80 ± 0.85	53.32 ± 0.04	36.55 ± 2.87	-30.73 ± 28.64	-54.92 ± 14.71	2.05 ± 1.82
4	ISP2+DMSO 3%	97.66 ± 0.65	95.57 ± 1.24	51.92 ± 1.61	54.79 ± 1.63	84.69 ± 6.56	-28.89 ± 14.62	90.11 ± 0.85
5	ISP2+Sc 25 µM	102.54 ± 0.11	95.19 ± 0.44	46.17 ± 3.42	12.31 ± 0.34	76.64 ± 12.29	-64.27 ± 23.91	84.98 ± 3.84
6	ISP2+Sc 50 µM	103.13 ± 1.94	94.30 ± 0.28	52.32 ± 1.08	53.61 ± 0.42	88.58 ± 6.49	-61.69 ± 19.26	89.56 ± 0.69
7	ISP2+BS cells	96.06 ± 0.07	90.94 ± 0.11	47.03 ± 0.89	52.57 ± 2.27	96.25 ± 3.17	-44.18 ± 25.01	89.88 ± 1.93
8	ISP2+EC cells	91.99 ± 0.85	92.82 ± 0.45	40.68 ± 0.48	54.26 ± 0.31	101.05 ± 0.15	-42.30 ± 21.15	73.41 ± 2.55
9	ISP2+AN filtrate	99.17 ± 0.08	98.11 ± 0.43	38.72 ± 0.17	63.22 ± 0.85	88.16 ± 1.73	-1.26 ± 7.55	36.39 ± 4.60
10	ISP2+GB filtrate	92.25 ± 0.53	95.76 ± 0.43	58.92 ± 0.98	65.02 ± 4.24	98.78 ± 0.76	-14.85 ± 13.64	90.76 ± 4.95
11	ISP2+PPB 5 mM	91.20 ± 0.55	93.73 ± 0.19	52.32 ± 1.77	57.12 ± 0.68	82.15 ± 7.57	-43.16 ± 23.60	90.27 ± 3.77
12	ISP2+PPB 30 mM	101.79 ± 0.14	99.02 ± 0.24	52.58 ± 0.23	38.89 ± 1.92	48.17 ± 6.57	-67.46 ± 1.83	-16.04 ± 7.29
13	ISP2 37°C	94.27 ± 0.67	54.74 ± 1.37	54.55 ± 0.37	8.07 ± 1.70	23.19 ± 5.33	-41.43 ± 1.58	-11.55 ± 3.28
14	ISP2 pH 9	88.86 ± 0.84	91.53 ± 0.14	63.78 ± 0.62	51.25 ± 0.87	96.29 ± 0.75	-29.78 ± 13.94	95.78 ± 3.36
15	1/2 ISP2	93.86 ± 0.70	96.46 ± 0.80	54.63 ± 4.67	44.58 ± 0.22	93.83 ± 0.51	-59.20 ± 6.37	92.08 ± 3.58
16	1/2 ISP2+GlcNAc 25 mM	88.98 ± 0.22	94.96 ± 0.53	61.56 ± 1.51	34.49 ± 2.47	56.46 ± 5.76	-74.31 ± 4.63	68.80 ± 2.79
17	ISP2/GYM	98.76 ± 0.33	98.26 ± 1.21	61.22 ± 1.73	20.15 ± 3.19	67.25 ± 1.32	-26.90 ± 2.66	22.80 ± 1.82
18	CP100	81.96 ± 3.43	78.44 ± 1.49	NT	NT	NT	NT	NT
19	CP400	NT	NT	99.13 ± 0.66	98.68 0.67	NT	NT	NT
20	NYS100	NT	NT	NT	NT	100.98 ± 1.25	104.12 ± 0.65	NT
21	MCZ100	NT	NT	NT	NT	NT	NT	103.04 ± 0.44

NT = Not tested; CP100 = chloramphenicol, 100 ppm; CP400 = chloramphenicol, 400 ppm; NYS100 = nystatin, 100 ppm; MCZ100 = miconazol, 100 ppm.

All crude extracts tested showed strong antimicrobial activity against *B. subtilis*, which resulted in over 80% growth reduction of the test strain (Figure 4.5). Based on the one-way ANOVA, there was a significant difference in the inhibition of *B. subtilis* by the crude extracts ($F_{(16,34)} = 41.612$, $p = 0.000$). The strongest inhibition was recorded for the crude extract of ISP2 supplemented with 50 µM scandium chloride ($103.13 \pm 1.94\%$). The antimicrobial activity was significantly stronger as compared to the crude extract of the basal medium ($p < 0.05$). Meanwhile, the crude extract from ISP2 medium supplemented with 1.5% NaCl resulted in the lowest growth reduction of $87.23\pm0.45\%$, which was significantly lower compared to ISP2 ($p < 0.05$).

Figure 4.5: Growth inhibition of *B. subtilis* resulted by the crude extracts of KBS50 in the MTP assay. *Significantly stronger inhibition as compared to ISP2 extract ($p < 0.05$). **No significant difference in growth inhibition as compared to ISP2 extract ($p > 0.05$).

Fifteen crude extracts showed strong antimicrobial activity against *S. aureus* which resulted in over 90% growth inhibition (Figure 4.6). Based on the one-way ANOVA, there was a significant difference in the inhibition of *S. aureus* by the crude extracts ($F_{(16,34)} = 262.429$, $p = 0.000$). Five extracts exhibited significantly weaker antimicrobial activity against *S. aureus* as compared to the basal medium ($p < 0.05$). These extracts include ISP2 + EC cells (92.82 ± 0.45%), ISP2 pH 9 (91.53 ± 0.14%), ISP2 + BS cells (90.94 ± 0.11), ISP2 + NaCl 1.5% (75.80 ± 0.85%) and ISP2 37°C (54.74 ± 1.37%). Meanwhile, the antimicrobial activity of other crude extracts against *S. aureus* did not show any significant difference as compared to the antimicrobial activity of the crude extract of ISP2 medium ($p > 0.05$).

Figure 4.6: Growth inhibition of *S. aureus* resulted by the crude extracts of KBS50 in the MTP assay. *No significant difference in growth inhibition as compared to ISP2 extract (*p* > 0.05).

The antimicrobial activity of KBS50 crude extracts against the Gram-negative test strains also increased significantly as compared to the crude extract of ISP2 medium (Figure 4.7 and 4.8). Based on the one-way ANOVA, there was a significant difference in the inhibition of *E. coli* ($F_{(16,34)} = 21.682$, $p = 0.000$) and *P. aeruginosa* ($F_{(16,34)} = 78.686$, $p = 0.000$). However, these extracts only exhibited weak antimicrobial activities against these Gram-negative bacteria, as indicated by the growth reduction lower than 80%. The strongest inhibition recorded against *E. coli* resulted from the crude extract of ISP2 pH 9 ($63.78 \pm 0.62\%$), while the strongest inhibition recorded against *P. aeruginosa* resulted from the crude extract of ISP2 supplemented with the culture filtrate of *G. boninense* ($65.02 \pm 4.24\%$). Both results were significantly stronger as compared to the inhibition of ISP2 extract ($p < 0.05$).

Figure 4.7: Growth inhibition of *E. coli* resulted by the crude extracts of KBS50 in the MTP assay. *Significantly stronger inhibition as compared to ISP2 extract ($p < 0.05$). **No significant difference in growth inhibition as compared to ISP2 extract ($p > 0.05$).
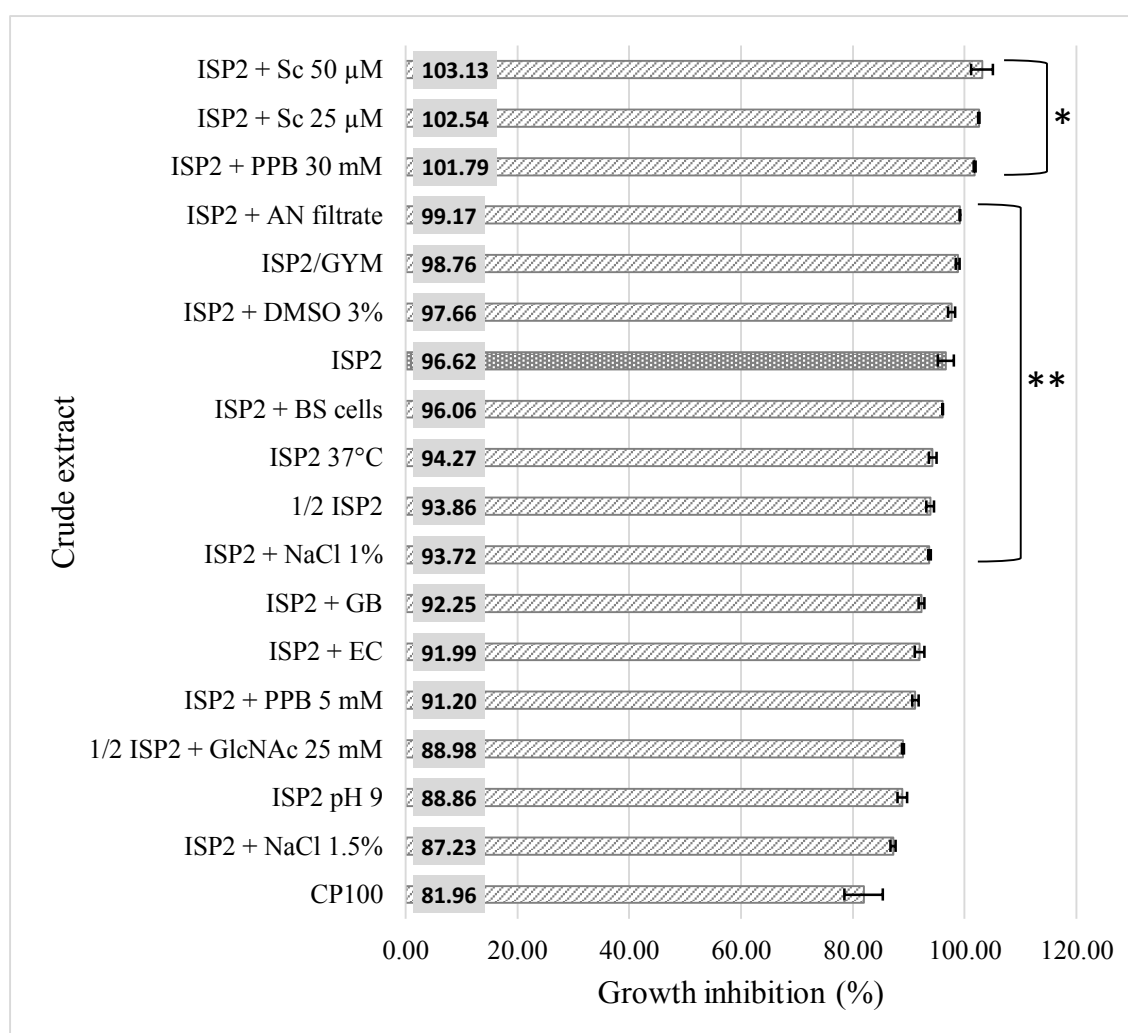
Figure 4.8: Growth inhibition of *P. aeruginosa* resulted by the crude extracts of KBS50 in the MTP assay. *Significantly stronger inhibition as compared to ISP2 extract ($p < 0.05$). **No significant difference in growth inhibition as compared to ISP2 extract ($p > 0.05$).

Ten extracts, including ISP2, exhibited strong antifungal activity against *A. niger*, where the growth reduction of the test strain was recorded at more than 80% (Figure 4.9). Based on the one-way ANOVA, there was a significant difference in the inhibition of *A. niger* ($F(16,34) = 17.617$, $p = 0.000$). Four extracts did not exhibit any antifungal activity, where the growth reduction was lower than 50% and significantly different as compared to ISP2 ($p < 0.05$). These extracts include ISP2 + NaCl 1.5%, ISP2 37°C, ISP2 + NaCl 1%, and ISP2 + PPB 30 mM. Only ISP2 medium supplemented with *E. coli* cells exhibit stronger antifungal activity as compared to ISP2 medium, however, there was no significant difference in growth inhibition between the two crude extracts ($p < 0.05$). Other crude extracts also showed no significant difference in inhibition as compared to ISP2 extract ($p < 0.05$).
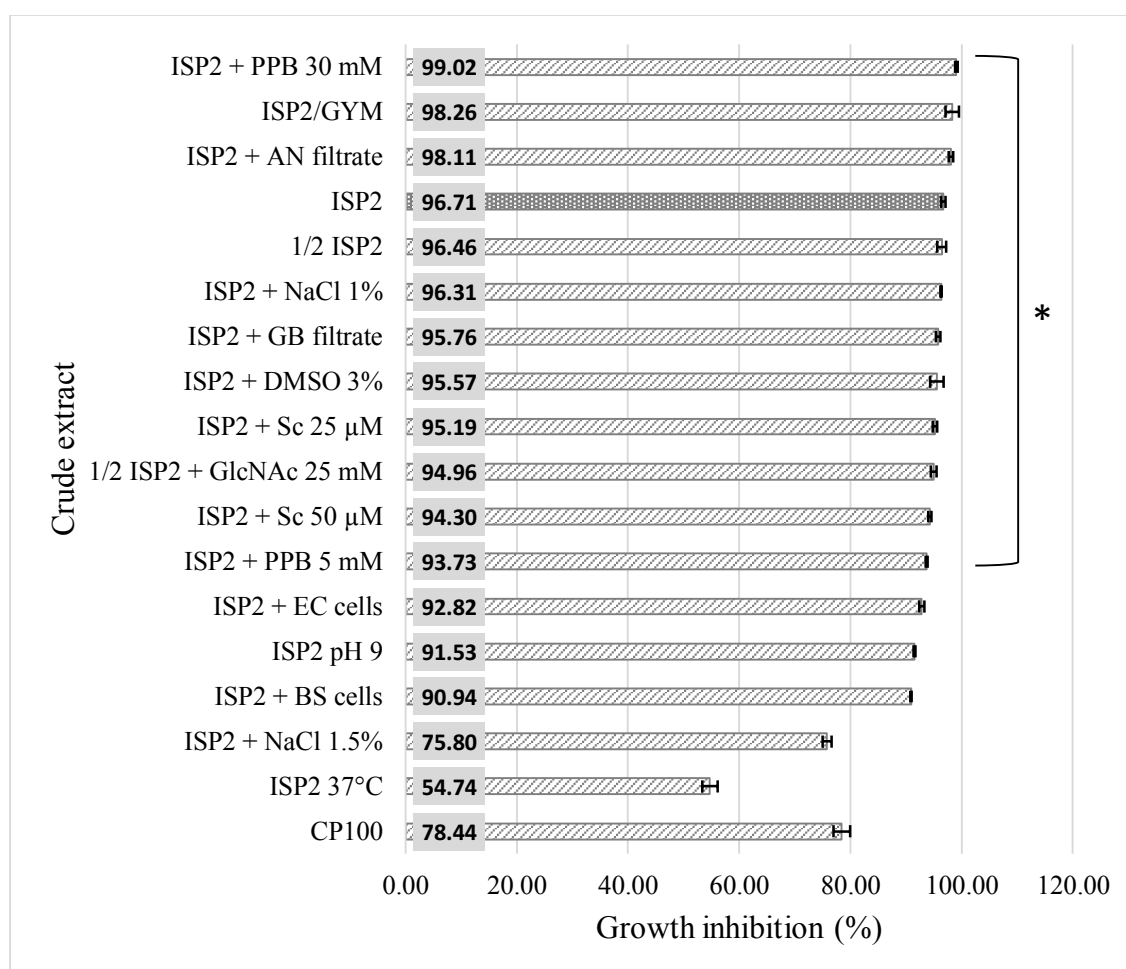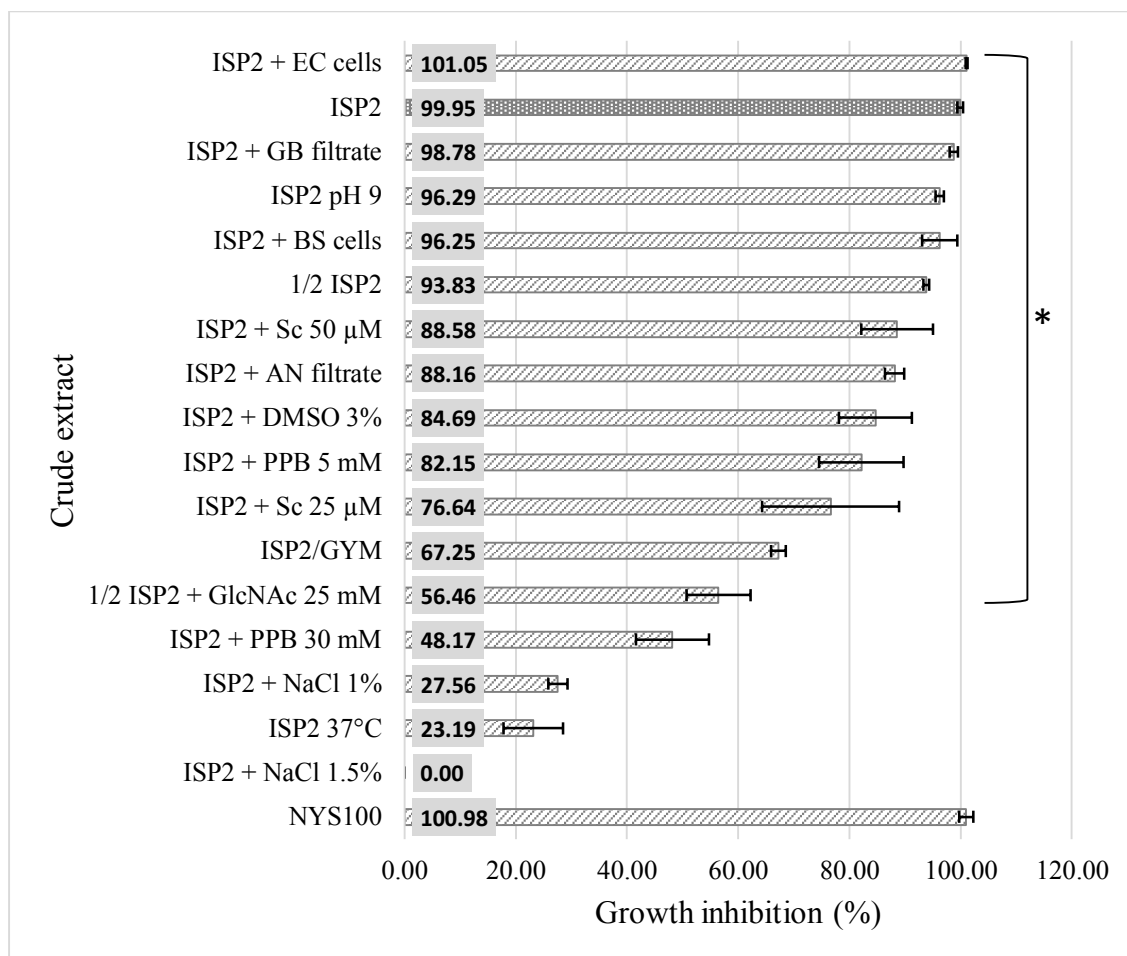
Figure 4.9: Growth inhibition of *A. niger* resulted by the crude extracts of KBS50 in the MTP assay. *No significant difference in growth inhibition as compared to ISP2 extract ($p > 0.05$). Growth inhibition resulted from the crude extract of ISP2 + NaCl 1.5% was represented as 0.00% in the bar chart due to the negative value (-30.73 ± 28.64%).

