Bioactive Compounds against Multidrug Resistant Bacteria Produced by an Endophytic *Actinomadura* sp.

by

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SUMMARY

Antibiotics are remain the frontline drugs to treat infectious diseases. However, their efficacy has been eroded by the increasing number of resistant and multidrug resistant pathogenic bacteria. Resistance to antibiotics has posed clinical, economic and social burdens as it increases morbidity as well as mortality, billions of dollars more in healthcare cost and loss of productivity. Unfortunately, even though a number of antibiotics are close to approval, the antibiotic pipeline for new drug applications is getting dry. Therefore, greater efforts to search for novel antibiotics are crucial to counter resistance.

Actinobacteria are proven as prolific producers of antimicrobial compounds and will continue to deliver new ones. As the main habitat of actinobacteria, soil has been screened massively for decades leading to the rediscovery of known compounds. Therefore, strategies are essential to the successful screening for new antimicrobial compounds. In an effort to discover novel antibiotics active against multidrug resistant bacteria, screening was conducted in the present study using unexploited samples and a well-defined strain with multidrug overexpressing efflux pumps as the target. The screening strategy employing unexplored samples and a novel target was recommended to avoid the discovery of known compounds.

Isolation of actinobacteria from Australian trees has not been done as extensively as herbaceous plants, and are one of the unique niches of actinobacteria and relatively unexplored. Therefore, the opportunity to obtain uncommon actinobacteria from trees that potentially produce novel antibiotics is greater. Roots, leaves and stems are part of plants commonly used as sources of isolations. In this study, the isolation sources were wood from deep within the trunks of trees. A rational approach to improve the isolation of actinobacteria was applied by extending the incubation of isolation plates for 16 weeks, using isolation media with low nutrients, and plating more samples on a larger number of isolation plates.

A total of 59 endophytic actinobacteria were isolated from wood of Australian eucalyptus trees- Sydney blue gum (*Eucalyptus saligna*), Lemon scented gum (*Corimbia citriodora*) and a tentatively classified eucalyptus as stringy bark (*Eucalyptus obliqua*). Most of them (51) were obtained from wood powder of stringy bark eucalyptus. The significant difference in the number of isolated actinobacteria among the three trees was proposed to correlate with the form of wood sample. The powder gave a larger surface area than the wood shavings thus showing a greater probability that more endophytic actinobacteria residing in the wood would grow on the isolation media. Powdering the wood also assisted in getting the actinobacteria out of the intercellular spaces.

Diverse genera of actinobacteria were identified among 21 isolates, including *Streptomyces*, *Asanoa*, *Actinoplanes*, *Actinoallomurus*, and *Actinomadura*. It is interesting that four of them are rare genera. In addition, 7 isolates are likely to be new species and two of them belong to the genus of *Streptomyces*. The polyphasic taxonomy of three selected isolates designated as DG1, AA9.2 and AA9(a)10 demonstrated that they are different from their closest type strains, *Actinomadura citrea*, *Streptomyces niveus*, and *Streptomyces alboniger*, respectively. However, DNA-DNA hybridization is required to confirm their novelty. This finding suggested that wood of native trees that represents a unique niche of actinobacteria are potential sources for the isolation of uncommon actinobacteria, including the slow emerging *Streptomyces*.

The antimicrobial activity test using whole cell target based assay revealed that strain DG1 that is an *Actinomadura* species produced active compounds against multidrug resistant *S. aureus* overexpressing *NorA* efflux pump. Early dereplication based on HPLC/DAD analysis showed that strain DG1 was predicted to produce unknown compounds worthy of further investigation. Active compounds were detected on Thin Layer Chromatography (TLC) plates after mycelial extract from scaled up submerged fermentation was purified using High Performance Centrifugal Partition Chromatography (HPCPC). However, it was difficult for further purification due to its instability and resultant low quantity of active compound. More stable active compounds were detected in the agar culture derived extract. Based on TLC patterns, the active compounds produced in solid-state

fermentation were different from those produced in submerged fermentation but retained their higher activity against the multidrug resistant *Staphylococcus aureus* compared to the sensitive strain. It is interesting that the LC/MS of the ethyl acetate extract after liquid partition of solid fermentation derived extract showed the presence of stable active compounds with unique UV spectra compared to a data base of compounds produced by actinobacteria. Larger up scale of the solid state growth conditions are required to generate sufficient pure compounds for structure elucidation.

Despite the lack of structure elucidation of the pure active compounds, this study suggested that uncommon actinobacteria are potential producers of novel antimicrobial compounds to reverse antibiotic resistance. The whole cell-target based screening employing a well-defined resistant strain overexpressing efflux pumps led to the detection of potentially new antibiotics active against multidrug resistant bacteria. The isolation of actinobacteria from overlooked habitats has enormous promise for the discovery of new antibiotics, subsequently allowing the antibiotic pipeline to flow once more.

DECLARATION

I certify that this thesis does not contain without acknowledgement any material previously accepted for a degree or diploma in any university or other tertiary institution, and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference has been made in the text.

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CHAPTER 1

LITERATURE REVIEW

1.1 Antibiotic resistance: Its emergence and spread

The use of antibacterial agents in the 1950s has significantly influenced the development of modern infectious disease therapy. Antibacterial agents have become life saving for the previously life-threatening and untreatable infectious diseases (O'Connell *et al.*, 2013, Butler *et al.*, 2017). Therefore, it was thought that infectious diseases had been defeated until the emergence of many resistant pathogenic bacteria was realized (Donadio *et al.*, 2002). In fact, the resistance to antibiotics has been observed since the early 1940s (Levy and Marshall, 2004). Resistance to antibiotics limit their clinical lifetime, as it occurs successively after the introduction of antibiotics in the clinical setting. The emergence of significantly clinical resistance varies between antibiotics. Resistance to penicillin was identified 5 years after it reached the market while chloramphenicol, streptomycin, and vancomycin had longer lifetimes of between 10-20 years before resistance to these agents was detected. However, daptomycin that was launched in 2005 showed resistance rapidly in about a year (O'Connell *et al.*, 2013).

Resistance is thought to exist intrinsically in bacterial species (Cox and Wright, 2013). Over hundreds of millions of years, bacteria have been exposed to a large variety of antibiotics and their derivatives. They have developed elegant mechanisms to encounter toxic compounds produced by fungi, plants and other organisms (Wright and Poinar, 2012). Thus, the genes responsible for antibiotic resistance were present before the introduction of antibiotics as was proved by the work of D'Costa *et al.* (2011). In addition to intrinsic resistance, bacteria have the ability to acquire transferable resistance genes that are present on plasmids, transposons, and other genetic elements (Debahov, 2013). These genes can be transferred between bacteria from the same or different species (Hooper, 2005).

The resistance to antibiotics arises when an antimicrobial compound makes contact with bacteria conferring resistant genes selected by the antimicrobial. The susceptible bacteria will be inhibited

whereas the resistant ones will be selected. Continuous antimicrobial selection increases and spreads the selected resistance genes and the hosts (Levy and Marshall, 2004).

It was proposed that exposure to antibiotics is the most important factor for the emergence and spread of antibiotic resistance. The level of antimicrobial exposure determines the pharmacokinetic and pharmacodynamics of the drug which can influence the elimination of the pathogen or the selection of resistant strains in the patient (Andersson and Hughes, 2010). The emergence and the spread of antibiotic resistance also has links with antibiotic use in the hospital and clinical settings (Canton and Morosini, 2011). The enormous usage of antibiotics kills the susceptible strains, but not the resistant ones. Thus, the mechanism of antibiotic resistant selection decreased when the use of antibiotic is restricted (Levy and Marshall, 2004).

1.2 The clinical and economic impact of antibacterial resistance

In general, resistant bacteria-associated infections often result in the significantly higher adverse outcomes compared to infections caused by susceptible bacteria. Clinically and economically adverse outcomes could arise due to antibiotic resistance. The clinically negative outcomes include either death or treatment failure while the economic negative outcomes stem from either treatment cost or length of stay (LoS) (Friedman *et al.*, 2016).