Strong antimicrobial activity against the yeast strain, *C. albicans,* was recorded, where 8 crude extracts resulted in over 80% growth reduction of the test strain (Figure 4.10). The inhibitions were significantly stronger compared to the ISP2 extract ($p < 0.05$). The highest inhibition was recorded from the crude extract of ISP2 pH 9, with 95.78 ± 3.36% growth inhibition. Meanwhile, the crude extract of ISP2 supplemented with *E. coli* cells resulted in weaker antimicrobial activity (73.41 ± 2.55% growth reduction) against *C. albicans*, but still significantly higher than the antimicrobial activity of ISP2 crude extract ($p < 0.05$). There was no significant difference in growth reduction resulted from the crude extract of ISP2 and ½ ISP2 medium supplemented with 25 mM GlcNAc.
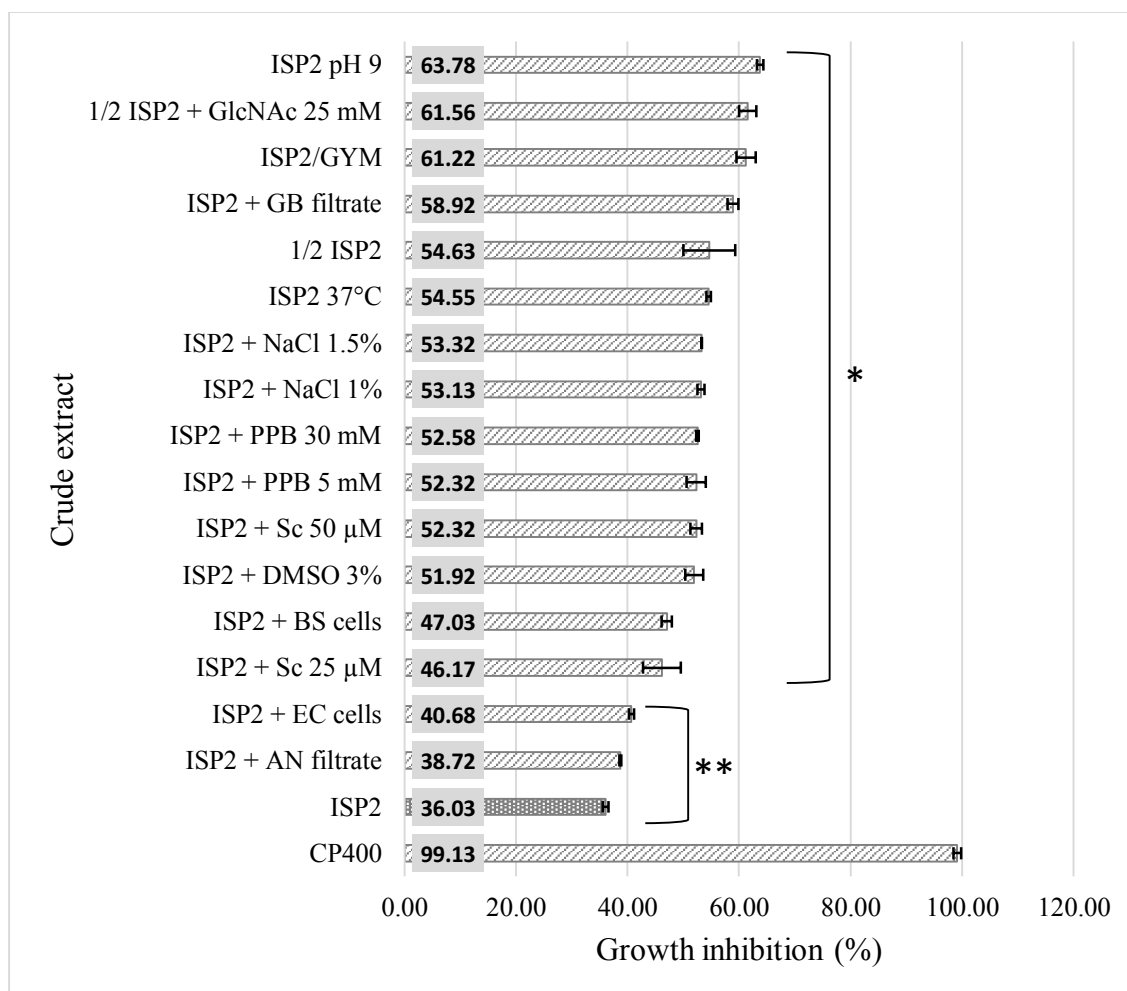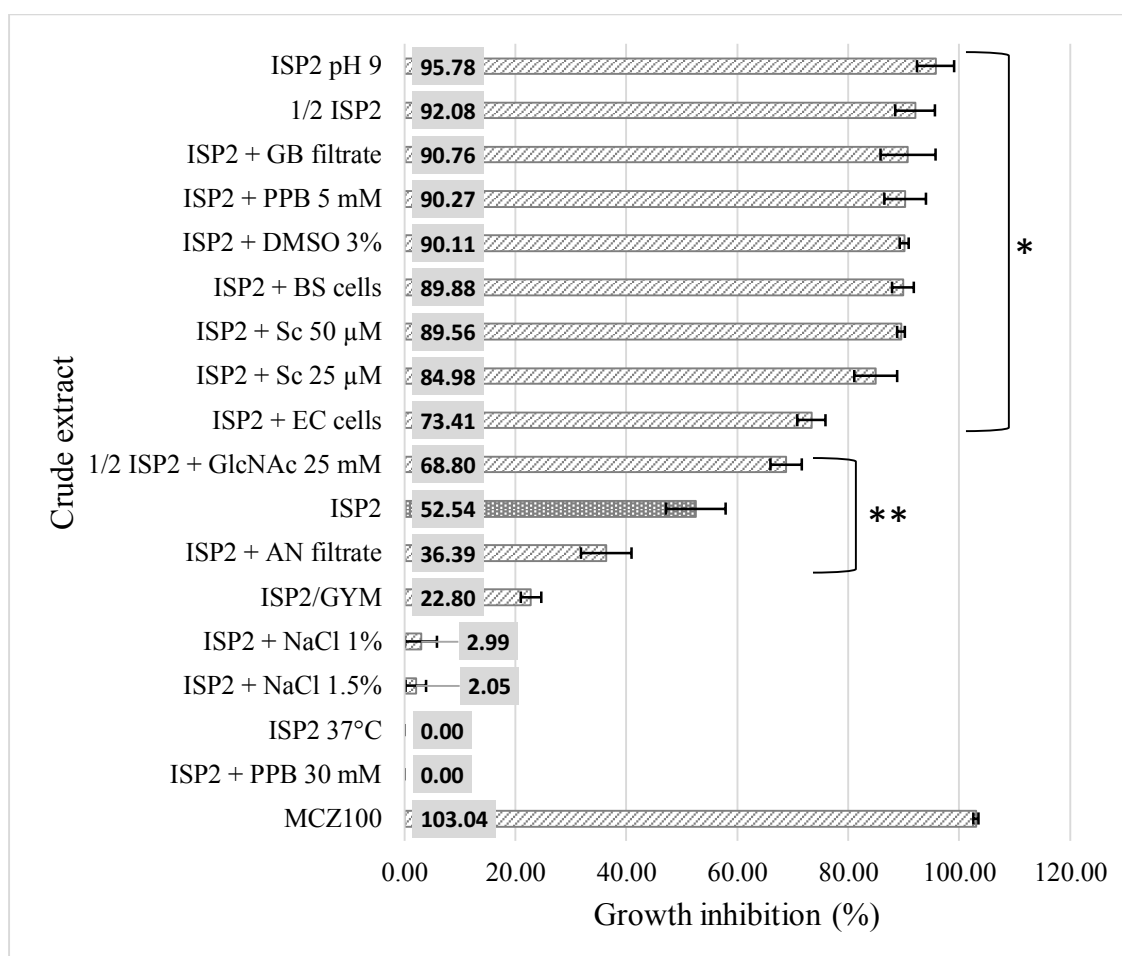
Figure 4.10: Growth inhibition of *C. albicans* resulted by the crude extracts of KBS50 in the MTP assay. *Significantly stronger inhibition as compared to ISP2 extract ($p < 0.05$). **No significant difference in growth inhib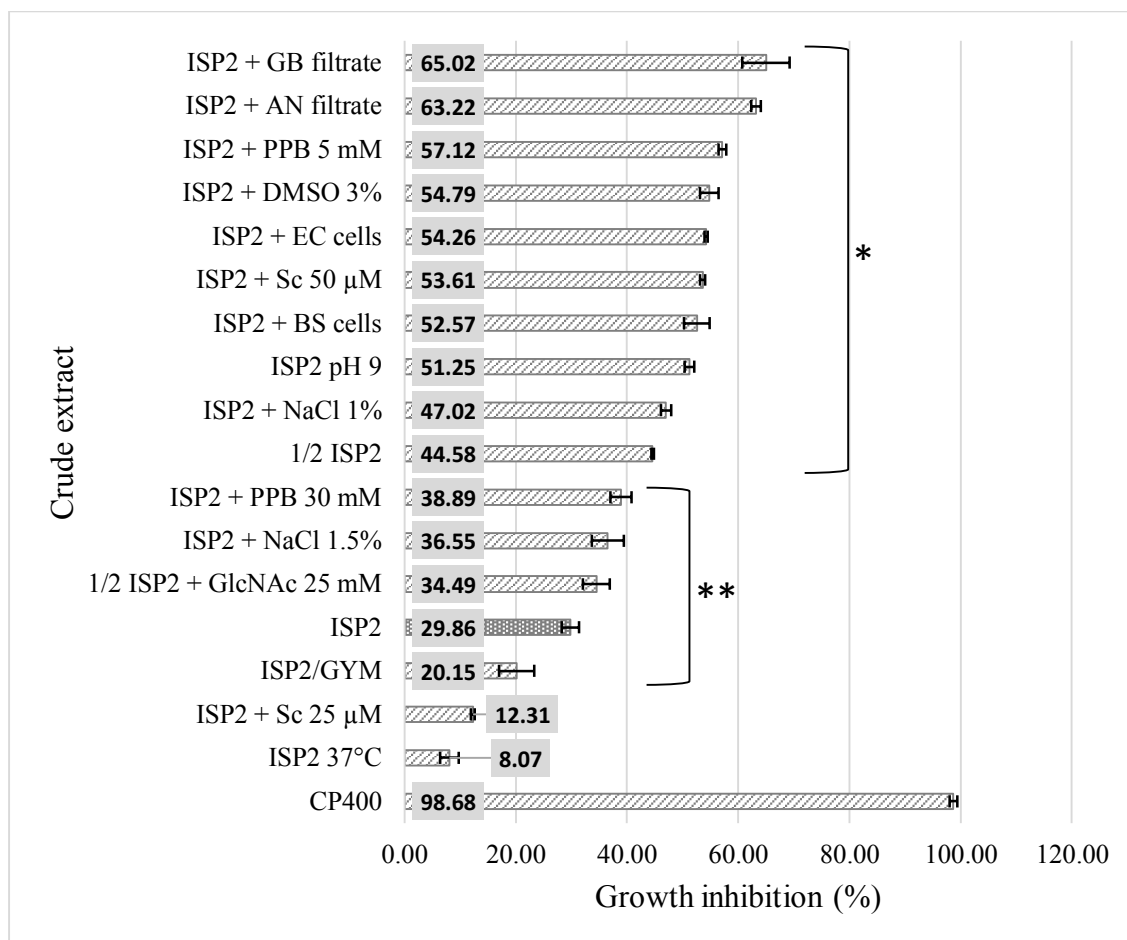ition as compared to ISP2 extract ($p > 0.05$). Growth inhibition resulted from the crude extract of ISP2 37°C (-11.55 ± 3.28%) and ISP2 + PPB 30 mM (-16.04 ± 7.29%) were represented as 0.00% in the bar chart due to the negative values.

Both antimicrobial screenings using the CFBs and crude extracts showed an enhanced detection of antimicrobial activity against the test strains. The biological and chemical elicitors that were incorporated into the fermentation broths had significantly increased the antimicrobial activity, especially against the yeast (*C. albicans*) and the Gram-negative test strains (*E. coli* and *P. aeruginosa*). Whereas the higher pH (ISP2 pH 9) significantly increases the antimicrobial activity against *E. coli*, the growth inhibition of *P. aeruginosa* was significantly affected by the crude extracts of ISP2 supplemented with the culture filtrate of *G. boninense* (ISP2+GB filtrate). The opposite effect of *E. coli* cells

and the culture filtrate of *A. niger* toward the antimicrobial activity against both Gram-negative strain was also recorded. While these biological elicitors significantly increase the antimicrobial activity against *P. aeruginosa*, they did not result in any significant difference in growth inhibition of *E. coli*, as compared to the extract of the basal medium, ISP2. A similar observation was recorded for the antimicrobial activity of the crude extract of ISP2 medium supplemented with 25 µM scandium chloride (ISP2+Sc 25 µM) and ISP2 cultivated at 37°C. Both extracts exhibited significantly lower antimicrobial activity against *P. aeruginosa*, whereas no significant difference was recorded for the antimicrobial activity of these extracts against *E. coli*, as compared to the basal medium.

Meanwhile, incubation temperature of 37°C and addition of NaCl (1% and 1.5%) had significantly reduced the antifungal activity against *A. niger*, as compared to the basal medium. Interestingly, the addition of NaCl (1 and 1.5%) in the fermentation medium and the incubation temperature of 37°C also had a similar effect on the antimicrobial activity against *C. albicans*, where no antimicrobial activity was recorded from the crude extracts of these media. A lower concentration of phosphate buffer in the fermentation medium had a positive effect towards the antifungal activity as opposed to a higher concentration of phosphate buffer. At a 5 mM concentration (ISP2+PPB 5 mM), the growth of *A. niger* was reduced by over 80%, while at 30 mM concentration (ISP2+5PPB 30 mM) the growth inhibition was lower than 50%. A similar observation was recorded for the growth inhibition of *C. albicans*, where a higher concentration of phosphate buffer in the fermentation medium (ISP2 + PPB 30 mM) had a negative effect on the antimicrobial activity.

4.3.3. Comparative secondary metabolites profiling of the crude extracts

Changes in secondary metabolites profile as a result of the OSMAC fermentation approach was evaluated qualitatively by comparative analysis of the HPLC chromatograms. The analysis revealed that the OSMAC approach had generally enhanced the detection of secondary metabolites by the actinomycete, as compared to the basal medium, ISP2. At least four main compounds were detected from the extract of ISP2 medium (Figure 4.11). The production of these metabolites appeared to be affected by the elicitors and the cultivation conditions. The yields of the metabolites were estimated based on the peaks heights measured as the UV absorbance value (mAU). In addition to

the main peaks detected from the basal medium, 9 unique peaks were detected from the crude extracts of ISP2 medium supplemented with 1% NaCl, and with the culture filtrate of *A. niger*. A total of 13 unique peaks or secondary metabolites were detected from the OSMAC fermentation approach, including those from the extract of ISP2 (Table 4.4).

An incubation temperature of 37°C and the medium with the pH adjusted to pH 9 increases the production of compound 1 and 2, respectively, while the addition of 3% DMSO in the fermentation medium increases the production of compound 3 and 4 (Table 4.4). However, there was no additional peak detected from the crude extracts of these media. The addition of 1% NaCl in the ISP2 medium stimulated the production of 3 new metabolites (Figure 4.11 and 4.12). Compound 5 and 6 were only detected from the extracts of ISP2 medium supplemented with 1% and 1.5% NaCl, while compound 7 was also detected from the extracts of ISP2 medium supplemented with 5 mM phosphate buffer, and with the culture filtrate of *A. niger*. The highest number of potentially new metabolites were detected from the extract of ISP2 medium supplemented with the culture filtrate of *A. niger*, whereby 6 unique peaks were detected (Figure 4.13).

In contrast, the addition of elicitors also had an inhibitory effect on secondary metabolites production by KBS50 (Figure 4.14). Fermentation in ISP2 medium supplemented with 30 mM phosphate buffer and NaCl (1% and 1.5%) had negatively affected the production of compound 1, 3 and 4. A similar effect was observed for the fermentation media supplemented with the culture filtrate of *G. boninense,* and the incubation temperature at 37°C, where the production of compound 3 and 4 were negatively affected. The fermentation medium ISP2/GYM also showed a significant reduction in secondary metabolites production, as compared to the basal medium ISP2. Other fermentation media including the half-strength ISP2 (½ ISP2), ½ ISP2+GlcNAc 25 mM, ISP2+BS cells, ISP2+EC cells, and ISP2+Sc 50 μM did not have any significant effect on secondary metabolites production, as compared to the basal medium. The UV profiles of unique peaks detected are shown in Figure 4.15.

Table 4.4: List of unique secondary metabolite compounds detected from the crude extracts of KBS50 from the OSMAC fermentation approach.

Peak	Retention time (minute)	Peak height in ISP2 extract [mAU]	Highest peak detected (mAU)	Highest peak detected (extract)	Maximum UV signal (nm)
1	3.833	522.10	765.76	ISP2 37°C	200
2	6.217	68.54	176.81	ISP2 pH 9	273
3	7.735	585.70	1820.21	ISP2+DMSO 3%	273
4	7.866	421.98	1013.77	ISP2+DMSO 3%	273
5	7.359	-	22.81	ISP2+NaCl 1%	200
6	5.205	-	99.88	ISP2+NaCl 1.5%	273
7	4.665	-	41.11	ISP2+PPB 5 mM	400
8	7.102	-	80.57	ISP2+AN filtrate	200
9	7.270	-	75.67	ISP2+AN filtrate	200
10	9.046	-	152.98	ISP2+AN filtrate	200
11	10.015	-	122.28	ISP2+AN filtrate	200
12	10.288	-	128.82	ISP2+AN filtrate	200
13	10.829	-	92.99	ISP2+AN filtrate	200
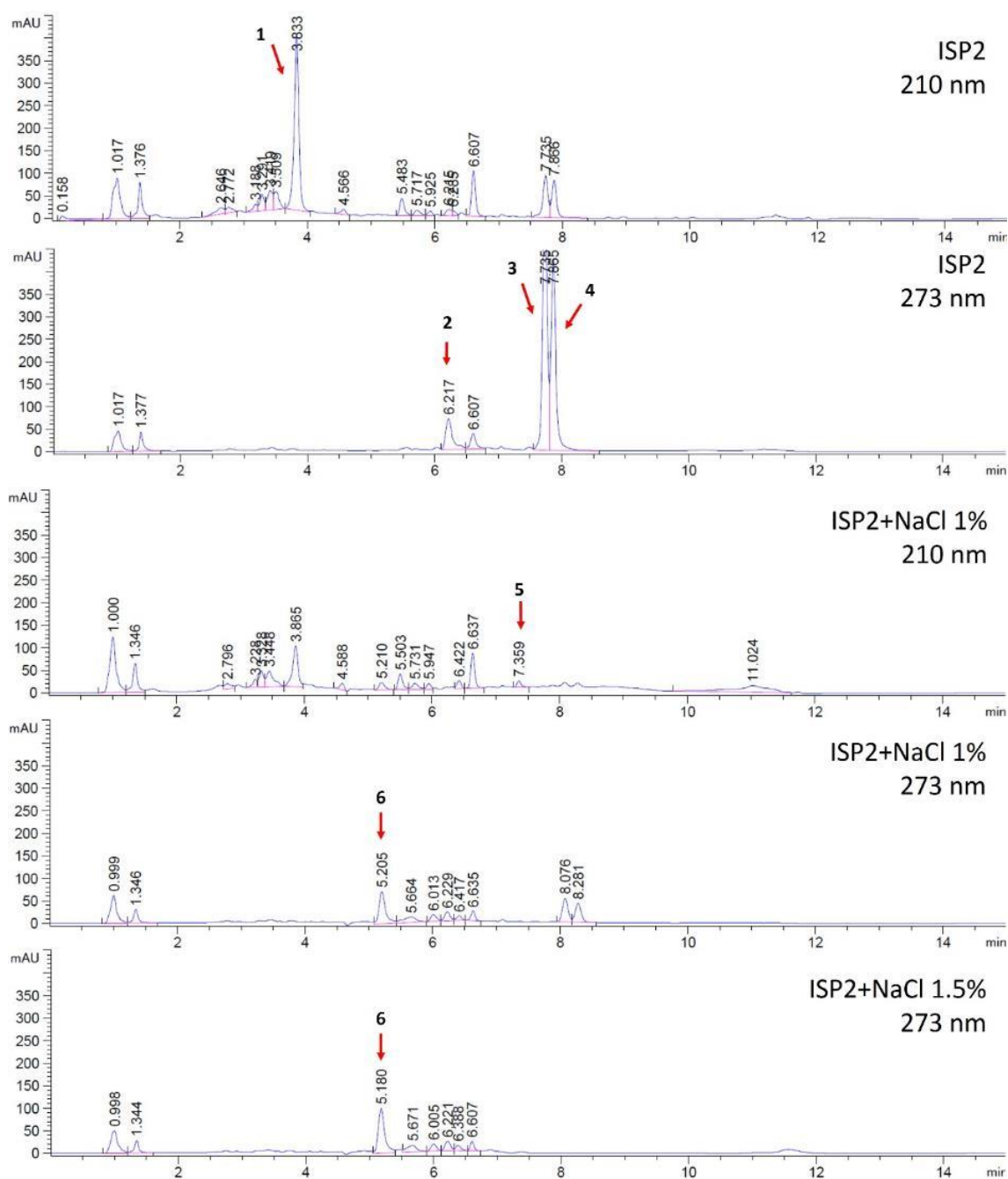
Figure 4.11: HPLC profiling shows unique peaks detected from the crude extract of ISP2 medium (compounds 1, 2, 3 and 4), and ISP2 medium supplemented with NaCl (compounds 5 and 6).
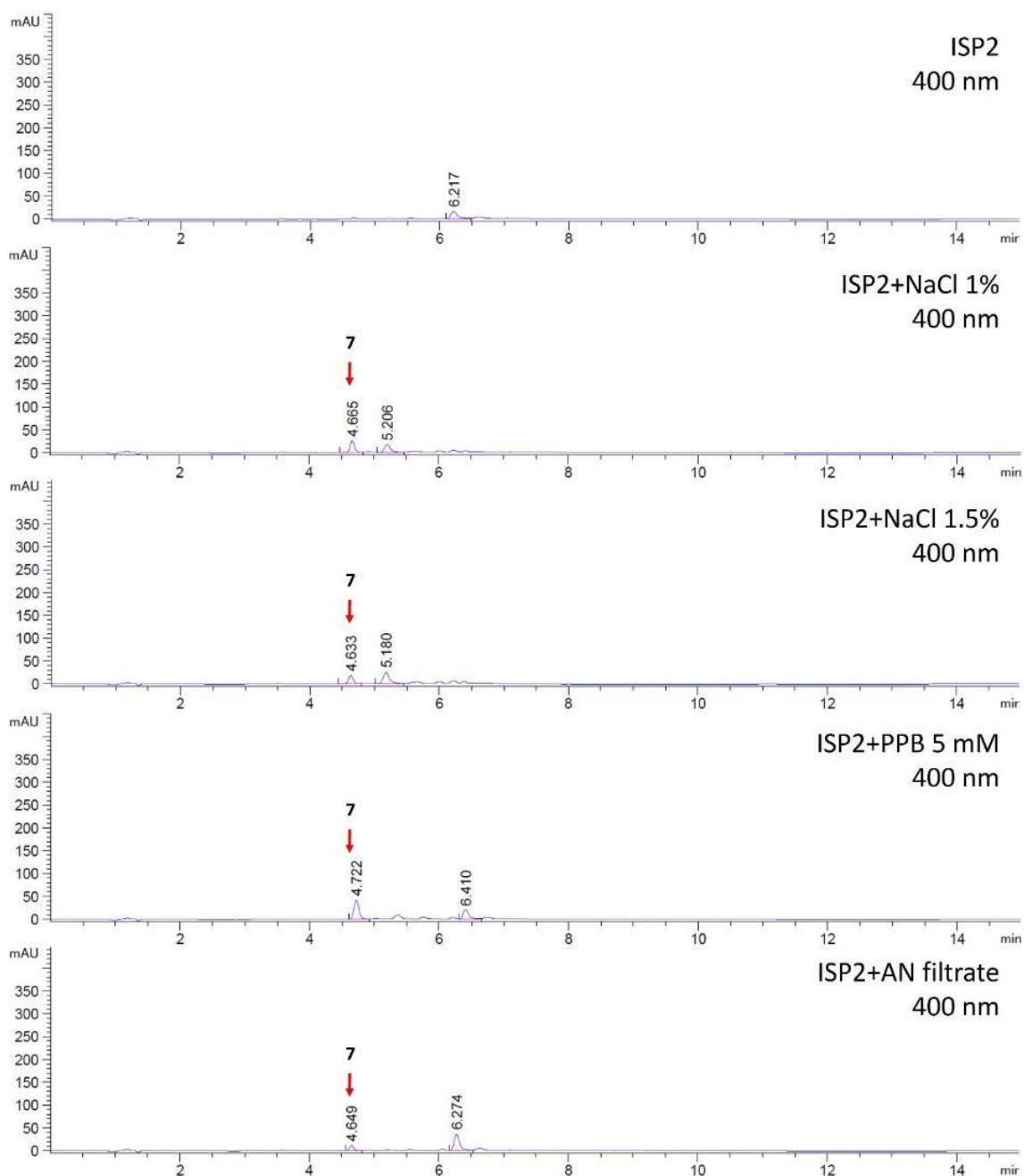
Figure 4.12: Comparative secondary metabolite profiling shows the unique peak (compound 7) detected from the crude extracts of ISP2 medium supplemented with NaCl (1% and 1.5%), 5 mM phosphate buffer, and the culture filtrate of *A. niger*.
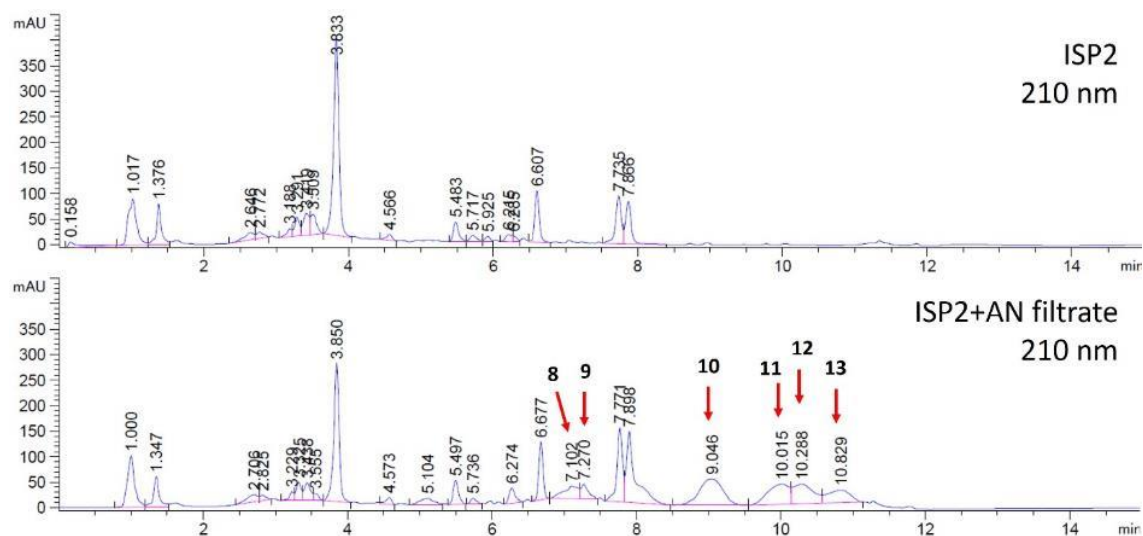
Figure 4.13: Comparative secondary metabolite profiling shows the unique peaks (compound 8-13) detected from the crude extract of ISP2 medium supplemented with the culture filtrate of *A. niger*.
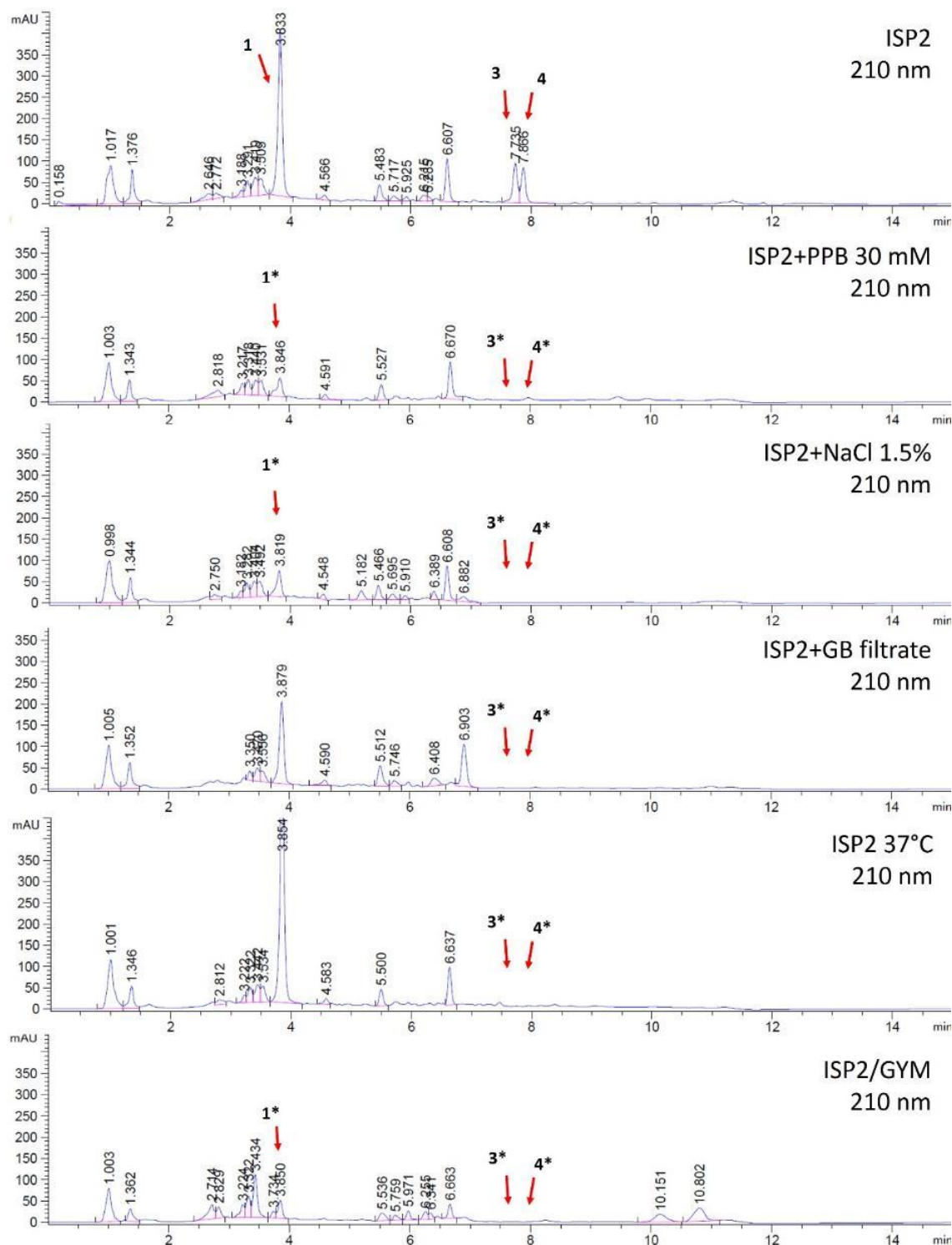
Figure 4.14: Comparative secondary metabolite profiling shows the inhibitory effects of elicitors (phosphate buffer, NaCl, and culture filtrate of *G. boninense*), cultivation temperature of 37°C, and peptone (in the ISP2/GYM medium) on the production of secondary metabolites by KBS50.
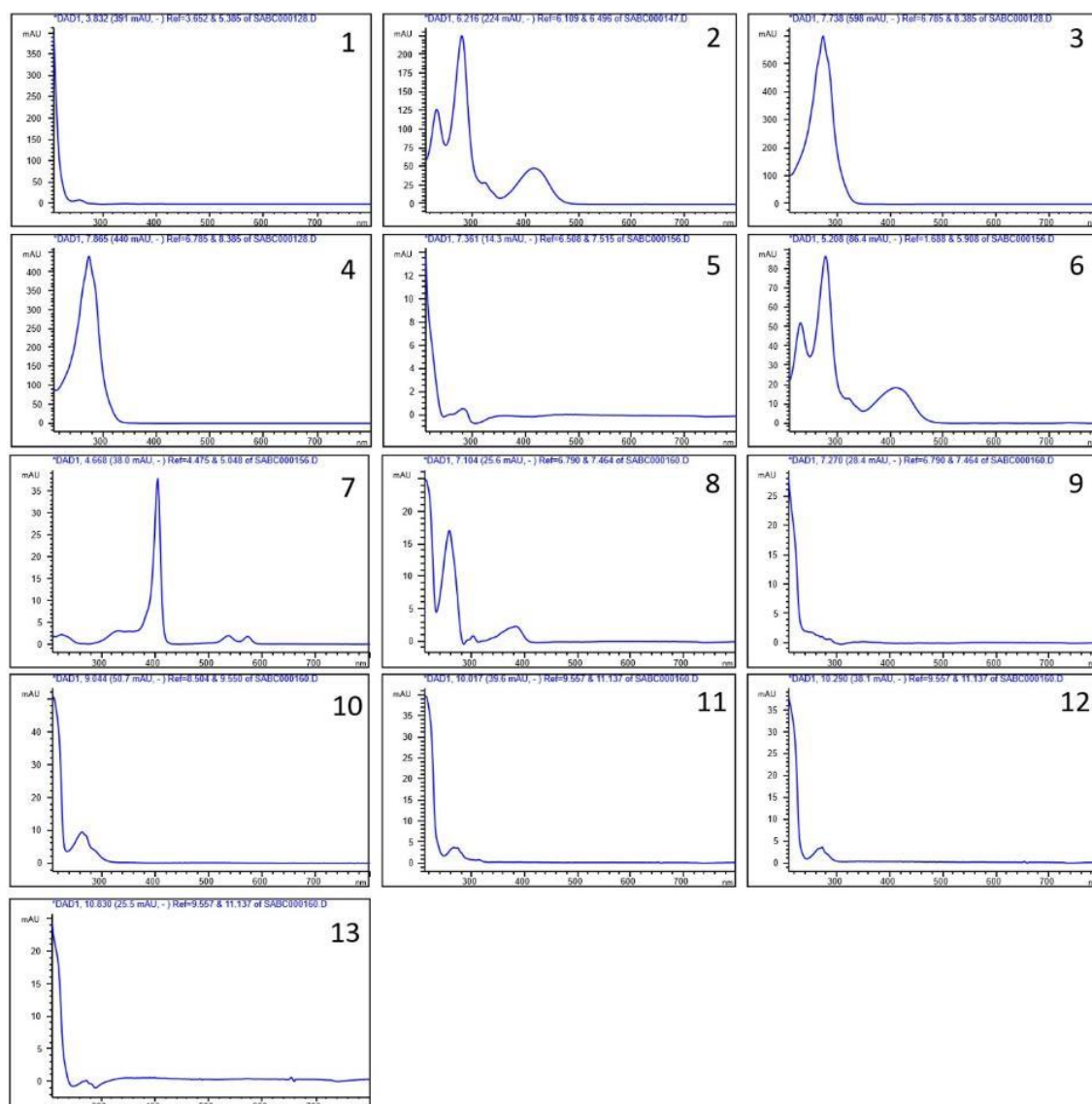
Figure 4.15: The UV profiles of unique peaks (compound 1-13) detected from the crude extract of the OSMAC fermentation media of KBS50.

4.3.4. Production of the antimicrobial compounds in 2 litre fermentation

The secondary metabolites were produced through fermentation in a 2 litre volume of the basal medium, ISP2. Extraction of secondary metabolites was carried out using ethyl acetate, which resulted in 190 mg of crude extract weight. Antimicrobial activity of the crude extract at 2.5 mg/ml concentration against *B. subtilis* and *S. aureus* was confirmed using the AwDA, with the mean diameter of inhibition zone recorded at 12.33 ± 0.33 mm and 8.00 ± 0.00 mm, respectively.

4.3.5. Bioassay-guided fractionation and purification of the antimicrobial compounds

Approximately 170 mg of the ethyl acetate crude extract was fractionated using the semi-preparative HPLC into 10 different fractions (Figure 4.16). The fractionation scheme is as shown in Figure 4.17. Fraction 3 to 10 were evaluated for antimicrobial activity against *B. subtilis* and *S. aureus* using the AwDA. Fraction 5, 6, 7, 8, 9, and 10 showed antimicrobial activity against *B. subtilis*, while only fraction 7 and 9 showed antimicrobial activity against *S. aureus* (Table 4.5). Fraction 1 and 2 were not tested for antimicrobial activity because HPLC analysis did not show the presence of any unique peak.

Table 4.5: Antimicrobial activity of the fractions in AwDA against Gram-positive test strains.

Fraction	Inhibition zone (mm) ± SEM	
	B. subtilis	*S. aureus*
3	-	-
4	-	-
5	10 ± 0.33	-
6	11.67 ± 0.33	-
7	15.33 ± 0.33	20 ± 0.00
8	11.33 ± 0.33	-
9	14.67 ± 0.33	9 ± 0.00
10	10 ± 0.00	-
Chloramphenicol (100 ppm)	21.33 ± 0.33	21.00 ± 0.58

The bioassay-guided fractionation showed that the ethyl acetate crude extract of KBS50 contains multiple compounds with antibacterial activity against the Gram-positive test strains, as demonstrated by the antimicrobial activity detected from several different fractions. The antimicrobial activities of fraction 5, 6, 8, and 10 were most likely contributed by minor peak(s). The bioactive compound(s) in these fractions only affected the growth of *B. subtilis*, which was presumably more susceptible towards the bioactive compound(s) as compared to *S. aureus*.

Fraction 7 exhibited the highest antimicrobial activity against both test strains with the mean diameter of inhibition of 15.33 ± 0.33 mm and 20.00 ± 0.33 mm for *B. subtilis* and *S. aureus*, respectively. However, HPLC analysis showed that the potential antimicrobial compound in this fraction might be present in very low quantity. The amount recovered in fraction 7 was only 3.1 mg, which would be insufficient for further fractionation and isolation of the antimicrobial compound. Meanwhile, fraction 9 contained two major peaks (Figure 4.18) which were predicted to exhibit the antimicrobial activity against the test strain. These two peaks, previously designated as compound 3 and compound 4 during the comparative HPLC analysis for the OSMAC fermentation approach, were present only in the crude extract of the fermented medium but were not detected in the crude extract of the non-inoculated broth. These two compounds were also produced in high yield based on their high UV absorbance values (mAU). Approximately 43 mg of fraction 9 was recovered from the crude extract, of which 30 mg was further fractionated to purify the two peaks using the semi-prep HPLC.