The clinical outcomes, i.e. treatment failure due to antibiotic-resistant pathogens could arise from different factors including the adjustment of pathogenic bacterial fitness because of the resistance genes, antibiotic resistant pathogens could cause delays in the administration of antibiotics. The population of resistant pathogens in the clinical setting correlate with the resistant genes. They are mostly the ones that are capable of surviving and spreading in the environment with high density of antibiotics (Cosgrove and Carmeli, 2003). The work of Zilberberg *et al.* (2014) revealed that multi-drug resistance (MDR) is an important determinant of inappropriate initial antibiotic therapy which is responsible for the increase up to three fold in the risk of hospital mortality. This inappropriate initial therapy includes inappropriate choice of therapy (Zilberberg *et al.*, 2014) and delay in the institution

of antibiotic therapy when empirical antibiotic therapy does not show the expected susceptibility results (Zilberberg *et al.*, 2014, Cosgrove and Carmeli, 2003)

In the case of nosocomial bacteremia caused by *Acenitobacter baumanii*, it was revealed that patients infected with MDR *A*. *baumanii* had a higher mortality rate, acquired greater care cost and longer hospital stay compare to those infected with susceptible *A*. *baumanii*. Using a match cohort study, the infections due to MDR *A*. *baumanii* led to 13.4 days longer hospitalization, the additional of $896 and $3,786 for antibiotic medication cost and hospitalization cost respectively (Lee *et al.*, 2007). Similarly, study of the negative impact of antibiotic resistance in MDR *Pseudomonas aeruginosa* demonstrated that MDR P. *aeruginosa* associated disease resulted in longer hospital stay, almost twice higher hospital cost, more than 3 fold higher medicinal cost and significantly higher proportion of in hospital mortality (Morales *et al.*, 2012).

1.3 Addressing antibiotic resistance

1.3.1 The need for novel antibiotics

Infections due to resistant bacteria is increasing around the world (CDC, 2013). This is worsened by the occurrence of multi-drug resistant bacteria where bacteria are resistant to more than one type of antibiotics. The resistant and multi-drug resistant bacteria spread very quickly causing a remarkable public health problems (Cars *et al.*, 2011). No less than 2 million people per year in the United States are infected with bacteria that are resistsnt to one or more antibiotics indicated for those infections. It was estimated that these resistant-antibiotic infections caused the death of at least 23 thousand people yearly. Other conditions complicated by infections due to resistant bacteria also led the death of many others (CDC, 2013)

A wide range of resistant/multidrug resistant Gram positive as well as Gram negative bacteria to currently available antibiotics have been reported. According to the World Health Organization (WHO), more than 50% *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* strains are resistant (WHO, 2014). In the United State, many other bacteria were found to confer resistance. Considering their threat to public health these resistant bacteria were classified into three groups

based on the level of concern. The first group was categorized as an urgent threat where the bacteria cause an immediate threat to public health thus urgent and aggressive action are mandatory. Included in this group are *Clostridium difficile*, carbapenem-resistant enterobacteriaceae, and drug resistant *Neisseria gonorrhoeae*. The second group was of serious concern and consists of 12 resistant bacteria including multidrug-resistant *A*. *baumanii*, Extended Spectrum β-lactamase producing Enterobacteriaceae (ESBLs), Vancomycin Resistant Enterococcus (VRE), multi-drug resistant *Pseudomonas aeruginosa*, and methicillin resistant *Staphylococcus aureus* (MRSA). Continuous actions are needed to avoid the development of resistance. The last group consisting of Vancomycin resistant *Staphylococcus aureus* (VRSA), Erythromycin resistant group A *Streptococcus*, and Clindamycin-resistant group B *Streptococcus* are at the level of concern where careful surveillance and anticipation are required (CDC, 2013).

Resistance to β-lactam antibiotics is mostly mediated by the enzymatic hydrolysis of these compounds (Bush and Jacoby, 2010). Following the increase in the number of β-lactamase producing strains (Bush and Jacoby, 2010), the resistance has increased significantly in the past two decades (Bush, 2010). Based on the substrate specificities, β-lactamases are classified into 4 main groups: penicillinases, AmpC type cephalosporinases, extended spectrum β-lactamase (ESBLs), and carbapenemases. Carbapenem-resistant enterobacteriaceae (CRE) are one of the most difficult causes of infections to treat. The prevalence of CRE is increasing worldwide (Livermore *et al.*, 2011, van Duin *et al.*, 2013). This bacteria cause about 9000 infections and led to 600 deaths in the United State (CDC, 2013). Livermore *et al.* (2011) evaluated the susceptibility of 81 CRE isolates from the UK against a number of antibiotics and found that more than 75% were resistant to chloramphenicol, ciprofloxacin and nitrofurantoin. Tigecycline was active, but resistance was found in about 50% of isolates, so limited choice of treatment for CRE is available at present. CRE in general is still susceptible to polymyxins, some aminoglycosides, and tigecycline *in vitro*, which are considered as the drugs of last resort. However, their use in clinical practice is restricted due the adverse profiles and uncertain efficacy *in vivo* (van Duin *et al.*, 2013).

Multi-drug resistant *Pseudomonas aeruginosa* is another Gram negative bacterium that produces ESBLs to confer resistance. *P*. *aeruginosa* causes a number of healthcare related infections such as pneumonia, bloodstream infection, urinary tract infection, and surgical site infection. Resistance to almost all antibiotics including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems have been detected in some strains. More than 13% of healthcare associated pseudomonas infections were due to multidrug resistance causing about 400 deaths each year (CDC, 2013). A study on the prevalence of multidrug resistant *P*. *aeruginosa* producing ESBLs in a tertiary care hospital showed the occurrence of resistance in his bacteria to the third generation of cephalosporin, cefotaxime, ceftazidime, cefazolin, cefepime, cepoferazone, and ceftriaxone, with the highest resistance was detected against ceftazidime. Tigecycline, a tetracycline antibiotic launched in 2005 (Butler *et al.*, 2017) also seemed to be less effective against this MDR bacteria as 70% resistance to this antibiotic was detected.

Among Gram positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most recalcitrant bacteria to antibiotic therapy. It developed resistance to β-lactam antibiotics soon after the establishment of penicillin, and further resistance to methicillin. By 2003, MRSA was found in more than 50% of *S. aureus* recovered in U.S hospitals. *S. aureus* then continued to develop resistance to the newer antimicrobial agents. Mediated by undefined mutations, it evolved to the so-called Vancomycin intermediate-resistant *Staphylococcus aureus* (VISA). The isolate has low-level resistance to vancomycin, a glycopeptide antibiotic. Next generation of true methicillin resistant MRSA arose later with high-level resistance to vancomycin designated as vancomycin resistance *Staphylococcus aureus* (VRSA). It was not susceptible anymore to various antibiotics such as clindamycin, aminoglycosides, trimethoprim-sulfamethoxazol, rifampicin, and fluoroquinolones (Arias, 2009). MRSA has become the more common cause of healthcare-associated infections worldwide. MRSA produce β-lactamase to break down β-lactam antibiotics (Arias, 2009) and all MRSA strains can produce these enzymes. They also are armed with low affinity Penicillin Binding Protein (PBP) 2a (Arias, 2009) encoded by the *mecA* gene that has been acquired from unknown sources causing resistance to all available β-lactam antibiotics (Fuda *et al.*, 2004).

Duplessis and Crum-Cianflone (2011) reviewed that ceftaroline, a new fifth generation cephalosporin, demonstrated potent activity against resistant *S. aureus* including MRSA-conferring PBP2a. VISA, hVISA, VRSE, and daptomycin resistant strains as well as linezolid resistant strains were also susceptible to ceftaroline. Recently, however, high level ceftaroline resistance MRSA was isolated from a patient with cystic fibrosis. Further analysis showed that mutation occurred at PBP2a, generating MRSA resistance to ceftaroline (Long *et al.*, 2014).