Compound 3 and 4 were recovered as single peak compounds with the purity of 99.86% and 93.24%, respectively, as determined from the analysis using the analytical HPLC (Figure 4.19). The amount recovered was 1.9 mg of compound 3, and 3.1 mg of compound 4. The antimicrobial activity of the compounds at 1 mg/ml concentration was evaluated using AwDA against *S. aureus*, as the representative of the Gram-positive test strain. The test was carried out in triplicates. Both compounds exhibited antimicrobial activity against *S. aureus*, with the mean inhibition diameter of 12.00 ± 0.00 mm and 13.00 ± 0.00 mm for compound 3 and 4, respectively (Figure 4.20).
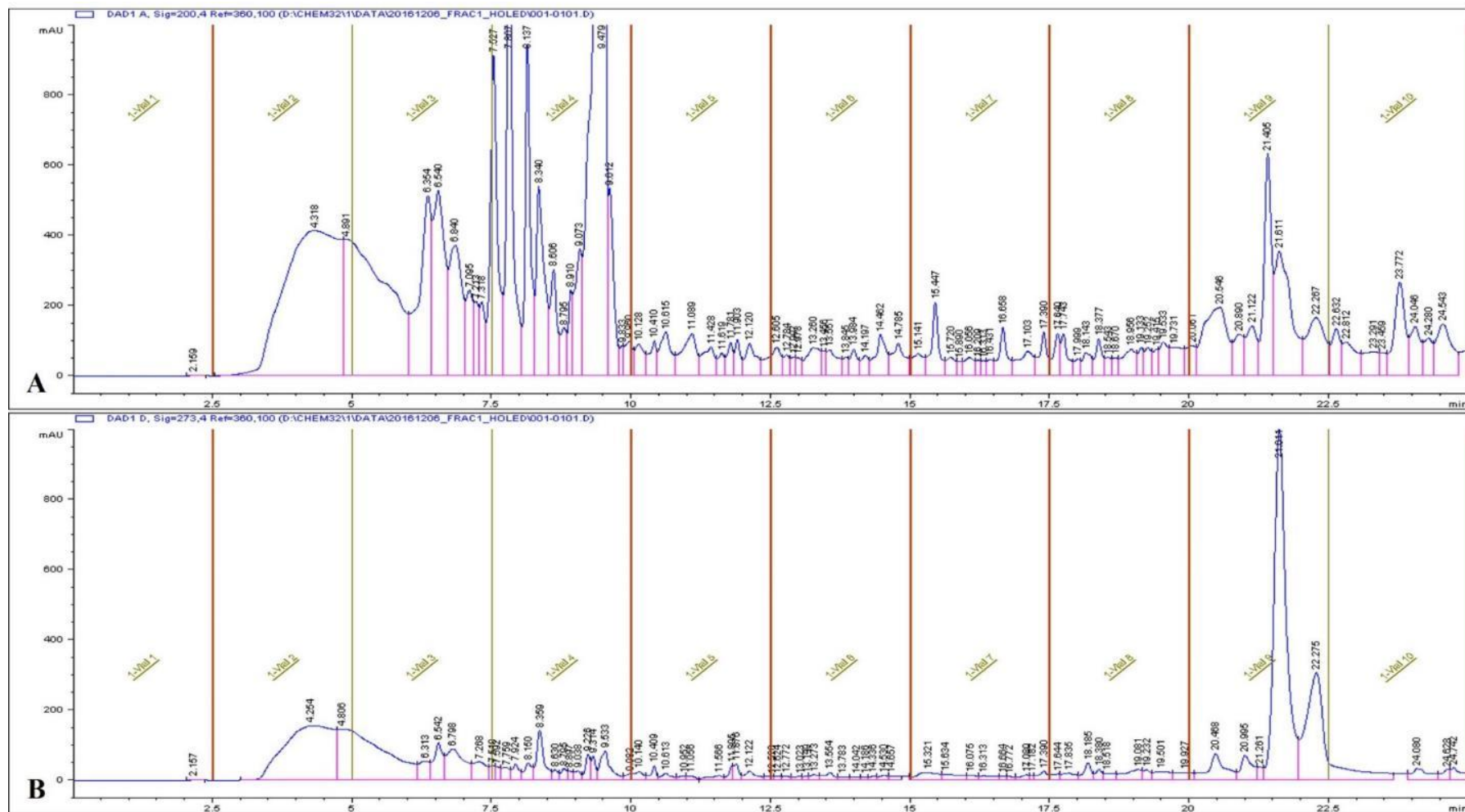
Figure 4.16: HPLC profiles of the crude extract from 2 litre fermentation broth at UV detection at 200 nm (A) and 273 nm (B). HPLC profiling was performed using the semi-preparative HPLC.
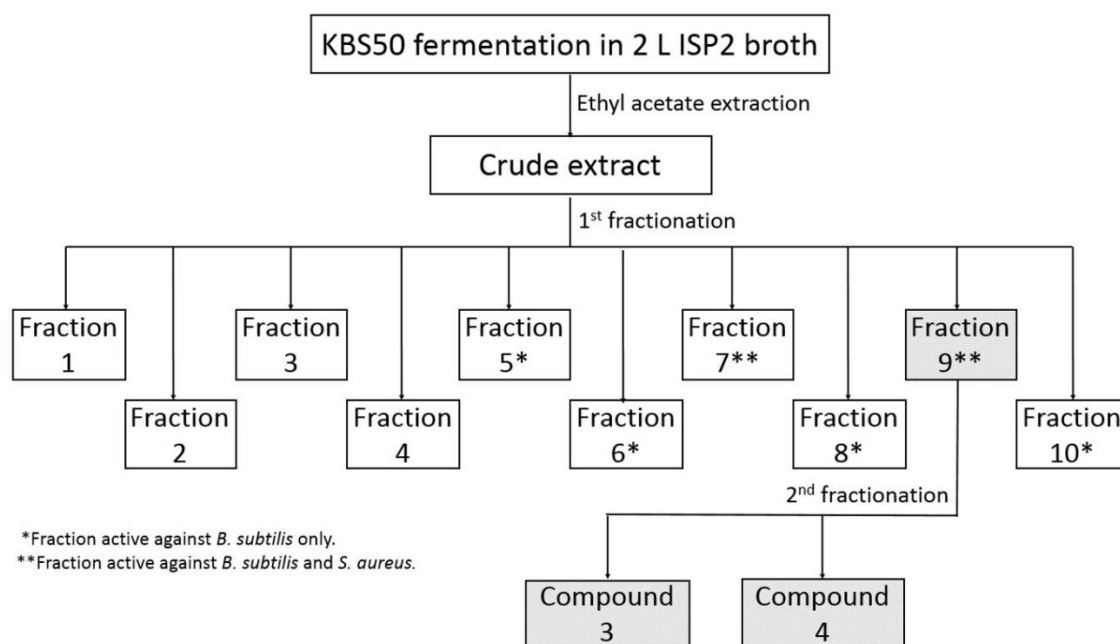
Figure 4.17: Schematic representation for the bioassay-guided fractionation of bioactive compounds from the ethyl acetate crude extract of KBS50.
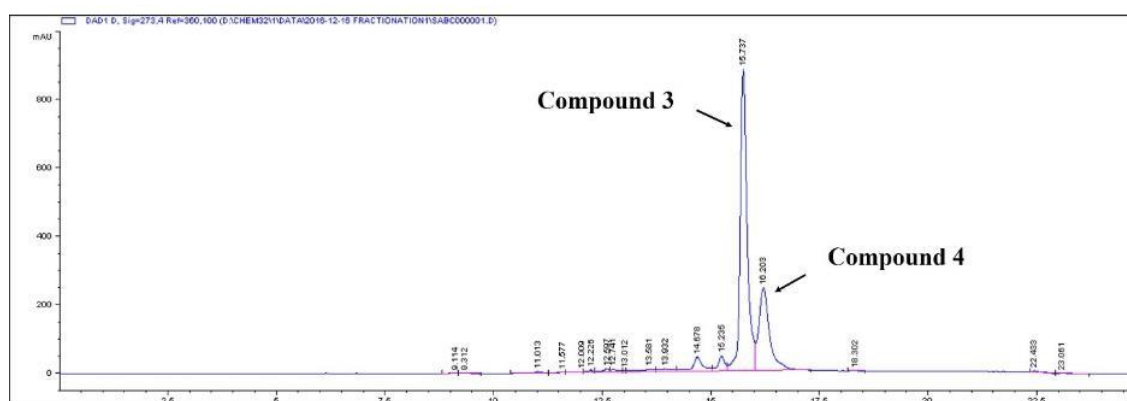
Figure 4.18: HPLC profile of fraction 9 showing two major peaks identified as compounds 3 and 4. HPLC profiling of the fraction was performed on the semi-preparative HPLC using the following solvent gradient: 0-8 minute, 10% acetonitrile and 90% water; 8-18 minute, 100% acetonitrile; 18-25 minute, 10% acetonitrile and 90% water.
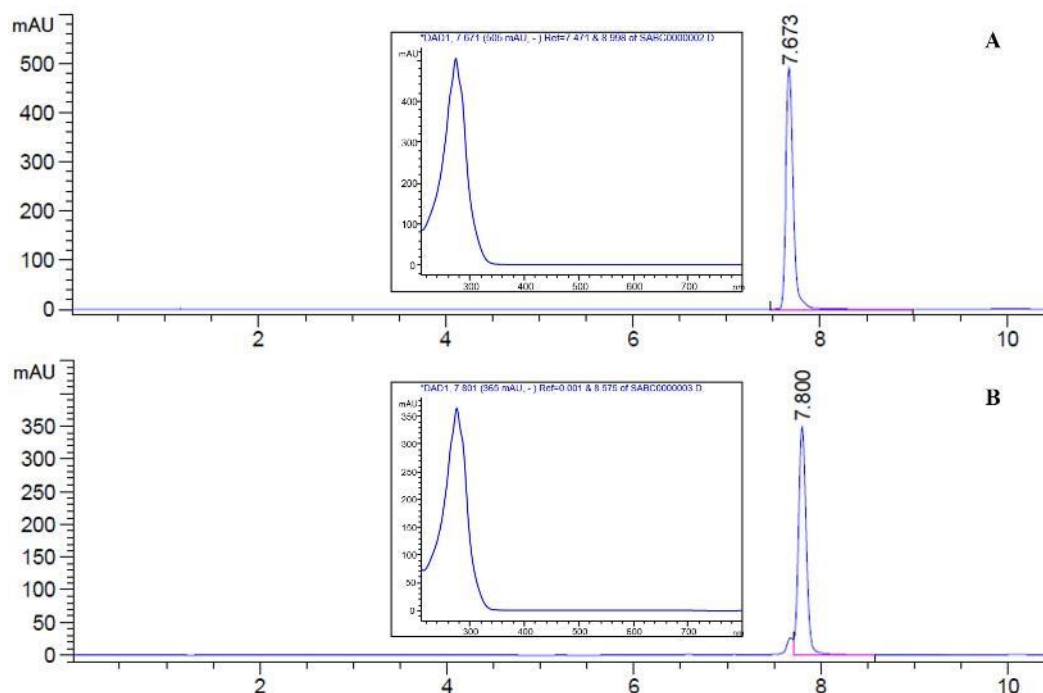
Figure 4.19: HPLC profile of compound 3 (A) and compound 4 (B) at 273 nm UV wavelength. Their UV profiles are shown next to the peak. HPLC profiling was performed on the Analytical HPLC Agilent 1100 system with the injection volume of 30 µl and compound concentration of 0.4 mg/ml.
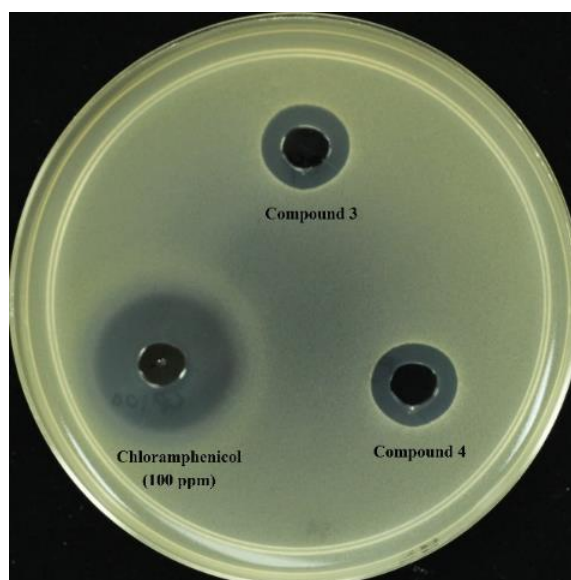
Figure 4.20: Growth inhibition of *S. aureus* on the AwDA by compound 3 and compound 4. The positive control was 100 ppm chloramphenicol which produced 21.00 ± 0.58 mm diameter of the clear zone.

4.3.6. MIC and MBC of the bioactive compounds

The MIC of compound 3 and compound 4 against *S. aureus* was determined using the broth microdilution method. The lowest concentration of the compound in which no visible growth of *S. aureus* detected was set as the MIC value. Compound 3 showed the lowest MIC of 7.81 ± 0.00 µg/ml which resulted in $100.41 \pm 0.39\%$ growth inhibition, while compound 4 showed higher MIC at 62.50 ± 0.00 µg/ml that resulted in $100.88 \pm 0.23\%$ growth inhibition (Table 4.6). The growth of the test strain on 96-well plates was also confirmed by observation using the naked eyes, where no visible growth was detected in the wells with the MIC values, as well as at a higher concentration of the compound above the MIC value (Figure 4.21). As for the control (chloramphenicol), the MIC against *S. aureus* was recorded at 3.91 ± 0.00 µg/ml.

Table 4.6: The growth inhibition of S*. aureus* in the MIC test for compound 3 and 4.

No.	Concentration (µg/ml)	Growth inhibition (%) ± SEM		
		Compound 3	Compound 4	Chloramphenicol
1	500.00	96.66 ± 0.60	96.97 ± 1.54	NT
2	250.00	96.04 ± 0.35	99.24 ± 0.26	NT
3	125.00	93.88 ± 0.78	100.46 ± 0.45	98.65 ± 0.28
4	62.50	97.63 ± 0.88	100.88 ± 0.23*	99.02 ± 0.21
5	31.25	100.58 ± 1.14	12.62 ± 13.76	99.35 ± 0.10
6	15.63	100.42 ± 0.35	-10.31 ± 14.12	99.32 ± 0.05
7	7.81	100.41 ± 0.39*	-11.34 ± 14.94	98.90 ± 0.14
8	3.91	85.37 ± 2.50	-7.21 ± 11.65	93.52 ± 0.04*
9	1.95	9.14 ± 10.44	-7.55 ± 12.56	59.04 ± 0.60
10	0.98	-29.26 ± 8.74	-15.99 ± 14.04	34.28 ± 0.79
11	0.49	-69.06 ± 7.81	-43.28 ± 22.45	23.01 ± 0.18
12	0.24	-143.91 ± 24.04	-78.84 ± 10.77	8.24 ± 2.58

NT = Not tested. *The MIC value for the compounds and antibiotics.
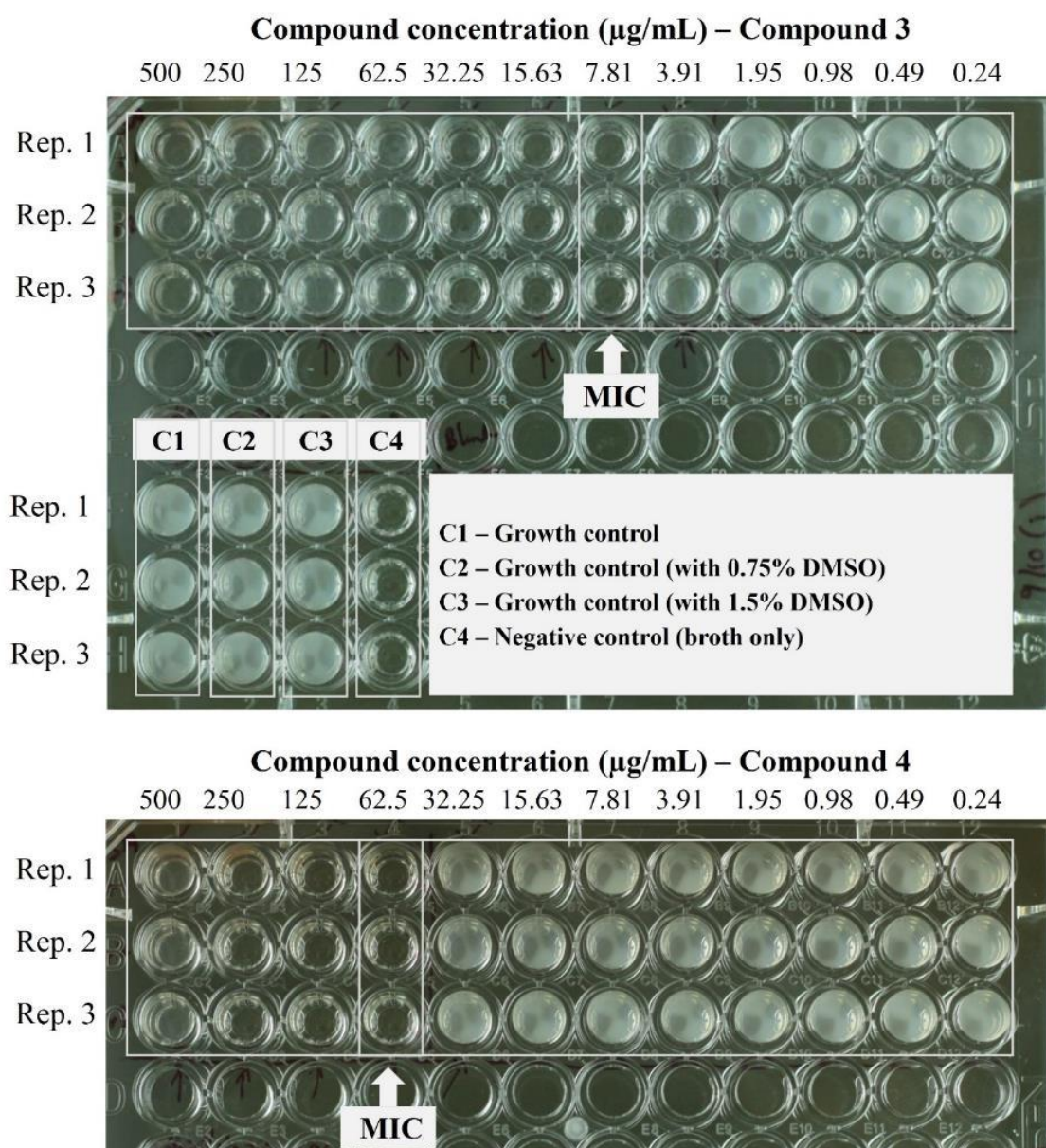
Figure 4.21: MTP assay for the determination of MIC of compound 3 and compound 4 against *S. aureus*.

The MBC for compound 3 was 15.63 µg/ml while the MBC for compound 4 was 125.00 µg/ml. At these respective concentrations as well as at a higher concentration of the compounds, no bacterial colony was detected on the MH agar plates after 24-h incubation at 37°C. Meanwhile, the culture broth from the wells of the MIC value of compound 3 and compound 4 resulted in 366.67 ± 66.67 CFUs and 67.00 ± 41.94 CFUs, respectively. Compound 3 and 4 can be considered as bactericidal since its MBC did not exceed four times the MIC (French 2006), where in this case the MBC was only two times of the MIC for both compounds. Both of these compounds are potential drug candidate for anti-infective against the Gram-positive bacteria.

4.4. Discussion

The main objective of the study in this chapter was to enhance the antibiotics and secondary metabolites detection from KBS50 using the OSMAC fermentation approach. This approach was seen as a viable strategy as it has been used successfully to increase secondary metabolites production in other actinomycetes as well as fungi (Bills et al. 2008; Bode et al. 2002; Hewage et al. 2014; Wang, QX et al. 2013). As stated by Bode et al. (2002), the OSMAC approach is "a random approach" whereby the cultivation parameters for a single strain can be altered in many possible ways that may lead to the discovery of new secondary metabolites. In that sense, there could be hundreds of possible growth conditions that can be manipulated to evaluate the antibiotics production from KBS50 using the OSMAC approach. However, only a small number of growth conditions were tested in this study to minimise the number of extracts to be screened. Although it is unlikely that small changes in the cultivation parameters can trigger the production of all the potential secondary metabolites from KBS50, the OSMAC fermentation strategy used in this study resulted in an increased in antimicrobial activities against the Gram-positive and Gram-negative bacteria, yeast and fungi. The secondary metabolites detection from the crude extracts was also enhanced, as demonstrated by the comparative HPLC analysis results. While current study did not mean to be a comprehensive screening of many possible growth conditions for the fermentation of KBS50, future study may include various combination of nutrient compositions, elicitors, and other cultivation parameters which can be used to activate secondary metabolites production (Bode et al. 2002; Reen et al. 2015; Zhu et al. 2014).

Incubation temperature and pH of a growth medium are two parameters that can greatly affect the growth of actinomycetes that consequently affect the antibiotics and secondary metabolites production (Bundale et al. 2015; James et al. 1991; Ripa et al. 2009). A slight increase in pH (pH 9) and incubation temperature (37°C) caused a significant shift in the antimicrobial activity and secondary metabolites profiles of KBS50, as compared to the control (ISP2 basal medium at pH 7.2 and incubation temperature of 28°C). Antimicrobial activities against *S. aureus*, *A. niger* and *C. albicans* were reduced significantly by the incubation temperature of 37°C, whereas the antimicrobial activity against *E. coli* was increased. The production of compound 3 and 4 was repressed at the elevated temperature, although the highest level of compound 1 was recorded. Meanwhile, the

strain showed a significant increase in antimicrobial activities against the Gram-negative bacteria and *C. albicans* when it was cultivated at pH 9. The slightly basic medium also increases the production level of compound 2.

The possibility that nutrient-rich medium negatively affecting the secondary metabolism of KBS50 was discussed in Chapter 2. In the current study, the strain also showed similar response even with a small increase in the amount of peptone added into the ISP2/GYM medium. A significant decrease in antimicrobial activity against *A. niger* and *C. albicans* was recorded. The comparative HPLC analysis also showed a significant reduction in the levels of major compounds detected from the crude extract of this medium. In contrast, the metabolites profile of the strain was relatively unchanged when it was cultivated in the half-strength ISP2 medium (½ ISP2). In this nutrient-poor medium, the strain showed an overall increase in antimicrobial activities. Previous study reported that N-acetylglucosamine (GlcNAc) may induce antibiotics biosynthesis in *Streptomyces* under poor nutrient conditions (Rigali et al. 2008). However, adding the (GlcNac) into the half-strength ISP2 medium did not result in any major changes to the metabolites profile and antimicrobial activity of KBS50.

The incorporation of elicitors into the basal medium provided additional cues that further stimulate secondary metabolites production. These elicitors were selected based on the literature survey that reported their effect towards secondary metabolite biosynthesis. The addition of DMSO in fermentation media was reported to increase antibiotics production in actinomycetes by 2 to 3 folds (Chen et al. 2000). Similarly, the rare earth element (scandium) was also reported to increase antibiotics production by as much as 25 folds (Kawai et al. 2007). It also increased the expression level of secondary metabolites biosynthetic genes in *S. coelicolor* (Tanaka et al. 2010). Similarly, in this study, a significant increase in antimicrobial activities against the Gram-negative bacteria and *C. albicans* was recorded as the results of elicitation using DMSO and scandium. The exact mechanism of action of DMSO and scandium in eliciting the secondary metabolites production in actinomycetes is still unknown, although it could potentially affect the ribosome which consequently alters the mechanism of genes expression and translation (Chen et al. 2000; Kawai et al. 2007).

The dead cells of *B. subtilis* and *E. coli* (Luti & Mavituna 2011; Luti & Yonis 2013), and the culture filtrate of fungi (Wang, D et al. 2013) were reported to increase antibiotics production in actinomycetes and other microorganisms. These elicitors mimic the biological interaction between different microorganisms in the natural environment which may potentially lead to the biosynthesis of antibiotics as a mean of self-defense mechanism (Abdelmohsen et al. 2015; Luti & Yonis 2013). Other researchers have reported the uses of live cells in a fermentation approach called co-cultivation (Luti & Yonis 2013; Wang, D et al. 2013). This strategy is theoretically more representative of the actual microbial interaction in the environment, as live cells may directly interact with each other and provide various signalling molecules that may affect secondary metabolite production at the molecular level (Abdelmohsen et al. 2015). However, the incorporation of live cells might affect the growth of actinomycetes due to the competition over nutrient components as actinomycetes are generally grown slower than the competing bacteria. In most cases, the dead cells or culture filtrates provided the same elicitation effect on antibiotics production by the producer strain (Luti & Yonis 2013; Wang, D et al. 2013). In this study, the culture filtrate of *A. niger* was found to have the most profound effect on KBS50 where several new metabolites were produced, while the culture filtrate of *G. boninense* seems to inhibit the production of the major metabolites. In previous studies, it was shown that the concentration of elicitors could affect antibiotics production (Luti & Mavituna 2011; Luti & Yonis 2013; Wang, D et al. 2013). Hence, it would be interesting to test different concentration of these elicitors in a future study to determine their influence on the production of secondary metabolites by KBS50.

Phosphate can either enhance or repress secondary metabolites production depending on its concentration in the cultivation medium (Bode et al. 2002; Martín 2004). According to Martin et al. (1977), phosphate may suppress antibiotics biosynthesis by inhibiting the formation of precursors and limiting the availability of inducers for secondary metabolite biosynthetic pathway. High concentration of phosphate negatively affecting secondary metabolites production at the translational level (Bibb 2005; Martín 2004). In this study, the extract of ISP2 medium supplemented with 5 mM phosphate buffer exhibited strong antimicrobial activity against *A. niger* and *C. albicans*. However, at a higher concentration (30 mM), the antimicrobial activities against both test strains were reduced significantly while the production of major compounds was repressed. Similar observation on the effect of phosphate on antibiotics production by actinomycetes has been reported (Doull

& Vining 1990; Giardina et al. 2014; Hobbs et al. 1992; Hobbs et al. 1990; Lounès et al. 1996). Based on this result, future design of fermentation media for KBS50 should take into consideration the limit of inorganic phosphate concentration that should be added. The minimum amount of phosphate would be necessary as it may promote the growth of actinomycetes (Giardina et al. 2014).

Plantactinospora sp. KBS50 can tolerate high salinity of up to 7% although it does not require NaCl for growth. The previous study in actinomycetes and fungi has shown that NaCl can affect the growth and secondary metabolites production due to its effect on the osmotic pressure of the medium (Huang et al. 2011; Ng et al. 2014; Wang et al. 2011). Studies on *Salinispora* species, which requires salt for growth, showed that the antibiotics production correlates to the growth rate of the strains under different concentration of NaCl (Ng et al. 2014). The addition of 1-1.5% NaCl in the fermentation medium might have increased the biomass, although this effect was not measured in the current study. The addition of NaCl resulted in a contrasting effect on antibiotics and secondary metabolites production. The comparative HPLC analysis showed that compound 5 and 6 were only detected from medium supplemented with 1% and 1.5% NaCl. A higher level of compound 6 was detected from the medium with 1.5% NaCl. Increasing the concentration of NaCl might increase the production level of compound 6. In contrast, the production of major metabolites (compound 1, 3 and 4) was suppressed in the presence of NaCl. Coincidently, the antimicrobial activity, especially against *A. niger* and *C. albicans*, was reduced significantly with the presence of NaCl. These contrasting results indicate that silent secondary metabolites genes were activated in the presence of NaCl, while it suppresses or limiting the expression of other biosynthetic genes.

Initial antimicrobial screening of the crude extracts from the standard fermentation media ISP2, MB, FM1 and FM8 only showed narrow spectrum antibacterial activity against the Gram-positive bacteria. Under these standard cultivation conditions, there could be a chance that some bioactive compounds with broad spectrum activities were present in a very small quantity that consequently limits the detection of their antimicrobial activity. Therefore, the sensitivity of the bioassay screening method is important to enable the detection of bioactivity of low concentration compound (Rutledge & Challis 2015). For an instant, the antifungal activity of the crude extract of the ISP2 medium was detected using the MTP assay in this study. However, similar activity was not detected using the

AwDA during the initial screening. The MTP assay was more sensitive probably because the assay provides direct interactions between the bioactive compound and the test strain, whereas AwDA relies on the diffusion of the bioactive compound through the agar matrix which consequently further dilute the concentration and the potency of bioactive compound (Jenkins & Schuetz 2012). The sensitivity of AwDA can also be affected by the solubility or the diffusibility of the bioactive compounds in the agar, where the non-polar compound would be less soluble and difficult to diffuse (Jenkins & Schuetz 2012; Valgas et al. 2007). Regardless of the assay method employed for antimicrobial screening, it is imperative to increase the level of secondary metabolites production so that their bioactivity can be detected easily in antimicrobial screening.

4.5. Summary

In this study, the antibiotics and secondary metabolites production by KBS50 was further evaluated using the OSMAC fermentation approach. Under the OSMAC scheme, the actinomycete was fermented in a basal medium (ISP2) supplemented with selected biological and chemical elicitors including NaCl, DMSO, rare earth element (scandium), dead cells of *B. subtilis* and *E. coli*, culture filtrate of *A. niger* and *G. boninense*, potassium phosphate buffer, and N-acetylglucosamine. In addition to the elicitor elements, the strain was also cultivated at a higher temperature of 37°C, pH 9, half-strength ISP2, and the ISP2/GYM medium. The main objective of this fermentation strategy was to enhance the antibiotics and secondary metabolites detection, which was assessed using the antimicrobial screening and comparative HPLC analysis. The antimicrobial screening of the CFBs and the ethyl acetate crude extracts from the OSMAC fermentation media recorded a broad spectrum antimicrobial activities against the Gram-positive and Gram-negative bacteria, yeast and fungi test strains. Statistical analysis revealed that the antimicrobial activities were significantly increased, as compared to the basal medium, ISP2. Similarly, the comparative HPLC analysis showed an increased in secondary metabolites production, as well as the detection of potentially new metabolites, particularly from the crude extracts of ISP2 medium supplemented with 1% NaCl and with the culture filtrate of *A. niger*. To evaluate the antimicrobial potential of the antibiotics as well as to estimate the possible number of the bioactive compound produced by KBS50 against the Gram-positive bacteria, the ethyl acetate crude extract from a 2 liter fermentation broth (ISP2) was subjected to a bioassay-guided fractionation. Six out

of eight fractions tested exhibited antimicrobial activity against *B. subtilis*, while only two fractions (fraction 7 and 9) exhibited the antimicrobial activity against *B. subtilis* and *S. aureus*. The result showed that KBS50 produces a different type of bioactive compounds which can be isolated from the crude extract. Further fractionation of two major peaks from fraction 9 resulted in the isolation of two compounds (compound 3 and 4) with potent antimicrobial activity against *S. aureus*. Compound 3 recorded the strongest antimicrobial activity against *S. aureus*, with the MIC and MBC recorded at 7.81 μg/ml and 15.63 μg/ml, respectively. In summary, the OSMAC fermentation approach used in this study had successfully enhanced the detection of antibiotics and secondary metabolites from *Plantactinospora* sp. KBS50. The bioassay-guided fractionation further established the capability of KBS50 as a promising source of bioactive secondary metabolite compounds with a potent antimicrobial activity which can be developed as new drug candidates.

General Summary and Conclusions

5.1   Overall Summary

Finding a new source of bioactive compounds, especially the antibiotics, is an urgent need amid the increasing incident involving drug-resistant pathogens (Boucher et al. 2009; Demain 2014). At the same time, the full potential of rare actinomycetes from Sarawak as a source of novel natural product drugs has not been fully explored. Hence, this research project is an opportunity to highlight the value of rare actinomycetes from Sarawak in the wake of an increasing demand for a new source of bioactive natural product compounds. The aim of the present study was to evaluate the capability of a rare actinomycete strain, *Plantactinospora* sp. KBS50, to produce the antibiotics and secondary metabolites compounds. KBS50 was selected for this study primarily due to its potential novelty and genetic capability to synthesise secondary metabolite compounds. The fact that marine actinomycetes have been increasingly recognised as a prolific producer of natural product drugs in recent years (Jensen et al. 2005; Zotchev 2012) further justify the selection of this particular strain among other unique actinomycetes available from the Sarawak Biodiversity Centre's microbial culture collection.

The present study was divided into three main components. In the first component of the project as reported in Chapter 2, the identity of KBS50 was established using a near-complete 16S rRNA gene sequence for BLAST and phylogenetic analysis. The basic morphological and physiological characteristics were studied and compared with other closely related species. The identification and characterization provides a valuable insight into the taxonomical and ecological perspective of KBS50 and is particularly important to distinguish the strain from its closest relatives. The preliminary antimicrobial screening was carried out to determine its antagonistic properties and antimicrobial activities against representatives of Gram-positive and Gram-negative bacteria, yeast and fungi test strains. The extracellular enzyme activity was also evaluated.

The capabilities of KBS50 to produce secondary metabolite compounds was assessed using a genome mining approach in Chapter 3. The genomic DNA was sequenced using the third generation sequencing technology, then assembled to produce a complete circular chromosome. The genome sequence was subsequently analysed for the identification of secondary metabolites BGCs using bioinformatics tools. The genome analysis further reveals the full biosynthetic capabilities of KBS50 as a potential producer of various types of secondary metabolite compounds.

The antibiotics and secondary metabolites production from KBS50 was further evaluated using the OSMAC fermentation approach in Chapter 4. This strategy was intended to enhance the detection of antimicrobial activities of KBS50 in antimicrobial screenings, as well as to enhance the detection of secondary metabolites from its crude extracts using HPLC analysis. Finally, the bioassay-guided fractionation was utilised to isolate two bioactive compounds from the fermentation product of KBS50. The potency of these compounds was tested to demonstrate their potential as new drug candidates. The salient findings of this study are summarised.

5.1.1 Identity and characteristics of *Plantactinospora* sp. KBS50

The actinomycete strain, KBS50, was identified as a new species of *Plantactinospora* primarily from the BLAST and phylogenetic analysis of the near-complete 16S rRNA gene sequence (GenBank accession number KY348801). *Plantactinospora* is a relatively new genus in the *Micromonosporaceae* family. The genus currently comprised of 6 validly described species. Most species were isolated from plant tissues and only one species was isolated from peat swamp forest soil. As such, KBS50 could be the first member of this genus originated from the marine-associated environment, as well as the first species reported from Sarawak, Malaysia. The near-complete 16S rRNA sequence analysis showed that KBS50 had the highest sequence identity of 97.9% with *P. mayteni*, while it shared between 96.9-97.7% sequences identities with other species of *Plantactinospora*. Phylogenetic analysis also showed that KBS50 was only distantly related to the genus *Salinispora*, a group of true marine-actinomycetes that are well-known as a prolific producer of bioactive compounds.

KBS50 is a Gram-positive actinomycete. It formed circular and slightly convex colonies on ISP2 agar. The strain formed extensively branched substrate mycelia on agar media, while aerial mycelia were absent. The colonies on ISP2 agar were orange-yellow colour without formation of soluble pigments. The most notable physiological differences that clearly distinguished strain KBS50 from other species of *Plantactinospora* includes its ability to tolerate high concentration of NaCl (up to 7% w/v) and tolerance to high pH medium (up to pH 12), while it was unable to grow at incubation temperature of 40°C and above. The incubation temperature for KBS50 is in the range of 20-37°C with the optimum growth was observed between 25-30°C. Suitable pH for the cultivation of KBS50 was shown to be in the range of pH 6-11. Unlike the true marine actinomycetes *Salinispora*, KBS50 does not require NaCl for growth. KBS50 was also tested positive for proteolytic activity on SMA medium, whereas no glucanase activity was detected.

5.1.2 The antagonistic and antimicrobial activities of KBS50

KBS50 demonstrated strong antagonistic activities against the Gram-positive bacteria (*B. subtilis* and *S. aureus*) and fungi (*A. niger*, *G. boninense* and *R. solani*) on the perpendicular streak assay. No antagonistic activity was recorded against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and yeast (*S. cerevisiae*). The perpendicular streak assay demonstrated that the type of agar media could affect the antagonistic activity of KBS50. The strongest antagonistic activities against *B. subtilis* and *S. aureus* were recorded on MB agar. Similarly, the strongest antagonistic activities against fungi test strains were also recorded on MB agar.

The secondary screening of the CFBs and crude extracts from the fermentation media ISP2, MB, FM1 and FM8 using the agar well diffusion assay method showed antimicrobial activities against *B. subtilis* and *S. aureus* only. For both types of samples, the strongest antimicrobial activities were recorded from the ISP2. The results indicate that the antimicrobial compound(s) was produced at the highest level in ISP2 broth as compared to other media. The MIC of the ISP2 crude extract against *B. subtilis* and *S. aureus* was determined at 5.21 ± 1.30 µg/ml and 15.63 ± 0.00 µg/ml, respectively.

Comparative HPLC analysis showed that the crude extract from ISP2 medium showed the highest number of major peaks with a higher level of production, as compared to other

media (MB, FM1 and FM8). Based on the results of antimicrobial screening and comparative HPLC analysis, the ISP2 medium was identified as the best fermentation broth for the production of bioactive secondary metabolites from KBS50.

The OSMAC strategy had successfully enhanced the detection of antimicrobial activities from KBS50. The use of MTP Assay format for antimicrobial screening also increases the sensitivity of the test strains towards the antimicrobial compounds produced by KBS50 in the OSMAC fermentation. This was demonstrated by a strong antimicrobial activity against *A. niger* recorded from the crude extract of ISP2. Other than the antimicrobial activities against *B. subtilis*, *S. aureus* and *A. niger*, the crude extracts from the OSMAC fermentation strategy also recorded strong antimicrobial activity against the yeast, *C. albicans*, although no activity was recorded against *S. cerevisiae*. In addition, moderate antimicrobial activities were also recorded against the Gram-negative test strains, *E .coli* and *P. aeruginosa*. Based on these findings, it was shown that *Plantactinospora* sp. KBS50 could potentially produce broad-spectrum antimicrobial compounds when grown under specific cultivation conditions or with the presence of particular elicitor elements.

5.1.3 Cultivation conditions affecting the antibiotics and secondary metabolites production

The antimicrobial screening of the crude extracts from the OSMAC fermentation approach had demonstrated the capability of KBS50 to produce broad-spectrum antimicrobial compounds. As compared to the basal medium, ISP2, significant increase or decrease in antimicrobial activities were recorded, especially against *A. niger* and *C. albicans*. Similarly, the comparative HPLC analysis also showed significant changes in secondary metabolites profile as the result of the OSMAC fermentation strategy. The results showed that the production of antibiotics and other secondary metabolites by KBS50 can be influenced by the cultivation conditions such as pH of the medium, incubation temperature, as well as the presence of biological or chemical elicitors in its cultivation media.

KBS50 exhibited strong antimicrobial activities against *B. subtilis* and *S. aureus* in nearly all cultivation conditions used under the OSMAC approach. Most notable increased in

antimicrobial activity against *E. coli* was recorded from the extract of ISP2 broth at pH 9, while the highest activity against *P. aeruginosa* was recorded from the extract of ISP2 supplemented with the culture filtrate of *G. boninense*. Meanwhile, the highest antimicrobial activity against *A. niger* and *C. albicans* was recorded from ISP2 broth supplemented with the dead cells of *E. coli* and ISP2 broth at pH 9, respectively.

On the other hand, the OSMAC fermentation approach also resulted in significant reductions of antimicrobial activities against the test strains. Most notably was the incubation temperature of 37°C which had negatively affected the antimicrobial activities against *S. aureus*, *A. niger* and *C. albicans*. The addition of NaCl (1% and 1.5%) and a higher concentration of phosphate (30 mM) also negatively affecting the antimicrobial activities against *A. niger* and *C. albicans*.

Comparative HPLC analysis revealed that the production of secondary metabolites by KBS50 was affected by various cultivation parameters used in the OSMAC fermentation approach. The incubation temperature of 37°C and pH 9 had a positive effect on the production of compound 1 and 2, respectively. The production level of compound 3 and 4 was increased in the presence of 3% DMSO. New metabolites were also detected from the crude extracts of fermentation media that were supplemented with NaCl (1% and 1.5%), 5 mM phosphate buffer and the culture filtrate of *A. niger*. Meanwhile, the secondary metabolites production, especially compound 1, 3 and 4, was negatively affected by the presence of 30 mM phosphate buffer and NaCl (1% and 1.5%). The incubation temperature of 37°C and the culture filtrate of *G. boninense* also inhibited the production of compound 3 and 4.

5.1.4 Secondary metabolites and antimicrobial compounds produced by KBS50

Plantactinospora sp. KBS50 produced at least 13 secondary metabolites compounds as detected by the HPLC analysis. Four of these major compounds (compound 1-4) were detected from the crude extract of ISP2, while other compounds were detected from the crude extracts of ISP2 broth supplemented with NaCl (1% and 1.5%), 5 mM phosphate and the culture filtrate of *A. niger*.

The bioassay-guided fractionation of the crude extract from the 2 litre fermentation broth (ISP2) demonstrated that KBS50 produced more than one compound that was active against the Gram-positive bacteria. From 8 fractions tested, 6 fractions exhibited antimicrobial activity against *B. subtilis*, while only 2 fractions (fraction 7 and 9) exhibited antimicrobial activity against *S. aureus*. Fraction 7 recorded the highest antimicrobial activity against both test strains. This result indicates the presence of strong antimicrobial compound(s) in fraction 7.

Two major peaks were successfully isolated from fraction 9, with the purity of 99.86% and 93.24% for compound 3 and 4, respectively. Both compounds demonstrated strong antimicrobial activity against *S. aureus*. The MIC for compound 3 and 4 was determined at 7.81 µg/ml and 62.50 µg/ml, respectively, while the MBC of compound 3 and 4 was determined at 15.63 µg/ml and 125 µg/ml. Both compounds were tested to be bactericidal against the *S. aureus*.

5.1.5 Genome properties and gene clusters for secondary metabolites

The genome sequence of *Plantactinospora* sp. KBS50 consist of a single circular chromosome with a length of 6,689,726 bp and a G+C content of 72.9%. The RAST annotation predicted a total of 5,820 CDS with the coding capacity of 87.8%. The majority of the CDS (70.3%) were annotated with a putative function while the remaining CDS were assigned as hypothetical proteins. A total of 56 RNAs were identified, with 47 of them were tRNAs and 9 were rRNAs (3 operons). KBS50 devoted up to 6.6% of its genome sequence for regulatory functions. Twelve genes encoding for CRISPR or CRISPR-associated proteins were also identified from the genome sequence. The analysis revealed that KBS50's genome contains many genes for protein and carbohydrate metabolism. These include 332 genes for protein metabolism and 526 genes for carbohydrate metabolism. A large number of genes for protein and carbohydrate metabolism indicates that KBS50 can utilise a wide variety of carbon and nitrogen sources as energy.

The secondary metabolites BGCs from KBS50 genome sequence were identified using the antiSMASH software. From the total of 60 BGCs identified, 37 are putative clusters while the other 23 comprised of the major types of BGCs including PKS (7), NRPS (7),

PKS-NRPS hybrid (2), bacteriocin (1), lantipeptide (2), terpene (3) and siderophore (1). The antiSMASH analysis also predicted 11 core structures of polyketides and peptides product of the modular type-1 PKS and NRPS, based on the substrate specificity of the acyltransferase and the adenylation domains of PKS and NRPS gene clusters, respectively.

Most of the BGCs identified from KBS50 have low gene similarity to any known gene clusters, with the exception of cluster 47 (putative), cluster 6 (terpene) and cluster 56 (type-3 PKS) which share between 71-100% gene similarity with known gene clusters. Cluster 6 showed a remarkably similar gene cluster organisation with the sioxanthin gene cluster found in *S. tropic*a, with 100% gene similarity. Comparative analysis of secondary metabolites BGCs between KBS50 and other related species revealed comparable biosynthetic capabilities in term of the number of major types of gene clusters. KBS50 has the highest number of NRPS-associated gene clusters (9) as compared to *S. arenicola* (6), *S. tropica* (6), *M. narathiwatensis* (8), and *S. coelicolor* (4).

5.2   Conclusions

The continuous search for a new source of bioactive natural product compounds as drug candidates is essential to enable a constant supply of effective treatments for infectious diseases and other ailments (Boucher et al. 2009). It was the aim of the present study to assess the natural product biosynthetic capabilities of *Plantactinospora* sp. KBS50, a rare marine-derived actinomycete from Sarawak, as a potential source of novel bioactive compounds. KBS50 is a unique actinomycete strain that represents only a small fraction of the hidden microbial diversity in Sarawak that is yet to be explored. Nonetheless, this study which had focused solely on a single strain may enhance our knowledge of the natural product biosynthetic capabilities of the rare actinomycetes from Sarawak, particularly the *Plantactinospora* genus. This study also showed that the marine-associated environment such as the coastal area in Sarawak can be a valuable source of unique actinomycetes with huge potential that can be exploited for natural product discovery.