Bacteria seem to find ways to encounter antibiotics (Arias, 2009). The development of resistance in *Staphylococcus aureus* described previously showed how bacteria could deal with newer antibiotics. In addition, effective antibiotics are crucial for current surgery, transplantation, cancer chemotherapy and severe HIV-associated infections. However, resistance to antibiotics has limited the availability of effective antibiotics (Levy and Marshall, 2004). This suggests that new antimicrobial agents are needed to overcome resistance. Infectious Diseases Society of America (IDSA) declare 10 X '20 initiative to develop 10 new, safe and effective antibiotics through either the discovery of new drug classes or modifying the existing classes to deliver possible new drug (IDSA, 2010). Between 2000 and 2015, 30 new antibiotic approvals have been made, six of them represent new class of antibiotics (Butler *et al.*, 2017). Among the six new classes, 3 classes were approved since 10 x '20 initiative was launched. Thus, continuing effort in drug discovery has to be done to fill the antibiotic pipeline and to realize the 10 x '20 initiative. This is expected to finally fulfil the demand for new antibiotics to overcome resistance.

1.3.2 Rejuvenating ineffective existing antibiotics

Reviving the activity of existing antibiotics that have lost their activity due to resistance is another potential way to overcome antibiotic resistances (Poole and Lomovskaya, 2006, Lynch, 2006, Lomovskaya and Bostian, 2006). Blocking the activity of efflux mechanisms will allow the antibiotics which are substrates for certain efflux systems to enter the cells and reach their target site to exert the activity. Thus, efflux pump inhibitors should be used in combination with antibiotics to restore their activity, despite the existence of efflux pump system (Poole and Lomovskaya, 2006) .

Efflux pump inhibitors (EPIs) enable inhibition of the efflux activity at different targets including the substrate-binding site, pump modulation site, proton transport, exit pore, pump assembly, and efflux gene expression. By targeting the substrate binding site, EPIs act as competitive inhibitors, so it possibly will only potentiate a group of substrate antimicrobials. On the other hand, such EPIs might inhibit different efflux pumps that have the same substrate, and resistance to such compounds is less likely to develop. In contrast to EPIs that target substrate binding sites, the activity of all antimicrobial substrates of a pump are estimated to increase by targeting the pump modulation site. In this case, the EPIs do not act at the binding site, but they are able to disturb the whole pump activity. Pump modulation may develop resistance to inhibitors. Efflux pumps expel drugs using cellular energy generated from a proton gradient, i.e. drug transports are coupled to proton movement. Efflux pump should be inactivated by disconnecting the drug efflux from proton influx. Targeting the pump assembly is specifically directed to the Resistance Nodulation Division (RND) family that is build up by a cytoplasmic membrane unit, a channel forming outer membrane unit and periplasmic adapter that connect the two other units. The disruption of this system to integrate would block the pump activity.

Two methods can be employed to discover EPIs, chemistry based programs and cell based screening. A chemistry based program exploits the substrate specificity character of antibiotics for the efflux pump. EPIs can be derived from an antibiotic scaffold so they have a similar structure with certain antibiotics, but lack of antibiotic activity and can be used in combination with other antibiotics from the same group (Lynch, 2006). However, there would be a challenge to develop such EPIs for clinical application when a number of efflux pump sub-families are present (Marquez, 2005). Cell based screening involved growing bacteria in the presence of efflux pump substrates at the concentration under the minimal inhibitory concentration (MIC) (Kumar *et al.*, 2008) as well as compounds from a chemical library or a natural product extract. The decrease in the MIC value indicates the potentiation of an antibiotic due to the presence of an efflux pump inhibitor.

Intensive studies have been done towards the discovery of EPIs for Gram positive multi drug resistant bacteria. *Staphylococcus aureus* overexpressing *NorA* efflux pump are generally used in

pairs with the wild type parents as test organisms. The rational of targeting NorA efflux pump is the frequent existence of this pump in clinical isolates of *S. aureus*. A greater synergistic activity against resistant strains compared to the parent indicates the efflux pump inhibition (Schindler *et al.*, 2013). Many compounds originating from natural products and biosynthesis have been found demonstrating inhibitory activity on the NorA efflux pump. Among natural EPIs are polyphenolic amide, porphyrin, diterpenes and flavonoids whereas the synthetic ones include piperine analogs, fluoroquinolone analogs, indole based compounds, substituted dihydronaphtalene and mesoionic compounds. These EPIs have been reviewed in detail by Schindler *et al.* (2013)

The search for compounds that inhibit efflux pumps in Gram negative bacteria is increasing nowadays (Piddock *et al.*, 2010). Garvey *et al.* (2011) and Noumedem *et al.* (2013) detected that medicinal plant extracts and vegetable extracts, respectively, potentiate antibiotic activity against multidrug resistant Gram negative bacteria. Some identified EPIs for Gram negative bacteria have been reviewed in Kourtesi *et al.* (2013). Most recent, punigratane, a novel pyrrolidine alkaloid isolated from *Granatum rind*, was reported to inhibit the efflux pump of *Klebsiella pneumoniae* (Rafiq *et al.*, 2016). Opperman *et al.* (2014) synthetized a pyranopyridine EPI, MBX2319, an RND efflux pump modulator.

Various compounds inhibiting efflux pumps have been identified and this inhibition could restore and enhance the efficacy of ineffective antimicrobials. The combination of EPIs and antibiotics to overcome multi-drug resistant bacteria was affirmed *in vitro*. Nevertheless, no EPIs so far has been approved for clinical application. Co-administration of an EPI, MP-601,205 with fluoroquinolone to treat infection due to fluoroquinolone resistant bacteria in cancer disease has undergone preclinical trials. However, it was discontinued because of adverse effects arising from the combination therapy (Schindler *et al.*, 2013). The most challenging task to introduce the EPIs-antimicrobials combination therapy in the clinic is to modify the pharmacokinetics of both agents to obtain the maximum pharmacodynamic effects as well as minimizing the toxicity (Lomovskaya and Bostian, 2006, Lomovskaya *et al.*, 2007).

1.4 Antibiotic sources and development: Natural product scaffolds or Synthetic molecule scaffolds?

Antibiotics could be derived from natural products or synthetic molecules (Walsh, 2003, Singh and Barret, 2006). In fact, natural products have played a major role in antibiotic discovery that are currently used for therapeutic purposes (Singh *et al.*, 2011, Butler, 2005). However, in general in the 1990s big pharmaceutical companies diminished their efforts to mine natural products for new drug discovery. This is because natural products are present only in small amounts, they have complex molecular structures, and discovery of drugs from natural products has rediscovery issue with large numbers of known compounds. Despite these limitations, "the more important quantifiable advantages" of natural product scaffolds will keep them as the main source for new drug leads (Lam, 2007).

The first limitation of natural products for the source of drug discovery is that they are frequently synthesized in small amounts. A bigger quantity of natural products is required for preclinical development done by fermentation and this could prolong the time line in developing new drugs (Lam, 2007, Ortholand and Ganesan, 2004). The low yield of natural products often does not meet the economical requirement for industrial scale production (Huang, 2012).

Nevertheless, the advances in fermentation optimization and genetics are now able to address the problem. Instead of classical fermentation optimization, more advanced fermentation optimizations using sequential statistical design experiments have now been developed to achieve a better improvement of natural product yield (Shi *et al.*, 2006, Ren *et al.*, 2008, Mao *et al.*, 2007). Shi *et al.* (2006) studied the optimization of γ-polyglutamic acid production by *Bacillus subtilis* ZJU-7 using Respond Surface Methodology. In this study, a Fractional Factorial Design was applied to screen the significant factors for γ -polyglutamic acid production. The results were then subjected to Central Composite Design for media formulation. Two-fold increase of γ-polyglutamic acid production was observed in this study. A similar strategy to optimize fermentation medium was used by Ren *et al.* (2008) and Zhang *et al.* (2010) to enhance the bacterial nitrite oxidizing rate and the production of tallysomycin H-1 respectively. They applied Central Composite Design for medium optimization, but

instead of Fractional Factorial Design, a single factor optimization analyzed by Pluckett-Burman Design was used to screen the significant fermentation factors. The bacterial nitrite oxidizing rate and tallysomycin production increased 48% and 13 fold, respectively.