The work presented in this thesis reported on the application of a classical approach and the more advanced genome mining approach for natural product screening from a unique

actinomycete strain. The classical or traditional approach relies on the biological screening of extracts for bioactivity. The drawback is that actinomycetes may not produce bioactive secondary metabolites in sufficient quantity or may not produce the metabolites at all under the standard cultivation condition (Rutledge & Challis 2015), consequently limit the detection of their full biological potential. The present study demonstrated that the incorporation of the OSMAC fermentation strategy is advantageous in enhancing the detection of antibiotics and secondary metabolites from KBS50. The OSMAC fermentation approach had shown that different elicitors or parameters may affect the production of specific metabolites. This information can be used as the basis for the optimisation of cultivation conditions which can increase the compound yield for further isolation and purification. It is important to mention that only a limited number of possible growth conditions and elicitors were evaluated for the OSMAC fermentation. Further enhancement of antibiotics and secondary metabolites production and detection from KBS50 may be possible if more cultivation parameters are evaluated in the future study.

Nevertheless, exploitation of cultivation conditions alone may not guarantee the detection of all possible metabolites from KBS50, as the genetic factor play the most important role in determining how many compounds a particular microorganism could actually produce (Bode et al. 2002). Thus, the full biosynthetic capability of KBS50 was uncovered through the genome mining approach which involved the sequencing of its complete genome for the identification of secondary metabolites BGCs. The genome data provided an overview of the enzymatic mechanism underlying the biosynthetic pathway of the secondary metabolites produced by KBS50. Whether these genes were actually expressed during fermentation or they remained silent under the tested cultivation conditions, are remain to be seen. This aspect can be addressed in the future study through the investigation of the expression of the biosynthetic genes.

To the best of our knowledge, this study is the first reported genome of a species from the genus *Plantactinospora*. The genome sequence may serve as a reference genome for comparative analysis with other *Plantactinospora* species in the future. Such analysis will further improve our understanding of the secondary metabolism of this unique actinomycete genus.

In summary, the antibiotics and secondary metabolites biosynthetic potential of *Plantactinospora* sp. KBS50 has been successfully evaluated in the present study. The results of antimicrobial screening and identification of secondary metabolites biosynthetic genes from the genome sequence provided strong indication that KBS50 is indeed a prolific producer of bioactive natural product compounds. This study will, therefore, facilitate the discovery of new chemical entity from *Plantactinospora* sp. KBS50 which can be developed as potential drug candidates.

5.3   Future directions and recommendations

The study presented in this thesis is part of an ongoing natural product bioprospecting program with the long-term goal is to discover new chemical entity with potent biological activity from the microorganism isolated from Sarawak which can be developed as potential drug candidates. Through this study, it was established that *Plantactinospora* sp. KBS50 is a prolific source of secondary metabolites. Therefore more efforts should be put towards the screening, isolation, identification and characterization of bioactive compounds from KBS50. Future studies may include, but not limited to, the following:

a.   Identification and characterization of compound 2 and 3 using the LC-MS and NMR analysis. The bioactivity of these compounds can be further evaluated against broad spectrum microorganism, including the methicillin-resistant *S. aureus* strain. Optimisation of fermentation media and condition, as well as fermentation in larger scale, may be necessary to increase the yield of these compounds for further isolation and purification.

b.   Further assessment of the effect of different cultivation parameters towards secondary metabolites production by KBS50 based on the data from the OSMAC fermentation approach presented in this study. The testing may include a different range of incubation temperature, pH or concentration of elicitors which have been shown to positively enhance the antibiotics and secondary metabolites production by KBS50.

c.   Gene expression analysis of the secondary metabolites BGCs to identify which gene cluster is expressed under different cultivation conditions. Gene expression study in combination with comparative metabolic profiling can be used to investigate which gene cluster is responsible for the production of a particular

metabolite. In addition, gene expression analysis will allow the identification of silent BGCs, of which can then be manipulated through various genetic engineering methods. These may include ribosomal engineering, heterologous gene expression and the manipulation of regulatory genes to activate the expression of the silent gene clusters (Zerikly & Challis 2009).

This study has shown that the marine-associated environment such as the beach area in Sarawak can be a valuable source of genetically talented rare actinomycetes species. Further exploration of similar habitats including the mangrove area, is highly recommended in the continuous search of rare actinomycetes for natural product screening. Last but not least, the OSMAC fermentation approach and the genome mining approach applied in this study should be extended to evaluate the capabilities of other rare actinomycetes species already available in the Sarawak Biodiversity Centre' microbial culture collection. A further enhancement to this strategy may include the use of LC-MS equipment for compound dereplication and identification. In addition, the molecular networking and the pattern-based genome mining approach (Duncan et al. 2015) can link the metabolites detected by the mass spectrometry analysis to its respective BGCs. This promising strategy if incorporated into the natural product screening workflow can greatly facilitate the discovery of novel compounds from the rare actinomycetes from Sarawak.

REFERENCES

Abdelmohsen, UR, Grkovic, T, Balasubramanian, S, Kamel, MS, Quinn, RJ & Hentschel, U 2015, 'Elicitation of secondary metabolism in actinomycetes', *Biotechnology Advances*, vol. 33, no. 6, pp. 798–811.

Adegboye, MF & Babalola, OO 2013, 'Actinomycetes: A yet inexhaustive source of bioactive secondary metabolites', in A Méndez-Vilas (ed), *Microbial pathogens and strategies for combating them: Science, technology and education*, Formatex Research Center, Badajoz, pp. 786-795.

Alnahdi, HS 2012, 'Isolation and screening of extracellular proteases produced by new isolated *Bacillus* sp.', *Journal of Applied Pharmaceutical Science*, vol. 2, no. 9, pp. 71–74.

Amrita, K, Nitin, J & Devi, CS 2012, 'Novel bioactive compounds from mangrove derived actinomycetes', *International Research Journal of Pharmacy*, vol. 3, no. 9, pp. 25–29.

Andrews, JM 2001, 'Determination of minimum inhibitory concentrations', *Journal of Antimicrobial Chemotherapy*, vol. 48, suppl. 1, pp. 5–16.

Anzai, Y, Saito, N, Tanaka, M, Kinoshita, K, Koyama, Y & Kato, F 2003, 'Organization of the biosynthetic gene cluster for the polyketide macrolide mycinamicin in *Micromonospora griseorubida*', *FEMS Microbiology Letters*, vol. 218, no. 1, pp. 135–141.

Ayuso-Sacido, A & Genilloud, O 2005, 'New PCR primers for the screening of NRPS and PKS-I systems in actinomycetes: Detection and distribution of these biosynthetic gene sequences in major taxonomic groups', *Microbial Ecology*, vol. 49, no. 1, pp. 10–24.

Aziz, RK, Bartels, D, Best, AA, DeJongh, M, Disz, T, Edwards, RA, … Zagnitko, O 2008, 'The RAST Server: Rapid Annotations using Subsystems Technology', *BMC Genomics*, vol. 9, no. 1, pp. 75.

Azman, AS, Othman, I, Velu, SS, Chan, KG & Lee, LH 2015, 'Mangrove rare actinobacteria: Taxonomy, natural compound, and discovery of bioactivity', *Frontiers in Microbiology*, vol. 6, pp. 1–15.

Balouiri, M, Sadiki, M & Ibnsouda, SK 2016, 'Methods for in vitro evaluating antimicrobial activity: A review', *Journal of Pharmaceutical Analysis*, vol. 6, no. 2, pp. 71–79.

Baltz, RH 2006, 'Marcel Faber Roundtable: Is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration?', *Journal of Industrial Microbiology and Biotechnology*, vol. 33, pp. 507–513.

Baltz, RH 2016, 'Genetic manipulation of secondary metabolite biosynthesis for improved production in *Streptomyces* and other actinomycetes', *Journal of Industrial Microbiology and Biotechnology*, vol. 43, pp. 343–370.

Bartholomew, JW & Finkelstein, H 1958, 'Relationship of cell wall staining to gram differentiation', *Journal of Bacteriology*, vol. 75, no. 1, pp. 77–84.

Begum, MF & Absar, N 2009, 'Purification and characterization of intracellular cellulase from *Aspergillus oryzae* ITCC-4857.01', *Mycobiology*, vol. 37, no. 2, p. 121.

Bentley, SD, Chater, KF, Cerdeño-Tárraga, AM, Challis, GL, Thomson, NR, James, KD, … Hopwood, DA 2002, 'Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)', *Nature*, vol. 417, pp. 141–147.

Bérdy, J 2005, 'Bioactive microbial metabolites', *The Journal of Antibiotics*, vol. 58, no. 1, pp. 1–26.

Bérdy, J 2012, 'Thoughts and facts about antibiotics: Where we are now and where we are heading', *The Journal of Antibiotics*, vol. 65, no. 8, pp. 441–441.

Bibb, MJ 2005, 'Regulation of secondary metabolism in streptomycetes', *Current Opinion in Microbiology, vol.* 8, no. 2, pp. 208-215.

Bills, GF, Platas, G, Fillola, A, Jiménez, MR, Collado, J, Vicente, F, … Peláez, F 2008, 'Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays', *Journal of Applied Microbiology*, vol. 104, no. 6, pp. 1644–1658.

Binnewies, TT, Motro, Y, Hallin, PF, Lund, O, Dunn, D, La, T, … Ussery, DW 2006, 'Ten years of bacterial genome sequencing: Comparative-genomics-based discoveries', *Functional and Integrative Genomics*, vol. 6, no. 3, pp. 165–185.

Bister, B, Bischoff, D, Ströbele, M, Riedlinger, J, Reicke, A, Wolter, F, … Süssmuth, RD 2004, 'Abyssomicin C - A polycyclic antibiotic from a marine *Verrucosispora* strain as an inhibitor of the p-aminobenzoic acid/tetrahydrofolate biosynthesis pathway', *Angewandte Chemie - International Edition*, vol. 43, no. 19, pp. 2574–2576.

Blin, K, Medema, MH, Kazempour, D, Fischbach, MA, Breitling, R, Takano, E & Weber, T 2013, 'antiSMASH 2.0 - a versatile platform for genome mining of secondary metabolite producers', *Nucleic Acids Research*, vol. 41, no. W1, pp. W204–W212.

Blin, K, Wolf, T, Chevrette, MG, Lu, X, Schwalen, CJ, Kautsar, SA, … Medema, MH

2017, 'antiSMASH 4.0 - improvements in chemistry prediction and gene cluster boundary identification', *Nucleic Acids Research*, vol. 45, no. W1, pp. W36-W41.

Bode, HB, Bethe, B, Höfs, R & Zeeck, A 2002, 'Big effects from small changes: Possible ways to explore nature's chemical diversity', *ChemBioChem*, vol. 3, no. 7, pp. 619–627.

Bode, HB & Müller, R 2005, 'The impact of bacterial genomics on natural product research', *Angewandte Chemie - International Edition*, vol. 44, no. 42, pp. 6828-6846.

Boucher, HW, Talbot, GH, Bradley, JS, Edwards, JE, Gilbert, D, Rice, LB, … Bartlett, J 2009, 'Bad bugs, no drugs: No ESKAPE! An update from the Infectious Diseases Society of America', *Clinical Infectious Diseases*, vol. 48, no. 1, pp. 1–12.

Bouslimani, A, Sanchez, LM, Garg, N & Dorrestein, PC 2014, 'Mass spectrometry of natural products: Current, emerging and future technologies', *Natural Product Reports*, vol. 31, no. 6, pp. 718–729.

Bredholt, H, Fjærvik, E, Johnsen, G & Zotchev, SB 2008, 'Actinomycetes from sediments in the Trondheim Fjord, Norway: Diversity and biological activity', *Marine Drugs*, vol. 6, no. 1, pp. 12–24.

Brötz, H & Sahl, H 2000, 'New insights into the mechanism of action of lantibiotics - diverse biological effects by binding to the same molecular target', *Journal of Antimicrobial Chemotherapy*, vol. 46, no. 1, pp. 1–6.

Buermans, HPJ & den Dunnen, JT 2014, 'Next generation sequencing technology: Advances and applications', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1842, no. 10, pp. 1932–1941.

Bundale, S, Begde, D, Nashikkar, N, Kadam, T & Upadhyay, A 2015, 'Optimization of culture conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from soil', *Advances in Microbiology*, vol. 5, no. 6, pp. 441–451.

Cane, DE & Walsh, CT 1999, 'The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases', *Chemistry and Biology*, vol. 6, no. 12, pp. R319–R325.

Carver, T, Thomson, N, Bleasby, A, Berriman, M & Parkhill, J 2009, 'DNAPlotter: Circular and linear interactive genome visualization', *Bioinformatics*, vol. 25, no. 1, pp. 119–120.

Challis, GL 2008, 'Mining microbial genomes for new natural products and biosynthetic pathways', *Microbiology*, vol. 154, no. 6, pp. 1555–1569.

Chaudhary, H S, Soni, B, Shrivastava, AR & Shrivastava, S 2013, 'Diversity and versatility of actinomycetes and its role in antibiotic production', *Journal of Applied Pharmaceutical Science*, vol. 3, pp. S83–S94.

Chawawisit, K, Bhoopong, P, Phupong, W & Lertcanawanichakul, M 2015, 'Antimicrobial and cytotoxic activities of bioactive compounds produced by *Streptomyces* sp. KB1', *International Journal of Pharmacy and Pharmaceutical Sciences*, vol. 7, no. 11, pp. 118-122.

Chen, G, Wang, GYS. Li, X, Watres, B & Davies, J 2000, 'Enhanced production of microbial metabolites in the presence of dimethyl sulfoxide', *The Journal of Antibiotics*, vol. 53, no. 10, pp. 1145–1153.

Chiang, YM, Chang, SL, Oakley, BR & Wang, CCC 2011, 'Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms', *Current Opinion in Chemical Biology*, vol. 15, no. 1, pp. 137–143.

Chin, CS, Alexander, DH, Marks, P, Klammer, AA, Drake, J, Heiner, C, … Korlach, J 2013, 'Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data', *Nature Methods*, vol. 10, no. 6, pp. 563–569.

Choi, SS, Kim, HJ, Lee, HS, Kim, P & Kim, ES 2015, 'Genome mining of rare actinomycetes and cryptic pathway awakening', *Process Biochemistry*, vol. 50, no. 8, pp. 1184–1193.

Chu, BC, Garcia-Herrero, A, Johanson, TH, Krewulak, KD, Lau, CK, Peacock, RS, … Vogel, HJ 2010, 'Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view', *Biometals*, vol. 23, no. 4, pp. 601–611.

Coates, AS, Hu, Y, Bax, R & Page, C 2002, 'The future challenges facing the development of new antimicrobial drugs', *Nature Reviews Drug Discovery*, vol. 1, no. 11, pp. 895–910.

Cotter, PD, Hill, C & Ross, RP 2005, 'Food microbiology: Bacteriocins: developing innate immunity for food', *Nature Reviews Microbiology*, vol. 3, no.. 10, pp. 777–788.

Cragg, GM & Newman, DJ 2013, 'Natural products: A continuing source of novel drug leads', *Biochimica et Biophysica Acta - General Subjects*, vol. 1830, no. 6, pp. 3670–3695.

D'Argenio, V, Petrillo, M, Pasanisi, D, Pagliarulo, C, Colicchio, R, Talà, A, … Alifano, P 2016, 'The complete 12 Mb genome and transcriptome of *Nonomuraea*

gerenzanensis with new insights into its duplicated "magic" RNA polymerase', *Scientific Reports*, vol. 6, no. 1, p. 18.

de Lima Procópio, RE, da Silva, IR, Martins, MK, de Azevedo, JL & de Araújo, JM 2012, 'Antibiotics produced by *Streptomyces*', *Brazilian Journal of Infectious Diseases*, vol. 16, no. 5, pp. 466–471.

de Lorenzo, V & Neilands, JB 1986, 'Characterization of iucA and iucC genes of the aerobactin system of plasmid ColV-K30 in *Escherichia coli*', *Journal of Bacteriology*, vol. 167, no. 1, pp. 350–355.

Demain, AL 2014, 'Importance of microbial natural products and the need to revitalize their discovery', *Journal of Industrial Microbiology and Biotechnology*, vol. 41, no. 2, pp. 185–201.

Ding, D, Chen, G, Wang, B, Wang, Q, Liu, D, Peng, M & Shi, P 2013, 'Culturable actinomycetes from desert ecosystem in northeast of Qinghai-Tibet Plateau', *Annals of Microbiology*, vol. 63, no. 1, pp. 259–266.

Doull, JL & Vining, LC 1990, 'Nutritional control of actinorhodin production by *Streptomyces coelicolor* A3(2): Suppressive effects of nitrogen and phosphate', *Applied Microbiology and Biotechnology*, vol. 32, no. 4, pp. 449–454.

Duncan, KR, Crüsemann, M, Lechner, A, Sarkar, A, Li, J, Ziemert, N, … Jensen, PR 2015, 'Molecular networking and pattern-based genome mining improves discovery of biosynthetic gene clusters and their products from *Salinispora* species', *Chemistry and Biology*, vol. 22, no. 4, pp. 460–471.

Ekblom, R & Wolf, JBW 2014, 'A field guide to whole-genome sequencing, assembly and annotation', *Evolutionary Applications*, vol. 7, no. 9, pp. 1026–1042.

Farnet, CM & Zazopoulos, E 2005, 'Improving drug discovery from microorganisms', in L. Zhang & AL Demain (eds), *Natural products: Drug discovery and therapeutic medicine*, Humana Press, Totowa, NJ, pp. 95–106.

Feling, RH, Buchanan, GO, Mincer, TJ, Kauffman, CA, Jensen, PR & Fenical, W 2003, 'Salinosporamide A: A highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus *Salinospora*', *Angewandte Chemie - International Edition*, vol. 42, no. 3, pp. 355–357.

Felsenstein, J 1985, 'Confidence limits on phylogenies: An approach using the bootstrap', *Evolution*, vol. 39, no. 4, pp. 783–791.

Feng, Y, Zhang, Y, Ying, C, Wang, D & Du, C 2015, 'Nanopore-based fourth-generation DNA sequencing technology', *Genomics, Proteomics and Bioinformatics*, vol. 13,

no. 1, pp. 4–16.

Fenical, W & Jensen, PR 2006, 'Developing a new resource for drug discovery: Marine actinomycete bacteria', *Nature Chemical Biology*, vol. 2, no. 12, pp. 666–673.

Fiedler, HP, Bruntner, C, Bull, AT, Ward, AC, Goodfellow, M, Potterat, O, … Mihm, G 2005, 'Marine actinomycetes as a source of novel secondary metabolites', *Antonie van Leeuwenhoek*, vol. 87, no. 1, pp. 37–42.

Fleischmann, R, Adams, M, White, O, Clayton, R, Kirkness, E, Kerlavage, A, … Craig Venter, J 1995, 'Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd', *Science*, vol. 269, no. 5223, pp. 496–512.

Forde, BM & O'Toole, PW 2013, 'Next-generation sequencing technologies and their impact on microbial genomics', *Briefings in Functional Genomics*, vol. 12, no. 5, pp. 440–453.

Frank, AM, Bandeira, N, Shen, Z, Tanner, S, Briggs, SP, Smith, RD & Pevzner, PA 2008, 'Clustering millions of tandem mass spectra', *Journal of Proteome Research*, vol. 7, no. 1, pp. 113–122.

Fraser, CM, Eisen, JA & Salzberg, SL 2000, 'Microbial genome sequencing', *Nature*, vol. 406, pp. 799–803.

French, GL 2006, 'Bactericidal agents in the treatment of MRSA infections - the potential role of daptomycin', *Journal of Antimicrobial Chemotherapy*, vol. 58, no. 6, pp. 1107–1117.

Gao, F & Zhang, CT 2008, 'Ori-Finder: A web-based system for finding oriCs in unannotated bacterial genomes', *BMC Bioinformatics*, vol. 9, p. 79.

Gao, F & Zhang, CT 2007, 'DoriC: A database of oriC regions in bacterial genomes', *Bioinformatics*, vol. 23, no. 14, pp. 1866–1867.

Gebreyohannes, G, Moges, F, Sahile, S & Raja, N 2013, 'Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia', *Asian Pacific Journal of Tropical Biomedicine*, vol. 3, no. 6, pp. 426–435.

Genilloud, O, González, I, Salazar, O, Martín, J, Tormo, JR & Vicente, F 2011, 'Current approaches to exploit actinomycetes as a source of novel natural products', *Journal of Industrial Microbiology and Biotechnology*, vol. 38, no, 3, pp. 375–389.

Genilloud, O, Pelaez, F, Gonzalez, I & Diez, MT 1994, 'Diversity of actinomycetes and fungi on seaweeds from the Iberian coasts', *Microbiologia*, vol. 10, no. 4, pp. 413–422.

Gershenzon, J & Dudareva, N 2007, 'The function of terpene natural products in the natural world', *Nature Chemical Biology*, vol. 3, no. 7, pp. 408–414.

Giardina, A, Alduina, R, Gallo, G, Monciardini, P, Sosio, M & Puglia, AM 2014, 'Inorganic phosphate is a trigger factor for *Microbispora* sp. ATCC-PTA-5024 growth and NAI-107 production', *Microbial Cell Factories*, vol. 13, p. 133.