The yield of natural products can also be improved through the advanced in genetics. The biosynthetic pathway of antibiotics of interest can be manipulated genetically to increase the yield (Lam, 2007). It is known that the availability of biosynthetic precursors plays an important role in the biosynthesis of secondary metabolites and determines their production level. These precursors are mainly generated from the catabolism of various carbon substrates. Therefore, manipulating the genes encoding the key enzymes involved in metabolic network of carbon flux could increase the production of specific secondary metabolites such as antibiotics.

Cofactors (additional elements) of primary carbon metabolism could also be the targets for metabolic engineering due to their function as precursors for secondary metabolite biosynthesis (Olano *et al.*, 2008). Wang *et al.* (2007) studied the effect of heterologous gene expression encoding S-adenosylmethioninesynthetase (SAMs) on the production of erythromycin A, a clinically important macrolide antibiotic, by *Saccharopolyspora erythraea*. SAMs catalyzed the biosynthesis of S-adenosylmethionine (SAM) that is proposed to be an important factor for cell differentiation and antibiotic production in *Streptomyces* sp. This study revealed that the integration of the SAM gene from *Streptomyces spectabilis* into the chromosome of a *S. erythraea* enhanced the production of erythromycin from 920 IU/ml to 2000 IU/ml. The increased production of intracellular SAM in a *S. erythraea* recombinant strain promotes the methylation process of *ery B* to *ery A* and enhances *eryA* production. A similar study conducted by Zhao *et al.* (2010) showed that the overexpression of SAM genes in a heterologous novobiocin producer strain *Streptomyces coelicolor* increased the concentration of intracellular SAM significantly in the antibiotic production phase. The production of novobiocin by the heterologous strain is also enhanced up to 50% compared with the control strain.

The small amount of natural products synthesized naturally makes it present as minor constituent in the complex mixture of extracts consisting of hundreds different compounds. The extracts are

commonly prepared by extraction of fresh or dried material and medium after the fermentation process using different solvents prior to isolation and purification of specific compounds (Ortholand and Ganesan, 2004, Walsh, 2003). Bioassay Guided Fractionation is done to purify and identify the active compounds (Inui *et al.*, 2012). It is a time consuming process from an industrial point of view and often incompatible with the current "blitz" of screening campaign (Ortholand and Ganesan, 2004). This step may also lead to loss of identified potential activity. A compound could be irreversibly adsorbed by the solid phase in the separation process during the isolation (Inui *et al.*, 2012).

This problem, however, is now able to be addressed as extract preparation and bioassay guided fractionation have been automated. In addition, instead of using crude extracts, it is possible to employ fully or partially purified material straightaway for further analysis. The loss of activity during isolation and purification can now be lessened by applying a biochemometrics method. This method can also examine multiple active compounds in a complex mixture of natural products without previous isolation and structure elucidation (Inui *et al.*, 2012).

The second problem of natural products for drug discovery is due to their molecular structures that are commonly large and high in "sterical complexity". They tend to have more chiral centres (Ortholand and Ganesan, 2004), and have many functional groups to be protected during the synthesis that make it a challenging task to modify natural products by organic chemistry. It is easier to generate many synthetic chemical analogues than natural product analogues in the time same frame (Lam, 2007).

However, with their complex structure, natural products provide unrivalled diversity. Natural products can become sources of promising scaffolds as starting points in drug discovery due to their exquisite level of three-dimensional sophistication, the diversity of ring skeletons and the way their topological functional groups are present which is beyond the present capacity of medicinal chemistry (Lam, 2007, Ortholand and Ganesan, 2004). The recent advances in organic synthetic methodologies, biotransformation, combinatorial biosynthesis, and combination of these techniques have successfully derived and modified natural products by total synthesis in a shorter period of time.

The third limitation of natural products as sources for drug discovery is the experience of rediscovery of known compounds in the screening step. However, it is not because natural products are not a good source for drug discovery, but more innovative strategies are needed to mine natural products. Natural products have played an important role in the discovery and development of antibiotics that have been introduced into clinical use (Singh *et al.*, 2011). Many classes of antibiotics were discovered and derived from natural products during the 'Golden era" of antibiotic discovery (1940-1962) (Singh and Barret, 2006). After that, however, the rediscovery of known compounds has become a major problem in the screening efforts of antibiotics from natural products. The question remains: Have natural products as source of antibiotics become exhausted after being explored for more than half a century?

The majority of antibiotics are produced by actinobacteria (Berdy, 2005). These filamentous bacteria are one of the major inhabitants of the soil. It is estimated that one gram of soil harbor more than 1 million actinobacteria (Goodfellow and William, 1983). Soil actinobacteria have been explored intensively especially by the pharmaceutical industry for more than half a century (Baltz, 2008) because many of them produce antibiotics, including those that have a significant role in a clinical setting such as tetracycline, erythromycin, vancomycin, ß lactams, cephalosporin and rifampicin (Goodfellow and William, 1983, Osburne *et al.*, 2000).

Even though antibiotic producing actinobacteria are abundant in soil, the antibiotic metabolic pathways are present in different proportions. Some of them exist at higher frequency than others. Streptothricin was found in approximately 10% of random soil actinobacteria. The streptomycin metabolic pathway is present at around 1%, whereas tetracycline and actinomycin are present at the same proportion of 0.1% (Baltz, 2007). During the intensive period of screening, the antibiotics at higher frequency were reisolated as it is easier to isolate actinobacteria with the more abundant metabolic pathways compared to those that are less frequent, resulting in the rediscovery of known compounds (Payne *et al.*, 2007, Baltz, 2007). Nevertheless, there are still great possibilities to screen novel antibiotics produced by actinobacteria as only around 10^7 out of the10^{25}-10^{26} global soil actinobacteria have been screened for antibiotic activity in the past 50 years. It is estimated that

many different antibiotics at lower frequency wait to be discovered. To avoid rediscovery, at least three strategies should be taken into account: increase the throughput, screen only actinobacteria that are less explored, high throughput screening coupled with antibiotic selection (Baltz, 2006). In addition, dereplication by which the chemical and biological characteristics of the unknown compounds are compared with those of known compounds that have been identified previously, have to be conducted effectively using a fully integrated bioinformatics system (Berdy, 2005).

Natural products, with their characteristics, will continue to be the key in the search for novel antibiotics. In fact, only 3 classes of currently used clinical antibiotics have originated from synthetic molecules: oxazolidinones, quinolones and sulfonamides **(Singh and Barret, 2006, Singh *et al.*, 2011)**.

1.5 Actinobacteria: prolific producers of antibiotics

Actinobacteria are recognised as prolific producers of antibiotics. They are one of the major phyla in the domain bacteria and consist of a diverse group of species with a range of spore morphologies, physiologies, and metabolic characteristics. Thus, they are able to live in various environmental settings. At least 53 actinobacteria genomes have been sequenced completely and annotated. They have a big genome size between 1.93 Mb to 10.15 Mb consisting of 1605 to 8983 genes that encode proteins. Based on the homology search of the genes encoding for known proteins responsible for secondary metabolisms, Actinobacteria have a huge diversity of secondary metabolic gene clusters of up to 30 pathways. Microbes with small genome size between 2-4 Mb are unproductive for secondary metabolites (Nett *et al.*, 2009)

1.5.1 *Streptomyces* group

Within the order of Actinomycetales, the genus *Streptomyces* is the most prolific producer of various bioactive compounds, including antibiotics (Watve *et al.*, 2001, Marinelli, 2009). It also plays an important role in drug discovery as a rich source of lead compounds (Weber *et al.*, 2003). Over 10,000 bioactive secondary metabolites are produced by the order of Actinomycetales and about 76% of them are derived from *Streptomyces* (Berdy, 2005). According to the Antibiotic Literature

Database (ABL) that provides information on microbial products since 1900, actinobacteria produce around 61.6 % of the more than 8000 antimicrobial agents listed in the database, followed by fungi and bacteria that produce 21.5% and 16.9% of the antimicrobial compounds, respectively. Almost three-quarters (45.6%) of antimicrobial agents produced by actinobacteria are derived from the genus of *Streptomyces* and the rest (16%) are produced by other genera of actinobacteria, often called rare actinobacteria.

Continuous efforts have been made to screen antibiotics from *Streptomyces* since the discovery of streptomycin in 1942. It is easier to isolate *Streptomyces* from soil compared to other actinobacteria as they are present ubiquitously in soil. Following the discovery of streptomycin more new antibiotics were discovered from *Streptomyces*, so the chances of finding novel antibiotics from this genus decreases (Lazzarini *et al.*, 2000, Marinelli, 2009). However, it is believed that the existing antibiotics originating from *Streptomyces* are only a small portion of antibiotics they produce (Chater, 2006), leaving a large number of compounds to be discovered. A mathematic model of antibiotic discovery from *Streptomyces* between 1947 and 1999 proposed by Watve *et al.* (2001) revealed an exponential increase in the number of compounds discovered for almost 17 years and it continued to increase, but at a lower linear rate before it reached a peak in 1970. The discovery rate started to decrease rapidly in the late 1980s and 1990s. Based on the model, the diminishing rate in new compounds discovery from *Streptomyces* is mainly due to the decrease in screening effort rather than the exhaustion of the compounds. It is estimated that the total number of antibiotics present in this genus is in the order of 150,000-294,300. A huge number of compounds are waiting to be discovered since only 3% of the existing compounds, approximately, have been reported so far (Watve *et al.*, 2001).

Streptomyces have large genomes of 8-10 Mb that usually consist of more than 20 gene clusters responsible for secondary metabolism. The genomes of least 4 *Streptomyces* have been completed (Nett *et al.*, 2009). The complete genome sequence of two *Streptomyces*, *S. coelicolor* and *S. avermitilis* also showed that this genus has the potential to generate diverse compounds as they have large genomes, 8.7 and 9.03 Mb, respectively. About 23 gene clusters encoding secondary

metabolism correspond to only 5% of the *S. coelilocor* genome. These genes encode the biosynthesis of actinorhodin, prodiginin, siderophores, and others that have been identified before (Weber *et al.*, 2003). Thus, *Streptomyces* will continue to have an outstanding capacity to produce new clinically useful secondary metabolites (Berdy, 2005).

1.5.2 Rare actinobacteria group

Rare or uncommon actinobacteria are refer to the genera other than *Streptomyces*. Up to September 2010 about 220 genera of actinobacteria have been recorded (Tiwari and Gupta, 2012), including the more common ones such as *Actinomadura, Actinoplanes, Amycolatopsis, Dactylosporangium, Kibdeosporangium, Microbispora, Micromonospora, Planobispora, Saccharomonospora, Saccharopolyspora, Streptosporangium* and *Planomonospora* (Lazzarini *et al.*, 2000).

As the chance to discover novel antibiotic from the massively screened *Streptomyces* group decreases, the focus of screening effort moves to the rare/uncommon actinobacteria group (Tiwari and Gupta, 2012, Lazzarini *et al.*, 2000) because this group of actinobacteria are less explored compared to *Streptomyces* group so the probability of finding novel compounds for drug discovery programs is predicted to increase (Donadio *et al.*, 2007, Baltz, 2006).

These actinobacteria also produce a number of antibiotics currently used clinically. Erythromycin, rifamycin, teicoplanin, vancomycin, and gentamicin are produced by *Saccharoplyspora erythraea, Amycolatopsis mediterrannei, Actinoplanes teichomyceticus, Amycolatopsis orientalis* and *Micromonospora purpurea,* respectively. This group is even predicted to generate the most unique, unprecedented, and occasionally very complex compounds having potent antimicrobial activity with low toxicity. Various classes of antibiotic compounds are produced by rare actinobacteria strains as reviewed by Tiwari et.al. (2012), showing their capability to generate diverse structures of antibiotics.

Between 1975 and 1980, when the majority of antibiotics were produced by the genus of *Streptomyces* (Watve *et al.*, 2001), only 5% of the compounds originated from rare actinobacteria (Nisbet, 1982). The number of antibiotics produced by this group increased in the next two decades and they share up to 25-30% of total antibiotics (Berdy, 2005). Unfortunately, it is not easy to isolate

rare actinobacteria, as well as to culture and maintain under conventional conditions. They often grow slower than the *Streptomyces* group and little is known about their genetics and physiology. Therefore, using conventional methods, only a small proportion of them can be isolated (Baltz, 2006, Tiwari and Gupta, 2012).

Selective isolation techniques have been developed to increase the recovery rate of rare actinobacteria. Nowadays, more than 50 rare actinobacteria taxa have been identified as producers of 2500 bioactive compounds compared to 11 in 1970. Only 50 bioactive compounds were derived from these 11 rare actinobacteria.

1.6 The strategies in the search for novel compounds produced by actinobacteria

The emergence of antibiotic resistance compels the continuing efforts to discover new antibiotics (Fischbach and Walsh, 2009, Walsh, 2003). However, in fact, the antibiotic pipeline is starving (Baltz, 2006). The number of new antibiotic has declined in the past decades. Only four new classes of antibiotics have reached the market in the last ten year: oxazolidinone (linezolid), lipopeptide (daptomycin), pleoromutilin (retapamulin), and macrolactone (fidaxomicin) (Jabes, 2011).

One of the reasons that cause antibiotic discovery unproductive is abandoning natural products as sources for antibiotic discovery (Baltz, 2006). Natural products provide high biodiversity of chemical structures with different activities that are important sources for drug or lead compounds. Among millions of new organic chemical structures that have been synthesized, only about 0.001% became approved drugs. In contrast, about 0.2-0.3 % of 10 thousand microbial products became clinically useful drugs and approximately the same percentage became successful lead compounds (Berdy, 2005).Therefore, disregarding natural products means excluding the source of high quality compounds to be screened which is one of the keys for success in pharmaceutical discovery programs (Baltz, 2006).

A feasible strategy to address the lack productivity in antibiotic discovery is to return to natural products due to their capacity to produce candidate molecules. One of the major problems to be

addressed in antibiotic discovery from natural products is the rediscovery of known compounds due to the intensive screening of soil actinobacteria. About 10 million or more soil actinobacteria have been screened for antibiotic production until 2005 and thousands of antibiotics have been discovered from more abundant actinobacteria (Baltz, 2006). These actinobacteria are easier to reisolate resulting in the discovery of the same compounds. Thus, strategies are important to find actinobacteria that produced novel compounds. Three strategies are proposed to avoid the rediscovery of known compounds: (1) develop a technique to exclude the discovery of known compounds in the screening process, (2) search for actinobacteria from new niches which are relatively unexplored (3) screen the slow growing actinobacteria due to their genetic capabilities to produce new compounds (Baltz, 2006, Fischbach and Walsh, 2009).

1.7 Endophytic actinobacteria: diversity and productive capacity for novel antibiotics

Higher plants are potential niches for actinobacteria (Qin *et al.*, 2011), called endophytic actinobacteria. These actinobacteria are able to colonize the inner tissues of the plants without causing any harm to the host. (Stone *et al.*, 2000). It was proposed that each plant harbors one or more endophytes. Only a few of the approximately 300,000 plant species on earth have been explored for their endophytes (Strobel and Daisy, 2003). Therefore, these habitats are relatively unstudied compared to soils.