Golinska, P, Wypij, M, Agarkar, G, Rathod, D, Dahm, H & Rai, M 2015, 'Endophytic actinobacteria of medicinal plants: Diversity and bioactivity', *Antonie van Leeuwenhoek*, vol. 108, no. 2, pp. 267–289.

Gray, AI, Igoli, JO & Edrada-Ebel, R 2012, 'Natural products isolation in modern drug discovery programs', *Methods in Molecular Biology,* vol. 864, pp. 515–534.

Grissa, I, Vergnaud, G & Pourcel, C 2007, 'The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats', *BMC Bioinformatics*, vol. 8, p. 172.

Guo, X, Guan, X, Liu, C, Jia, F, Li, J, Li, J, … Xiang, W 2016, '*Plantactinospora soyae* sp. nov., an endophytic actinomycete isolated from soybean root [*Glycine* max (L.) Merr]', *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 7, pp. 2578–2584.

Hall, T 1999, 'BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT', *Nucleic Acids Symposium Series*, vol. 41, pp. 95–98.

Harvey, AL 2008, 'Natural products in drug discovery', *Drug Discovery Today,* vol. 13, pp. 894–901.

Harwani, D 2013, 'Biodiversity of rare thermophilic actinomycetes in The Great Indian Thar Desert: An overview', *Indo American Journal of Pharmaceutical Research,* vol. 3, no. 11, pp. 9349–9356.

Hayakawa, M 2008, 'Studies on the isolation and distribution of rare actinomycetes in soil', *Actinomycetologica*, vol. 22, no. 1, pp. 12–19.

Hayakawa, M & Nonomura, H 1987, 'Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes', *Journal of Fermentation Technology*, vol. 65, no. 5, pp. 501–509.

Hayashi, S, Ozaki, T, Asamizu, S, Ikeda, H, Omura, S, Oku, N, … Onaka, H 2014, 'Genome mining reveals a minimum gene set for the biosynthesis of 32-membered macrocyclic thiopeptides lactazoles', *Chemistry and Biology*, vol. 21, no. 5, pp. 679–688.

Hendricks, CW, Doyle, JD & Hugley, B 1995, 'A new solid medium for enumerating cellulose-utilizing bacteria in soil', *Applied and Environmental Microbiology*, vol. 61, no. 5, pp. 2016–2019.

Hewage, RT, Aree, T, Mahidol, C, Ruchirawat, S & Kittakoop, P 2014, 'One strain-many compounds (OSMAC) method for production of polyketides, azaphilones, and an isochromanone using the endophytic fungus *Dothideomycete* sp.', *Phytochemistry*, vol. 108, pp. 87–94.

Hobbs, G, Frazer, CM, Gardner, DCJ, Flett, F & Oliver, SG 1990, 'Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): Kinetics and the influence of nutrients', *Journal of General Microbiology*, vol. 136, no. 11, pp. 2291–2296.

Hobbs, G, Obanye, AIC, Petty, J, Mason, JC, Barratt, E, Gardner, DCJ, … Oliver, SG 1992, 'An integrated approach to studying regulation of production of the antibiotic methylenomycin by *Streptomyces coelicolor* A3(2)', *Journal of Bacteriology*, vol. 174, no. 5, pp. 1487–1494.

Hong, K, Gao, AH, Xie, QY, Gao, H, Zhuang, L, Lin, HP, … Ruan, JS 2009, 'Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China', *Marine Drugs*, vol. 7, no. 1, pp. 24–44.

Huang, J, Lu, C, Qian, X, Huang, Y, Zheng, Z & Shen, Y 2011, 'Effect of salinity on the growth, biological activity and secondary metabolites of some marine fungi', *Acta Oceanologica Sinica*, vol. 30, no. 3, pp. 118–123.

Huang, X, Zhou, S, Huang, D, Chen, J & Zhu, W 2016, '*Streptomyces spongiicola* sp. nov., an actinomycete derived from marine sponge', *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 2, pp. 738–743.

Hussain, A, Rather, MA, Dar, MS, Aga, MA, Ahmad, N, Manzoor, A, … Hassan, QP 2017, 'Novel bioactive molecules from *Lentzea violacea* strain AS 08 using one strain-many compounds (OSMAC) approach', *Bioorganic and Medicinal Chemistry Letters,* vol. 27, no. 11, pp. 2579–2582.

Ikeda, H, Ishikawa, J, Hanamoto, A, Shinose, M, Kikuchi, H, Shiba, T, … Ōmura, S 2003, 'Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*', *Nature Biotechnology*, vol. 21, no. 5, pp. 526–531.

Illakkiam, D, Anuj, NL, Ponraj, P, Shankar, M, Rajendhran, J & Gunasekaran, P 2013, 'Proteolytic enzyme mediated antagonistic potential of *Pseudomonas aeruginosa* against *Macrophomina phaseolina*', *Indian Journal of Experimental Biology*, vol.

51, no. 11, pp. 1024–1031.

Jain, M, Olsen, HE, Paten, B & Akeson, M 2016, 'The Oxford Nanopore MinION: Delivery of nanopore sequencing to the genomics community', *Genome Biology*, vol. 17, no. 1, p. 239.

James, PD, Edwards, C & Dawson, M 1991, 'The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat', *Journal of General Microbiology*, vol. 137, no. 7, pp. 1715–1720.

Janda, JM & Abbott, SL 2002, 'Bacterial identification for publication: When is enough enough?', *Journal of Clinical Microbiology*, vol. 40, no. 6, pp. 1887–1891.

Jeffrey, LSH 2008, 'Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak', *African Journal of Biotechnology*, vol. 7, no. 20, pp. 3697–3702.

Jeffrey, LSH & Halizah, H 2014, 'Biological active compounds from actinomycetes isolated from soil of Langkawi Island, Malaysia', *African Journal of Biotechnology*, vol. 13, no. 49, pp. 4523–4528.

Jenkins, SG & Schuetz, AN 2012, 'Current concepts in laboratory testing to guide antimicrobial therapy', *Mayo Clinic Proceedings*, vol. 87, no. 3, pp. 290–308.

Jensen, PR, Mincer, TJ, Williams, PG & Fenical, W 2005, 'Marine actinomycete diversity and natural product discovery', *Antonie van Leeuwenhoek*, vol. 87, no. 1, pp. 43–48.

Johnstone, TC & Nolan, EM 2015, 'Beyond iron: Non-classical biological functions of bacterial siderophores', *Dalton Transactions*, vol. 44, no. 14, pp. 6320–6339.

Jose, PA & Jebakumar, DSR 2013, 'Non-streptomycete actinomycetes nourish the current microbial antibiotic drug discovery', *Frontiers in Microbiology*, vol. 4, p. 240.

Ju, KS, Gao, J, Doroghazi, JR, Wang, KKA, Thibodeaux, CJ, Li, S, … Metcalf, WW 2015, 'Discovery of phosphonic acid natural products by mining the genomes of 10,000 actinomycetes', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 39, pp. 12175–12180.

Just-Baringo, X, Albericio, F & Álvarez, M. 2014, 'Thiopeptide antibiotics: Retrospective and recent advances', *Marine Drugs*, vol. 12, pp. 317-351.

Kasana, RC, Salwan, R, Dhar, H, Dutt, S & Gulati, A 2008, 'A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine', *Current Microbiology*, vol. 57, no. 5, pp. 503–507.

Kawai, K, Wang, G, Okamoto, S & Ochi, K 2007, 'The rare earth, scandium, causes antibiotic overproduction in *Streptomyces* spp', *FEMS Microbiology Letters*, vol.

274, no. 2, pp. 311–315.

Klitgaard, A, Nielsen, JB, Frandsen, RJN, Andersen, MR & Nielsen, KF 2015, 'Combining stable isotope labeling and molecular networking for biosynthetic pathway characterization', *Analytical Chemistry*, vol. 87, no. 13, pp. 6520–6526.

Knerr, PJ & van der Donk, WA 2012, 'Discovery, biosynthesis, and engineering of lantipeptides', *Annual Review of Biochemistry*, vol. 81, no. 1, pp. 479–505.

Koonin, EV & Galperin, MY 2003, 'Chapter 5 Genome Annotation and Analysis', *Sequence - Evolution - Function: Computational approaches in comparative genomics*, viewed 5 February 2016, <https://www.ncbi.nlm.nih.gov/books/NBK20253/>.

Koren, S, Harhay, GP, Smith, TP, Bono, JL, Harhay, DM, Mcvey, SD, … Phillippy, AM 2013, 'Reducing assembly complexity of microbial genomes with single-molecule sequencing', *Genome Biology*, vol. 14, no. 9, p. R101.

Koren, S & Phillippy, AM 2015, 'One chromosome, one contig: Complete microbial genomes from long-read sequencing and assembly', *Current Opinion in Microbiology*, vol. 23, pp. 110–120.

Krumsiek, J, Arnold, R & Rattei, T 2007, 'Gepard: A rapid and sensitive tool for creating dotplots on genome scale', *Bioinformatics*, vol. 23, no. 8, pp. 1026–1028.

Kwon, HC, Kauffman, CA, Jensen, PR & Fenical, W 2006, 'Marinomycins A-D, antitumor-antibiotics of a new structure class from a marine actinomycete of the recently discovered genus "*Marinispora*."', *Journal of the American Chemical Society*, vol. 128, no. 5, pp. 1622–1632.

Lam, KS 2006, 'Discovery of novel metabolites from marine actinomycetes', *Current Opinion in Microbiology*, vol. 9, no. 3, pp. 245–251.

Lam, KS 2007, 'New aspects of natural products in drug discovery', *Trends in Microbiology*, vol. 15, no. 6, pp. 279–289.

Land, M, Hauser, L, Jun, SR, Nookaew, I, Leuze, MR, Ahn, TH, … Ussery, DW 2015, 'Insights from 20 years of bacterial genome sequencing', *Functional & Integrative Genomics*, vol. 15, no. 2, pp. 141–161.

Laureti, L, Song, L, Huang, S, Corre, C, Leblond, P, Challis, GL & Aigle, B 2011, 'Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces ambofaciens*', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 15, pp. 6258–6263.

Lazzarini, A, Cavaletti, L, Toppo, G & Marinelli, F 2000, 'Rare genera of actinomycetes as potential producers of new antibiotics', *Antonie van Leeuwenhoek,* vol. 78, no. 3-4, pp. 399–405.

Lechevalier, HA & Lechevalier, MP 1967, 'Biology of actinomycetes', *Annual Review of Microbiology*, vol. 21, no. 1, pp. 71–100.

Lewis, K 2013, 'Platforms for antibiotic discovery', *Nature Reviews Drug Discovery*, vol. 12, no. 5, pp. 371–387.

Li, Q, Chen, X, Jiang, Y & Jiang, C 2016, 'Morphological identification of actinobacteria', in D Dhanasekaran & Y Jiang (Eds), *Actinobacteria - Basics and Biotechnological Applications*, InTech, Rijeka, pp. 59–86.

Liu, L, Li, Y, Li, S, Hu, N, He, Y, Pong, R, … Law, M 2012, 'Comparison of next-generation sequencing systems', *Journal of Biomedicine and Biotechnology*, vol. 2012, pp. 1–11.

Liu, Y & Yang, Q 2013, 'Cloning and heterologous expression of serine protease SL41 related to biocontrol in *Trichoderma harzianum*', *Journal of Molecular Microbiology and Biotechnology*, vol. 23, no. 6, pp. 431–439.

Lounès, A, Lebrihi, A, Benslimane, C, Lefebvre, G & Germain, P 1996, 'Regulation of spiramycin synthesis in *Streptomyces ambofaciens*: Effects of glucose and inorganic phosphate', *Applied Microbiology and Biotechnology*, vol. 45, pp. 204–211.

Ludwig, W, Mittenhuber, G & Friedrich, CG 1993, 'Transfer of *Thiosphaera pantotropha* to *Paracoccus denitrificans*', *International Journal of Systematic Bacteriology*, vol. 43, no. 2, pp. 363–367.

Luti, KJK & Mavituna, F 2011, 'Elicitation of *Streptomyces coelicolor* with dead cells of *Bacillus subtilis* and *Staphylococcus aureus* in a bioreactor increases production of undecylprodigiosin', *Applied Microbiology and Biotechnology*, vol. 90, no. 2, pp. 461–466.

Luti, KJK & Yonis, RW 2013, 'Elicitation of *Pseudomonas aeruginosa* with live and dead microbial cells enhances phenazine production', *Romanian Biotechnological Letters*, vol. 18, no. 6, pp. 7169–7178.

Ma, Z, Zhao, S, Cao, T, Liu, C, Huang, Y, Gao, Y, … Wang, X 2016, '*Verrucosispora sonchi* sp. nov., a novel endophytic actinobacterium isolated from the leaves of common sowthistle (*Sonchus oleraceus* L.)', *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 12, pp. 5430–5436.

Magarvey, NA, Keller, JM, Bernan, V, Dworkin, M & Sherman, DH 2004, 'Isolation and

characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites’, *Applied and Environmental Microbiology*, vol. 70, no. 12, pp. 7520–7529.

Maldonado, LA, Fenical, W, Jensen, PR, Kauffman, CA, Mincer, TJ, Ward, AC, … Goodfellow, M 2005, ‘*Salinispora arenicola* gen. nov., sp. nov. and *Salinispora tropica* sp. nov., obligate marine actinomycetes belonging to the family *Micromonosporaceae*’, *International Journal of Systematic and Evolutionary Microbiology*, vol. 55, no. 5, pp. 1759–1766.

Mardis, ER 2008, ‘Next-generation DNA sequencing methods’, *Annual Review of Genomics and Human Genetics*, vol. 9, no. 1, pp. 387-402.

Martín, JF 2004, ‘Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: An unfinished story’, *Journal of Bacteriology*, vol. 186, no. 16, pp. 5197–5201.

Martin, JF, Liras, P & Demain, AL 1977, ‘Inhibition by phosphate of the activity of candicidin synthases’, *FEMS Microbiology Letters*, vol. 2, no. 3, pp. 173–176.

Matsuda, S, Adachi, K, Matsuo, Y, Nukina, M & Shizuri, Y 2009, ‘Salinisporamycin, a novel metabolite from *Salinispora arenicola*’, *The Journal of Antibiotics*, vol. 62, no. 9, pp. 519–526.

Mcneil, MM & Brown, JM 1994, ‘The medically important aerobic actinomycetes: Epidemiology and microbiology’, *Clinical Microbiology Reviews*, vol. 7, no. 3, pp. 357-417.

Medema, MH, Blin, K, Cimermancic, P, De Jager, V, Zakrzewski, P, Fischbach, MA, … Breitling, R 2011, ‘AntiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences’, *Nucleic Acids Research*, vol. 39, pp. W339–W346.

Medema, MH & Fischbach, MA 2015, ‘Computational approaches to natural product discovery’, *Nature Chemical Biology*, vol. 11, no. 9, pp. 639–648.

Mincer, TJ, Jensen, PR, Kauffman, CA & Fenical, W 2002, ‘Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments’, *Applied and Environmental Microbiology*, vol. 68, no. 10, pp. 5005–5011.

Moore, E, Arnscheidt, A, KrÜger, A, StrÖmpl, C & Mau, M 2008, ‘Simplified protocols for the preparation of genomic DNA from bacterial cultures’, in GA Kowalchuk, FJ Bruijn, IM Head, AD Akkermans & JD Elsas (Eds), *Molecular Microbial Ecology Manual Second Edition*, Kluwer Academic Publishers, Dodrecht, pp. 3-18.

Mootz, HD & Marahiel, MA 1997, 'Biosynthetic systems for nonribosomal peptide antibiotic assembly', *Current Opinion in Chemical Biology*, vol. 1, no. 4, pp. 543–551.

Moran, MA, Rutherford, LT & Hodson, RE 1995, 'Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe', *Applied and Environmental Microbiology*, vol. 61, no. 10, pp. 3695–3700.

Muramatsu, H 2008, 'Development of a simple-identification method for actinomycetes based on partial 16S rDNA sequences as exemplified by a comparative study of Malaysian and Japanese actinomycetes', *Actinomycetologica*, vol. 22, pp. 30–33.

Muramatsu, H, Murakami, R, Ibrahim, ZH, Murakami, K, Shahab, N & Nagai, K 2011, 'Phylogenetic diversity of acidophilic actinomycetes from Malaysia', *The Journal of Antibiotics*, vol. 64, no. 9, pp. 621–624.

NCBI 2017, 'Genome information by organism', *National Center for Biotechnology Information*, viewed 11 June 2017, <http://www.ncbi.nlm.nih.gov/genome/browse/>.

Neilands, JB 1995, 'Siderophores: Structure and function of microbial iron transport compounds', *Journal of Biological Chemistry*, vol. 270, no. 45, pp. 26723–26726.

Ng, YK, Hodson, MP, Hewavitharana, AK, Bose, U, Shaw, PN & Fuerst, JA 2014, 'Effects of salinity on antibiotic production in sponge-derived *Salinispora* actinobacteria', *Journal of Applied Microbiology*, vol. 117, pp. 109–125.

Nguyen, DD, Wu, CH, Moree, WJ, Lamsa, A, Medema, MH, Zhao, X, … Dorrestein, PC 2013, 'MS/MS networking guided analysis of molecule and gene cluster families', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 28, pp. E2611–E2620.

Nigh, GD, Ying, CC & Qian, H 2004, 'Climate and productivity of major conifer species in the interior of British Columbia, Canada', *Forest Science*, vol. 50, no. 5, pp. 659–671.

Numata, K & Nimura, S 2003, 'Access to soil actinomycetes in Malaysian tropical rain forests', *Actinomycetologica*, vol. 17, no. 2, pp. 54–56.

Ochi, K 1987, 'Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: Significance of the stringent response (ppGpp) and GTP content in relation to a factor', *Journal of Bacteriology*, vol. 169, no. 8, pp. 3608–3616.

Ochi, K & Hosaka, T 2013, 'New strategies for drug discovery: Activation of silent or

weakly expressed microbial gene clusters', *Applied Microbiology and Biotechnology*, vol. 97, pp. 87-98.

Ochi, K, Tanaka, Y & Tojo, S 2014, 'Activating the expression of bacterial cryptic genes by rpoB mutations in RNA polymerase or by rare earth elements', *Journal of Industrial Microbiology and Biotechnology*, vol. 41, no. 2, pp. 403–414.

Oliynyk, M, Samborskyy, M, Lester, JB, Mironenko, T, Scott, N, Dickens, S, … Leadlay, PF 2007, 'Complete genome sequence of the erythromycin-producing bacterium *Saccharopolyspora erythraea* NRRL23338', *Nature Biotechnology*, vol. 25, no. 4, pp. 447–453.

Omura, S, Ikeda, H, Ishikawa, J, Hanamoto, A, Takahashi, C, Shinose, M, … Hattori, M 2001, 'Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 21, pp. 12215–12220.

Ostash, BE, Ogonyan, SV, Luzhetskyy, AN, Bechthold, A & Fedorenko, VA 2005, 'The use of PCR for detecting genes that encode type I polyketide synthases in genomes of actinomycetes', *Russian Journal of Genetics*, vol. 41, no. 5, pp. 473–478.

Overbeek, R, Begley, T, Butler, RM, Choudhuri, JV, Chuang, HY, Cohoon, M, … Vonstein, V 2005, 'The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes', *Nucleic Acids Research*, vol. 33, no. 17, pp. 5691–5702.

Overbeek, R, Olson, R, Pusch, GD, Olsen, GJ, Davis, JJ, Disz, T, … Stevens, R 2014, 'The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST)', *Nucleic Acids Research*, vol. 42, pp. D206–214.

Penn, K & Jensen, PR 2012, 'Comparative genomics reveals evidence of marine adaptation in *Salinispora* species', *BMC Genomics*, vol. 13, p. 86.

Perić-Concha, N & Long, PF 2003, 'Mining the microbial metabolome: A new frontier for natural product lead discovery', *Drug Discovery Today,* vol. 8, no. 23, pp. 1078-1084.

Pham, HT, Nguyen, NP, Phi, TQ, Dang, PT & Le, HG 2014, 'The antibacterial and anticancer activity of marine actinomycete strain HP411 isolated in the northern coast of Vietnam', *International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering*, vol. 8, no. 12, pp. 830–834.

Phongsopitanun, W, Kudo, T, Mori, M, Shiomi, K, Pittayakhajonwut, P, Suwanborirux,

K & Tanasupawat, S 2015, '*Micromonospora fluostatini* sp. nov isolated from marine sediment', *International Journal of Systematic and Evolutionary Microbiology*, vol. 65, no. 12, pp. 4417–4423.

Prakash, D, Nawani, N, Prakash, M, Bodas, M, Mandal, A, Khetmalas, M & Kapadnis, B 2013, 'Actinomycetes: A repertory of green catalysts with a potential revenue resource', *BioMed Research International*, vol. 2013, p. 264020.