Thousands varieties of plants from different ecosystems provides a great chance to find new and interesting actinobacteria. (Strobel and Daisy, 2003). Especially those that have ethnobotanical history, native to certain interesting places or have extreme longevity, grow in unique niche**s** are potential sources for novel endophytes (Strobel, 2003). These endophytes are sources for relatively unexplored chemically diverse bioactive compounds and are likely to produce the novel ones, including antibiotics (Strobel, 2003, Strobel and Daisy, 2003). Matsumoto and Takahashi (2017) studied the secondary metabolite biosynthetic genes based on the presence of non-ribosomal peptide synthetase (NRPS), polyketide synthetase (PKS) type I, and enediyne biosynthesis PKS (PKSE) genes in actinobacteria isolated from plant roots and soils. The prevalence of NRPS genes

in endophytic and soil *Streptomyces* was almost the same while the prevalence of PKSE genes in plant *Streptomyces* was significantly higher compared to soil *Streptomyces*. In contrast, it was surprising that the prevalence of all three genes was higher in non-*Streptomyces* endophytes compared to non-*Streptomyces* actinobacteria isolated from soil. These results indicated the richness of endophytic actinobacteria for secondary metabolites.

Studies on endophytic actinobacteria revealed that higher plants present great diversity of these group of microbes, despite the fact that the genus *Streptomyces* still dominates the population of endophytic actinobacteria isolated from various plants including medicinal plants (Qin *et al.*, 2009a, Zhao *et al.*, 2011, Huang, 2012, Tanvir *et al.*, 2014), tropical rainforest plants (Li *et al.*, 2008), crops (Coombs and Franco, 2003, Cao *et al.*, 2004, de Oliveira *et al.*, 2010, Tian *et al.*, 2007). Novel species belong to rare genera such as *Asanoa, Actinoallomurus, Actinoplanes, Pseudonocardia*, are isolated continuously. A number of novel *Streptomyces* species have also been found. Qin *et al.* (2011) reviewed that more than 40 new taxa have been identified which included 4 new genera, *Plantactinospora, Actinophytocola, Phytohabitans,* and *Jishengella*. Another new genus was found later designated as *Allonocardiopsis* (Du *et al.*, 2013). Qin *et al.* (2009a) identified at least 19 new taxa, 10 suborders and 32 genera of actinobacteria isolated from tropical rainforest medicinal plants. The wide diversity of endophytic actinobacteria was also shown by the work of Janso and Carter (2010) who identified 17 possible genera that belong to six families of actinobacteria isolated from tropical plants. It was interesting that most strains fall into the family *Streptosporangiaceae* and represented 5 genera: *Nonomuraea, Streptosporangium, Microbispora, Sphaerisporangium and Planotetraspora*. This also showed that plants growing in an area with great biodiversity, tropical rainforest, for example potentially have a wide variety of endophytic actinobacteria as proposed by Strobel and Daisy (2003). In addition, A number of genera were also found to commonly inhabit plant tissues, e.g. *Microbispora, Micromonospora, Nocardioides, Nocardia*, and *Streptosporangium* (Qin *et al.*, 2011)

Having a large taxonomic diversity, endophytic actinobacteria are resources for novel bioactive compounds, including antibiotics. This is based on the concept that unique compounds may be

produced by novel species because secondary metabolism is mainly strain-specific but sometimes species-specific; it can represent a taxonomic feature for a certain species as described by the case of *Salinispora* where the species level is discerned by the difference in the secondary metabolite produced by each species (Czaran *et al.*, 2002). It was also hypothesised that taxonomic diversity denotes chemical diversity due to chemical alteration for survival in the environment (Jensen, 2010).

Recent studies reveal the potential of endophytic actinobacteria to produce compounds active against a wide range of pathogens. To mention a few of them, Qin *et al.* (2009a) found 52% of actinobacterial isolates obtained from medicinal plants exhibit antimicrobial activities against *S. aureus, B. subtilis* and *P. aeruginosa*. Antibacterial or antifungal activity was detected in 56 out of 333 endophytic actinobacteria isolated from various plant roots. It was interesting that 50% of *Streptosporangium* species showed antibiotic activity, while only 25% of *Streptomyces* showed antibiotic activity (Matsumoto and Takahashi, 2017). Janso and Carter found that 56 out of 105 unique endophytic actinobacterial strains isolated from tropical plants inhibit the growth of at least one of the test organisms.

Furthermore, many novel antibiotics derived from endophytic actinobacteria have been successfully identified structurally. At least 21 of them have been reviewed by Qin *et al.* (2011), including Munumbicins A-D,Munumbicins E-4 and E-5, kakadumycins and coronamycins which were produced by endophytic *Streptomyces* isolated from medicinal plants. More recently, a number of novel antibiotics have also been isolated from endophytic *Streptomyces*. Three new indolsesquiterpenes antibiotics, Xiamycin A (Ding *et al.*, 2011), indosespene and sespenine were produced by endophytic *Streptomyces* sp. HK10595 isolated from a mangrove tree, *Kandelia candel*. All of them showed moderate to strong inhibition of growth of some bacteria including methicillin-resistant *Staphylococcus* and vancomycin-resistant *Enterobacteriacea*. Xiamycin A has been isolated before from endophytic *Streptomyces* of *Bruguiera gymnorrhiza*, a different mangrove tree (Ding *et al.*, 2010). Yang *et al.* (2015) identified a novel benzamide compound as 2-amino-3,4 dihydroxy-5-methoxybenzamide produced by *Streptomyces* sp. YIM 6708, an endophyte of *Dysophylla stellata* that has activity against *E.coli* and *Candida albicans.* The fermentation broth of

an endophytic *Streptomyces* sp.K15 isolated from *Houttuynia cordata* Thunb generated a major pyrrole compound active against pathogenic bacteria, designated as 2-pyrrole formic acid (Chen *et al.*, 2015). A novel isoflavone was extracted and purified from fermentation broth filtrate of an endophytic *Streptomyces* sp. YIM 65408. The compounds was a daidzen derivative identified as 1"-*O*-methyl-8-hydroxymethyl daidzein and has antifungal activity (Yang *et al.*, 2013). A new cyclic tetrapeptide compound showing antimicrobial activity was produced by an endophytic *Streptomyces* sp. YIM 67005. Based on the structure elucidation it was determined as cyclo(L-Tyr-L-Pro-L-Phe-trans-4-hydroxy-L-Pro) (Zhou *et al.*, 2014). Zhang *et al.* (2014) isolated a new prenylated indole derivative 3-acetonylidene-7-prenylindolin-2-one from an endophytic *Streptomyces* sp. neau-D50. This compound had a strong activity against phytopathogenic fungi.

Among 21 novel bioactive compounds produced by endophytic actinobacteria as reviewed by Qin *et al.* (2011), 20 of them originated from endophytic *Streptomyces*. However, a number of bioactive compounds other than antibiotics have been reported to be produced by non-*Streptomyces* endophytes, including compounds with antitumor, antiparasitic, and anti-HIV activities. A novel *Streptosporangium oxazolinicum* K07-0460^{T} an endophyte isolated from the root of an orchid produced Spoxazomicins A-C, novel antitrypanosomal alkaloids (Inahashi *et al.*, 2011b, Inahashi *et al.*, 2011a). Other new antitrypanosomal compounds were isolated later from *Actinoallomurus fulvus* MK10-036, an endophyte of *Capsicum frutescens* and were identified as Actinoallollides A-E (Inahashi *et al.*, 2015). Nakashima *et al.* (2013) isolated novel photo oxidative hemolysis inhibitors from the culture broth of *Polymorphospora rubra* K07-0510. The compounds were determined as trehangelins A, B, and C. *Micromonospora lupini* sp.nov. was found to produce antitumor anthraquinones, lupinacidins A and B (Igarashi *et al.*, 2007).

The studies mentioned above revealed that the probability to discover novel antibiotics and other bioactive compounds from endophytic actinobacteria is high. So far, endophytic *Streptomyces* species still dominate as the producer of novel antibiotics and other bioactive compounds. Nonetheless, the detection of antimicrobial activities as well as the determination of novel antimicrobial compounds produced by non-*Streptomyces* endophytes and the detection of genes

responsible for secondary metabolite synthesis in such species strongly indicate that a myriad novel bioactive compounds produced by rare actinobacterial endophytes are awaiting to be discovered.

Novel species of endophytic actinobacteria belong to various genera especially the rare ones that have been isolated. These serve as tremendous libraries by providing a broad diversity of compounds structurally and often unpredictable. This kind of library, which has large numbers and varies of chemical substances, should afford a feasible chance to successfully discover, previously unknown valuable natural products (Donadio *et al.*, 2007, Silver, 2005), including antibiotics.

1.8 Screening strategy to reverse resistance

Screening technology is one of the crucial steps in drug discovery programs because it is directly related to the detection of new natural antibiotics. Innovation in the screening method is required to reduce or even eliminate the rate of rediscovery of known compounds which has decrease the finding novel antibiotics and significantly increased the discovery cost, leading some big pharmaceutical companies to move away from antibacterial drug discovery.

1.8.1 Whole cell-based assay and Cell free-based assay

The whole cell based assay has been used as the main tool to assess antimicrobial activity since the discovery of penicillin. This assay employs one or more test organism/s seeded to the agar plate. The fermentation broth is then added to the agar plate either as a solution in a well or added to a filter paper disc and the formation of clear zone is observed as an indication for the inhibition or killing of bacteria (Singh *et al.*, 2011). This approach has successfully generated most of antibiotics available currently.

Whole cell screening is the only method that assesses all targets required for bacterial growth (Gwynn *et al.*, 2010). The synergy between different macromolecules that are essential for cellular function are maintained (Donadio *et al.*, 2002). Thus, this approach directly selects compounds that are active biologically as only potentially druggable targets are screened concurently (Baltz, 2007). Many targets are not single enzymes (Baltz, 2007) so, their function may be lost when one pathway is assayed (Donadio *et al.*, 2002). In addition, only active compounds that are able to pass the outer

cell barriers are selected. However, a whole cell screening based assay may encounter some limitations. It cannot reveal the mode of action of the active compounds. It also does not distinguish between known and unknown antibacterial compounds. This give rise to a major problem of the rediscovery of known compounds which become a big challenge when employing whole cell screening method, especially if the screening source derived from niches that have been explored intensively. A robust method to dereplicate novel compounds from the known ones at an early stage is required to overcome the problem (Baltz, 2007, Singh *et al.*, 2011), particularly when the compounds have strong activity and are present in a small quantity (Singh). Using LC-MS analysis combined with Ultra violet profile of each active extract, (Genilloud *et al.*, 2011) successfully identified 22% of more than 28,150 active extracts derived from actinobacteria matched with 190 different data of known antibiotics listed in their privately own library.

The advance in molecular biology and the relatively low variation of structural families of compounds delivered by whole cell screening (Gwynn *et al.*, 2010) brought the research on antibacterial drug discovery, especially in big pharmaceutical companies, from whole cell based to cell free/ target based screening. A cell free-based screening approach is more attractive as it defines the mode of action of the active compounds (Singh *et al.*, 2011). A number of bacterial targets have been chosen for subjects in cell free screening including fatty acid synthesis, DNA replication, protein modification, protein elongation, protein termination, RNA elongation, cell division, and amino acid synthesis (Payne *et al.*, 2007). Despite many potent enzyme inhibitors being discovered using this contemporary approach, most of them lack whole cell activity as they are unable to enter the cell barrier and hinder the compounds to reach the target cell (Singh *et al.*, 2011). In addition, it is difficult to modify such compounds so they can pass through the cell and exert their activity (Silver, 2005, Gwynn *et al.*, 2010) and sometimes the targets for cell free assay are based on hypothesis (Baltz, 2007). GlaxoSmithKline (GSK) experienced unsatisfying results employing target based screening for antibacterial discovery. Only 5 leads were obtained from 70 high throughput screening (HTS) using 67 targets conducted for 7 years and the cost was about US$1 million per HTS. A number of

other companies also faced the same hurdle in delivering lead compounds using HTS (Payne *et al.*, 2007).

1.8.2 Target based whole cell assay

The cell free/target based screening does not afford better results in delivering new antibacterial agents. Therefore, novel approaches have been developed to improve the chance of finding new antibacterial leads. Molecular target based screening lack of tractable leads as well as the whole cell activity (Gwynn *et al.*, 2010). Furthermore, experience also revealed that finding the cellular target for compounds with antibacterial activity is easier than modifying penetrability to enzyme inhibitors (Payne *et al.*, 2007). Therefore, whole cell based screening was revisited (Gwynn *et al.*, 2010, Payne *et al.*, 2007). Considering that biochemical analysis and genetic studies are important to define the mode of action (Payne *et al.*, 2007), integrating the whole cell-based screening with the cell free/target-based screening would be a way to identify compounds having target specificity without losing the whole cell activity (Gwynn *et al.*, 2010).

The success of target based whole cell approach was well presented by the application of antisense based whole cell screening that delivered platensimycin with a unique mode of action. It is a potent antibacterial which selectively inhibits type II fatty Acid (FabF) biosynthesis. This method employs two agar plates, one was seeded with *Staphylococcus aureus* containing plasmid that harbours antisense to FabF, the other plate was seeded with control *S*. *aureus*. The activity was indicated by the formation of larger clear zone on the plate seeded with *S*. *aureus* harbouring antisense than the control (Young *et al.*, 2006). Despite the controversy on the validity of type II fatty acid synthesis as an antibacterial target, the development of antisense based whole screening is a promising approach to generate novel antibacterial compounds with specific mode of action. This also might inspire the development of other method using different target.

Sensitive-resistant pair of strains screening was another target directed whole cell screening that generated novel antibiotic philipimycin produced by *Actinoplanes philipinensis* (Singh *et al.*, 2011). One plate was seeded with *S*. *aureus*, the other plate was seeded with thiazomycin (thiazolyl

peptides antibiotic) resistant *S. aureus* that had 4 amino acid deletions in the gene encoding L11 protein. The formation of a larger clear zone on the plate seeded with resistant *S. aureus* compared to the sensitive one showed that the compound has similar mode of action with thiazomycin. It could be thiazolyl peptide or non- thiazolyl peptide compounds. The LCMS of the extract did not indicate the presence of known thiazolyl peptides and it was found that it produced novel thiazolyl peptide antibiotic identified as philipimycin. Three novel thiazolyl peptides were discovered by combining this sensitive-resistant pair technique with miniaturized-high through put SPE, high resolution Fourier transform liquid chromatography and enriched the variety of the screening source, e.g rare actinobacteria.

1.8.3 Target based whole cell assay using multi drug resistant bacteria overexpressing drug efflux pumps

A target directed whole cell assay using genetically modified resistant bacteria was developed to generate novel antibiotics. It was expected that the assay would enable elimination of most known compounds. *Escherichia coli* that harbours a chromosome inserted with well-characterized genes conferring resistance to a number of antibiotics including streptothricin, streptomycin, tetracycline, chloramphenicol, bleomycin, ampicillin, rifampin, and multiple aminoglycosides was used as a target assay. It was reported that insertion of resistant genes precluded many existing antibiotics (Baltz, 2007).