Qin, S, Li, J, Chen, HH, Zhao, GZ, Zhu, WY, Jiang, CL, … Li, WJ 2009a, 'Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna China', *Applied and Environmental Microbiology*, vol. 75, no. 19, pp. 6176–6186.

Qin, S, Li, J, Zhang, YQ, Zhu, WY, Zhao, GZ, Xu, LH & Li, WJ 2009b, '*Plantactinospora mayteni* gen. nov., sp. nov., a member of the family *Micromonosporaceae*', *International Journal of Systematic and Evolutionary Microbiology*, vol. 59, no. 10, pp. 2527–2533.

Ravali, R, Phaneendra, M, Bhanu Jyothi, K, Ramya Santhoshi, L & Sushma, K 2011, 'Recent trends in analytical techniques for the development of pharmaceutical drugs', *Journal of Bioanalysis and Biomedicine,* vol. 3, no. 3, p. S11.

Reen, FJ, Romano, S, Dobson, ADW & O'Gara, F 2015, 'The sound of silence: Activating silent biosynthetic gene clusters in marine microorganisms', *Marine Drugs*, vol. 13, no. 8, pp. 4754–4783.

Rees, RW, Flood, J, Hasan, Y, Potter, U & Cooper, RM 2009, 'Basal stem rot of oil palm (*Elaeis guineensis*); mode of root infection and lower stem invasion by *Ganoderma boninense*', *Plant Pathology*, vol. 58, no. 5, pp. 982–989.

Retchless, AC & Lawrence, JG 2012, 'Ecological adaptation in bacteria: Speciation driven by codon selection', *Molecular Biology and Evolution*, vol. 29, no. 12, pp. 3669–3683.

Rhoads, A & Au, KF 2015, 'PacBio sequencing and its applications', *Genomics, Proteomics and Bioinformatics*, vol. 13, no. 5, pp. 278–289.

Richter, TKS, Hughes, CC & Moore, BS 2015, 'Sioxanthin, a novel glycosylated carotenoid, reveals an unusual subclustered biosynthetic pathway', *Environmental Microbiology*, vol. 17, no. 6, pp. 2158–2171.

Rigali, S, Titgemeyer, F, Barends, S, Mulder, S, Thomae, AW, Hopwood, DA & van Wezel, GP 2008, 'Feast or famine: The global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*', *EMBO Reports*, vol. 9, no. 7, pp. 670-

675.

Riley, M, Abe, T, Arnaud, MB, Berlyn, MKB, Blattner, FR, Chaudhuri, RR, … Wanner, BL 2006, '*Escherichia coli* K-12: A cooperatively developed annotation snapshot – 2005', *Nucleic Acids Research*, vol. 34, no. 1, pp. 1–9.

Ripa, FA, Nikkon, F, Zaman, S & Khondkar, P 2009, 'Optimal conditions for antimicrobial metabolites production from a new *Streptomyces* sp. RUPA-08PR isolated from Bangladeshi soil', *Mycobiology*, vol. 37, no. 3, pp. 211-214.

Rutledge, PJ & Challis, GL 2015, 'Discovery of microbial natural products by activation of silent biosynthetic gene clusters', *Nature Reviews Microbiology*, vol. 13, no. 8, pp. 509–523.

Rydén, AC, Lindberg, M & Philipson, L 1973, 'Isolation and characterization of two protease-producing mutants from *Staphylococcus aureus*', *Journal of Bacteriology*, vol. 116, no. 1, pp. 25–32.

Saitou, N & Nei, M 1987, 'The neighbor-joining method: A new method for reconstructing phylogenetic trees', *Molecular Biology and Evolution*, vol. 4, no. 4, pp. 406–425.

Salzberg, SL, Phillippy, AM, Zimin, A, Puiu, D, Magoc, T, Koren, S, … Yorke, JA 2012, 'GAGE: A critical evaluation of genome assemblies and assembly algorithms', *Genome Research*, vol. 22, no. 3, pp. 557–567.

Sanchez, S & Demain, AL 2002, 'Metabolic regulation of fermentation processes', *Enzyme and Microbial Technology*, vol. 31, no. 7, pp. 895–906.

Sazci, A, Erenler, K & Radford, A 1986, 'Detection of cellulolytic fungi by using Congo red as an indicator: A comparative study with the dinitrosalicyclic acid reagent method', *Journal of Applied Bacteriology*, vol. 61, no 6, pp. 559–562.

Schatz, A, Bugle, E & Waksman, SA 1944, 'Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria', *Experimental Biology and Medicine*, vol. 55, no.1, pp. 66–69.

Schleifer, KH 2009, 'Classification of bacteria and archaea: Past, present and future', *Systematic and Applied Microbiology*, vol. 32, no. 8, pp. 533–542.

Schwecke, T, Aparicio, JF, Molnár, I, König, A, Khaw, LE, Haydock, SF, … Leadlay, PF 1995, 'The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 17, pp. 7839–7843.

Sernova, NV & Gelfand, MS 2008, 'Identification of replication origins in prokaryotic

genomes', *Briefings in Bioinformatics*, vol. 9, no. 5, pp. 376–391.

Shen, B 2003, 'Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms', *Current Opinion in Chemical Biology,* vol. 7, no. 2, pp. 285-295.

Shin, SC, Ahn, DH, Kim, SJ, Lee, H, Oh, TJ, Lee, JE & Park, H 2013, 'Advantages of Single-Molecule Real-Time sequencing in high-GC content genomes', *PLoS ONE*, vol. 8, no. 7, p. e68824.

Shirling, EB & Gottlieb, D 1966, 'Methods for characterization of *Streptomyces* species', *International Journal of Systematic and Evolutionary Microbiology*, vol. 16, no. 3, pp. 313–340.

Siddiqui, MR, AlOthman, ZA & Rahman, N 2017, 'Analytical techniques in pharmaceutical analysis: A review', *Arabian Journal of Chemistry*, vol. 10, pp. S1409–S1421.

Solanki, R, Khanna, M & Lal, R 2008, 'Bioactive compounds from marine actinomycetes', *Indian Journal of Microbiology*, vol. 48, no. 4, pp. 410–431.

Solecka, J, Zajko, J, Postek, M & Rajnisz, A 2012, 'Biologically active secondary metabolites from actinomycetes', *Open Life Sciences*, vol. 7, no. 3, pp. 373–390.

Sorek, R, Lawrence, CM & Wiedenheft, B 2013, 'CRISPR-mediated adaptive immune systems in bacteria and archaea', *Annual Review of Biochemistry*, vol. 82, no. 1, pp. 237–266.

Spraker, J & Keller, N 2014, 'Waking sleeping pathways in filamentous fungi', in A Osbourn, RJ Goss & GT Carter (Eds), *Natural Products: Discourse, Diversity, and Design*, John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 277–292.

Stach, JEM, Maldonado, LA, Masson, DG, Ward, AC, Goodfellow, M & Bull, AT 2003, 'Statistical approaches for estimating actinobacterial diversity in marine sediments', *Applied and Environmental Microbiology*, vol. 69, no. 10, pp. 6189–6200.

Stackebrandt, E, Frederiksen, W, Garrity, GM, Grimont, PAD, Kämpfer, P, Maiden, MCJ, … Whitman, WB 2002, 'Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology', *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, no. 3, pp. 1043–1047.

Stackebrandt, E & Goebel, BM 1994, 'Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology', *International Journal of Systematic and Evolutionary Microbiology*, vol. 44, no. 4, pp. 846–849.

Stackebrandt, E & Ebers, J 2006, 'Taxonomic parameters revisited: Tarnished gold

standards', *Microbiology Today*, vol. 33, pp. 152–155.

Starcevic, A, Zucko, J, Simunkovic, J, Long, PF, Cullum, J & Hranueli, D 2008, 'ClustScan: An integrated program package for the semi-automatic annotation of modular biosynthetic gene clusters and *in silico* prediction of novel chemical structures', *Nucleic Acids Research*, vol. 36, no. 21, pp. 6882–6892.

Staunton, J & Weissman, KJ 2001, 'Polyketide biosynthesis: A millennium review', *Natural Product Reports*, vol. 18, no. 4, pp. 380–416.

Strobel, G & Daisy, B 2003, 'Bioprospecting for microbial endophytes and their natural products', *Microbiology and Molecular Biology Reviews*, vol. 67, no. 4, pp. 491-502.

Subramani, R & Aalbersberg, W 2012, 'Marine actinomycetes: An ongoing source of novel bioactive metabolites', *Microbiological Research*, vol. 167, no. 10, pp. 571-580.

Supong, K, Suriyachadkun, C, Suwanborirux, K, Pittayakhajonwut, P & Thawai, C 2013, '*Verrucosispora andamanensis* sp. nov., isolated from a marine sponge', *International Journal of Systematic and Evolutionary Microbiology*, vol. 63, pp. 3970–3974.

Taechowisan, T, Peberdy, JF & Lumyong, S 2003, 'Isolation of endophytic actinomycetes from selected plants and their antifungal activity', *World Journal of Microbiology and Biotechnology*, vol. 19, no. 4, pp. 381–385.

Takahashi, Y, Matsumoto, A, Seino, A, Iwai, Y & Omura, S 1996, 'Rare actinomycetes isolated from desert soils', *Actinomycetologica*, vol. 10, no. 2, pp. 91–97.

Tamura, K, Nei, M & Kumar, S 2004, 'Prospects for inferring very large phylogenies by using the neighbor-joining method', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 30, pp. 11030–11035.

Tamura, K, Stecher, G, Peterson, D, Filipski, A & Kumar, S 2013, 'MEGA6: Molecular Evolutionary Genetics Analysis version 6.0', *Molecular Biology and Evolution*, vol. 30, no. 12, pp. 2725–2729.

Tan, CJ, How, KC, Loh-Mia, PP, Ismet, A, Getha, K, Seki, T & Vikineswary, S 2002, 'Bioactivity of selected actinomycetes against *Ganoderma boninense*', *Asia-Pacific Journal of Molecular Biology and Biotechnology*, vol. 10, no. 2, pp. 119–125.

Tanaka, Y, Hosaka, T & Ochi, K 2010, 'Rare earth elements activate the secondary metabolite–biosynthetic gene clusters in *Streptomyces coelicolor* A3(2)', *The Journal of Antibiotics*, vol. 63, no. 8, pp. 477–481.

Thawai, C, Tanasupawat, S, Suwanborirux, K & Kudo, T 2010, '*Actinaurisporasiamensis* gen. nov., sp. nov., a new member of the family *Micromonosporaceae*', *International Journal of Systematic and Evolutionary Microbiology*, vol. 60, no. 7, pp. 1660–1666.

Tian, XP, Tang, SK, Dong, JD, Zhang, YQ, Xu, LH, Zhang, S & Li, WJ 2009, '*Marinactinospora thermotolerans* gen. nov., sp. nov., a marine actinomycete isolated from a sediment in the northern South China Sea', *International Journal of Systematic and Evolutionary Microbiology*, vol. 59, no. 5, pp. 948–952.

Tiwari, K & Gupta, RK 2012, 'Rare actinomycetes: A potential storehouse for novel antibiotics', *Critical Reviews in Biotechnology*, vol. 32, no. 2, pp. 108–132.

Tiwari, K & Gupta, RK 2013, 'Diversity and isolation of rare actinomycetes: An overview', *Critical Reviews in Microbiology*, vol. 39, no. 3, pp. 256–294.

Tiwari, K & Gupta, RK 2014, 'Bioactive metabolites from rare actinomycetes', *Studies in Natural Products Chemistry*, vol. 41, pp. 419–512.

Trautman, EP & Crawford, JM 2016, 'Linking biosynthetic gene clusters to their metabolites via pathway-targeted molecular networking', *Current Topics in Medicinal Chemistry*, vol. 16, no. 15, pp. 1705-1716.

Trujillo, ME, Bacigalupe, R, Pujic, P, Igarashi, Y, Benito, P, Riesco, R, … Normand, P 2014, 'Genome features of the endophytic actinobacterium *Micromonospora lupini* strain Lupac 08: On the process of adaptation to an endophytic life style?', *PLoS ONE*, vol. 9, no. 9, p. e108522.

Tuli, HS, Chaudhary, P, Beniwal, V & Sharma, AK 2015, 'Microbial pigments as natural color sources: Current trends and future perspectives', *Journal of Food Science and Technology*, vol. 52, no. 8, pp. 4669–4678.

Udwary, DW, Zeigler, L, Asolkar, RN, Singan, V, Lapidus, A, Fenical, W, … Moore, BS 2007, 'Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 25, pp. 10376–10381.

Ul-Hassan, A & Wellington, EM 2009, 'Actinobacteria', in M Schaechter (Ed), *Encyclopedia of Microbiology*, Elsevier, Oxford, pp. 25–44.

Valgas, C, de Souza, SM, Smânia, EFA & Smânia, AJr 2007, 'Screening methods to determine antibacterial activity of natural products', *Brazilian Journal of Microbiology*, vol. 38, pp. 369–380.

Van Lanen, SG & Shen, B 2006, 'Microbial genomics for the improvement of natural

product discovery', *Current Opinion in Microbiology,* vol. 9, no. 3, pp. 252-260.

Vandamme, P, Pot, B, Gillis, M, de Vos, P, Kersters, K & Swings, J 1996, 'Polyphasic taxonomy, a consensus approach to bacterial systematics', *Microbiological Reviews*, vol. 60, no. 2, pp. 407–438.

Vandermolen, KM, Raja, HA, El-Elimat, T & Oberlies, NH 2013, 'Evaluation of culture media for the production of secondary metabolites in a natural products screening program', *AMB Express*, vol. 3, no. 71, pp. 1–7.

Velho-Pereira, S & Kamat, NM 2011, 'Antimicrobial screening of actinobacteria using a modified cross-streak method', *Indian Journal of Pharmaceutical Sciences*, vol. 73, no. 2, pp. 223–228.

Větrovský, T & Baldrian, P 2013, 'The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses', *PLoS ONE*, vol. 8, no. 2, p. e57923.

Veyisoglu, A, Carro, L, Cetin, D, Guven, K, Spröer, C, Pötter, G, … Goodfellow, M 2016, '*Micromonospora profundi* sp. nov., isolated from deep marine sediment', *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 11, pp. 4735–4743.

Wang, D, Yuan, J, Gu, S & Shi, Q 2013, 'Influence of fungal elicitors on biosynthesis of natamycin by *Streptomyces natalensis* HW-2', *Applied Microbiology and Biotechnology*, vol. 97, no. 12, pp. 5527–5534.

Wang, QX, Bao, L, Yang, XL, Guo, H, Ren, B, Guo, LD, … Zhang, LX 2013, 'Tricycloalternarenes F-H: Three new mixed terpenoids produced by an endolichenic fungus *Ulocladium* sp. using OSMAC method', *Fitoterapia*, vol. 85, no. 1, pp. 8–13.

Wang, Y, Lu, Z, Sun, K & Zhu, W 2011, 'Effects of high salt stress on secondary metabolite production in the marine-derived fungus *Spicaria elegans*', *Marine Drugs*, vol. 9, no. 4, pp. 535–542.

Watanabe, T, Izaki, K & Takahashi, H 1982, 'New polyenic antibiotics active against Gram-positive and -negative bacteria. II. Isolation and purification of antibiotics produced by *Gluconobacter* sp. W-315', *The Journal of Antibiotics*, vol. 35, no. 9, pp. 1141–1147.

Watrous, J, Roach, P, Alexandrov, T, Heath, BS, Yang, JY, Kersten, RD, … Dorrestein, PC 2012, 'Mass spectral molecular networking of living microbial colonies', *Proceedings of the National Academy of Sciences of the United States of America*,

vol. 109, no. 26, pp. E1743–E1752.

Watve, MG, Tickoo, R, Jog, MM & Bhole, BD 2001, 'How many antibiotics are produced by the genus *Streptomyces*?', *Archives of Microbiology*, vol. 176, no. 5, pp. 386-390.

Weber, T, Blin, K, Duddela, S, Krug, D, Kim, HU, Bruccoleri, R, … Medema, MH 2015, 'AntiSMASH 3.0-A comprehensive resource for the genome mining of biosynthetic gene clusters', *Nucleic Acids Research*, vol. 43, pp. W237–W243.

Weber, T & Kim, HU 2016, 'The secondary metabolite bioinformatics portal: Computational tools to facilitate synthetic biology of secondary metabolite production', *Synthetic and Systems Biotechnology*, vol. 1, no. 2, pp. 69–79.

Weber, T, Rausch, C, Lopez, P, Hoof, I, Gaykova, V, Huson, DH & Wohlleben, W 2009, 'CLUSEAN: A computer-based framework for the automated analysis of bacterial secondary metabolite biosynthetic gene clusters', *Journal of Biotechnology*, vol. 140, pp. 13–17.

Wilson, KH, Blitchington, RB & Greene, RC 1990, 'Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction', *Journal of Clinical Microbiology*, vol. 28, no. 9, pp. 1942–1946.

Xing, H, Liu, C, Zhang, Y, Zhao, J, Li, C, Liu, H, … Xiang, W 2015, '*Plantactinospora veratri* sp. nov., an actinomycete isolated from black false hellebore root (*Veratrum nigrum* L.)', *International Journal of Systematic and Evolutionary Microbiology*, vol. 65, no. 6, pp. 1799–1804.

Xu, DB, Ye, WW, Han, Y, Deng, ZX & Hong, K 2014, 'Natural products from mangrove actinomycetes', *Marine Drugs*, vol. 12, no. 5, pp. 2590–2613.

Yamada, Y, Kuzuyama, T, Komatsu, M, Shin-ya, K, Omura, S, Cane, DE & Ikeda, H 2015, 'Terpene synthases are widely distributed in bacteria', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 3, pp. 857–862.

Yang, JY, Sanchez, LM, Rath, CM, Liu, X, Boudreau, PD, Bruns, N, … Dorrestein, PC 2013, 'Molecular networking as a dereplication strategy', *Journal of Natural Products*, vol. 76, no. 9, pp. 1686–1699.

Yang, SC, Lin, CH, Sung, CT & Fang, JY 2014, 'Antibacterial activities of bacteriocins: Application in foods and pharmaceuticals', *Frontiers in Microbiology*, vol. 5, p. 241.

Yeo, TC, Naming, M & Manurung, R 2014, 'Building a discovery partnership with Sarawak Biodiversity Centre: A gateway to access natural products from the rainforests', *Combinatorial Chemistry & High Throughput Screening*, vol. 17, no.

3, pp. 192–200.

Yoon, V & Nodwell, JR 2014, 'Activating secondary metabolism with stress and chemicals', *Journal of Industrial Microbiology and Biotechnology*, vol. 41, no. 2, pp. 415–424.

Zerikly, M & Challis, GL 2009, 'Strategies for the discovery of new natural products by genome mining', *ChemBioChem*, vol. 10, no. 4, pp. 625–633.

Zhang, H, Zhang, W, Jin, Y, Jin, M & Yu, X 2008, 'A comparative study on the phylogenetic diversity of culturable actinobacteria isolated from five marine sponge species', *Antonie van Leeuwenhoek*, vol. 93, no. 3, pp. 241–248.

Zhang, Y, Huang, H, Xu, S, Wang, B, Ju, J, Tan, H & Li, W 2015, 'Activation and enhancement of Fredericamycin A production in deepsea-derived *Streptomyces somaliensis* SCSIO ZH66 by using ribosome engineering and response surface methodology', *Microbial Cell Factories*, vol. 14, p. 64.

Zhi, XY, Li, WJ & Stackebrandt, E 2009, 'An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class *Actinobacteria*, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa', *International Journal of Systematic and Evolutionary Microbiology*, vol. 59, no. 3, pp. 589–608.

Zhu, H, Sandiford, SK & Van Wezel, GP 2014, 'Triggers and cues that activate antibiotic production by actinomycetes', *Journal of Industrial Microbiology and Biotechnology*, vol. 41, pp. 371-386.

Zhu, WY, Zhao, LX, Zhao, GZ, Duan, XW, Qin, S, Li, J, … Li, WJ 2012, '*Plantactinospora endophytica* sp. nov., an actinomycete isolated from *Camptotheca acuminata* Decne., reclassification of *Actinaurispora siamensis* as *Plantactinospora siamensis* comb. nov. and emended descriptions of the genus *Plantactinospora* and *Plantactinospora mayteni*', *International Journal of Systematic and Evolutionary Microbiology*, vol. 62, no. 10, pp. 2435–2442.

Ziemert, N, Alanjary, M & Weber, T 2016, 'The evolution of genome mining in microbes - a review', *Natural Product Reports*, vol. 33, no. 8, pp. 988–1005.

Zotchev, SB 2012, 'Marine actinomycetes as an emerging resource for the drug development pipelines', *Journal of Biotechnology*, vol. 158, no. 4, pp. 168–175.

Zotchev, SB, Sekurova, ON & Katz, L 2012, 'Genome-based bioprospecting of microbes for new therapeutics', *Current Opinion in Biotechnology*, vol. 23, no. 6, pp. 941–947.