This study applied a target directed whole cell assay using well characterized multi drug resistant *Staphylococcus aureus* overexpressing the *NorA* efflux pump gene as a test microorganism. Efflux pumps are membrane protein systems that expel a wide range of structurally unrelated compounds by utilizing cellular energy (Paulsen *et al.*, 1996). These systems are present in gram negative and gram positive bacteria (Lomovskaya and Bostian, 2006) and are known as the major factor for the emergence of multidrug resistant bacteria. This approach is potentially more sophisticated as it simultaneously selects the active compounds that are capable of passing the cell barrier (Lomovskaya and Bostian, 2006), and thus assesses the antibacterial activity upfront (Genilloud *et al.*, 2011).

NorA gene encodes NorA protein, a membrane-based multidrug efflux protein that is able to discharge fluoroquinolones and a number of compounds that have different structure (Kaatz *et al.*, 1993). Overexpression of the *NorA* gene was known as one of the mechanisms that mediate the resistance to fluoroquinolone antibiotics. *S. aureus* overexpressing *NorA* gene has been used in an assay to screen efflux pump inhibitors. It also has been employed to evaluate the activity of some fluoroquinolones antibiotics against multidrug resistant bacteria such as moxifloxacin (Piddock and Jin, 1999), gemifloxacin (Ince *et al.*, 2003), gatifloxacin (Ince and Hooper, 2001), and garenoxacin (Ince *et al.*, 2002). Fluoroquinolone antibiotics exert their activity by targeting DNA gyrase and topoisomerase IV, but their activity might be reduced due to the presence of a NorA efflux pump. For example, premafloxacin is 32 fold more active compared to ciprofloxacin, but it is a substrate for NorA and the MIC increased by at least double against *S. aureus* overexpressing NorA (Ince and Hooper, 2000). However, to the best of my knowledge, there is no report so far on the use of this genetically modified *S. aureus* as a target directed whole cell assay to screen for novel antibiotics that reverse resistance to antibiotics.

1.9 Research plan

1.9.1 Hypothesis

The trunks of native Australian trees are unique habitats for various genera of actinobacteria that could produce novel bioactive compounds against resistant bacteria.

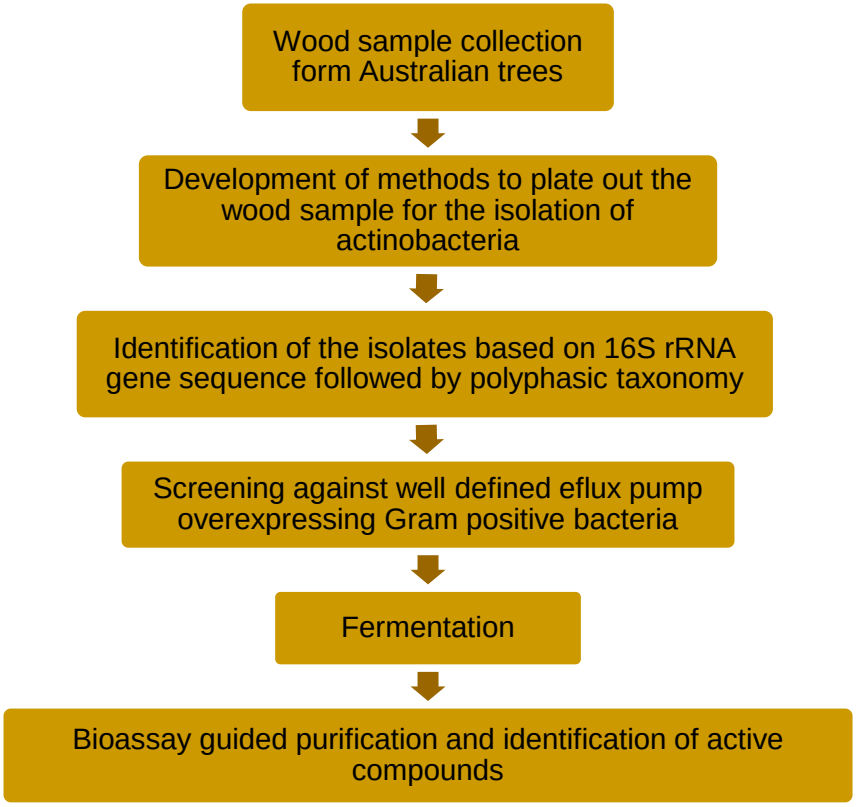
1.9.2 Aims

The aims of the project are:

1. To isolate endophytic actinobacteria living inside the wood of native Australian trees using different media combined with a larger plating on the isolation media and extended incubation times.
2. To identify the endophytic actinobacterial isolates based on 16S rRNA gene sequence followed by polyphasic taxonomy for selected potentially new isolates.

3. To screen the endophytic actinobacteria isolated from wood of Australian trees and endophytic actinobacteria in the Department of Medical Biotechnology culture collection isolated from roots, stems, and leaves of Australian trees against well-defined efflux pump overexpressing Gram positive bacteria.
4. To identify the active compounds against resistant bacteria.

1.9.3 Research project flowchart



CHAPTER 2

ISOLATION AND IDENTIFICATION OF WOOD ENDOPHYTIC ACTINOBACTERIA

2.1 Introduction

Research has been done to isolate endophytic actinobacteria from plants. A number of strains have been isolated from various plants including crops, herbs, medicinal plants, and native plants. It was reported that a higher proportion of endophytic actinobacteria are novel, therefore are expected to produce novel bioactive compounds. One of the proposed strategies to isolate novel endophytes and hence produce novel bioactive compounds is to select plants growing in broad biodiversity areas as sources for isolation (Strobel and Daisy, 2003).

A different approach has been taken to isolate endophytic actinobacteria from Australian medicinal plants. This involved the isolation of actinobacteria from roots, stems, and leaves of 4 Australian medicinal plants using 10 different isolation media with three isolation plates for each medium (Kaewkla and Franco, 2013). The incubation time of the isolation plates was also extended until 16 weeks, compared to 3-4 weeks incubation that is commonly applied. As soon as the actinobacteria emerged to form a minute colony on the isolation media they were removed and transferred to new medium to allow the slow growing ones to emerge. This approach resulted in the isolation of non-*Streptomyces* actinobacteria and at least 10 of them were identified as new species including one new genus (Kaewkla and Franco, 2013).

Most work on isolation of actinobacteria from plants has been done by using parts of plants such as roots, stems, and leaves as sources of isolation. In this study, isolation of actinobacteria was done using the wood of Australian tree trunks as the sources of isolation. Instead of using 10 different isolation media with three replicates for each medium as described by Kaewkla and Franco (2013), four different isolation media were used with 8 isolation plates for each medium. It was hypothesised that either novel slow growing streptomycetes or rare actinobacteria would be isolated and novel bioactive compounds against resistant *S. aureus* would be identified.

2.2 Methodology

2.2.1 Sample preparation and isolation media

2.2.1.1 Wood collection

Three native trees, Sydney blue gum (*Eucalyptus saligna*), Lemon scented gum (*Corymbia citriodora*), and an unidentified eucalyptus tree, tentatively classified as a stringy bark (*Eucalyptus obliqua)* were collected from tree cutters in the Adelaide Hills, South Australia. The tree trunks of 20-30 cm in diameter were used for the isolation source and were cut off with a 6 cm thickness. The samples were kept in sterile bags and were processed as soon as possible before they became dry.

2.2.1.2 Isolation media

Two sets of isolation media were used to isolate actinobacteria from native Australian wood. The first set was tap water yeast extract (TWYE) agar and humic acid vitamin (HV) agar medium. The second set included four different isolation media. Three of the media were VL70 gellan gum based medium: VL70 with a mixture of D-glucose, D-galactose, D-xylose, and L –arabinose (GGXA, 0.5 mM for each sugar), VL70 with a mixture of 17 amino acids (GGAA), and VL70 with 0.5% carboxy methyl cellulose (GGCMC). The composition of VL70 was adapted from Hudson et.al (1989), Joseph et.al (2003) and Schoenborn et.al (2004). The last medium was Humic Acid Vitamin Agar (Hayakawa). The composition of each isolation media is listed in Appendix 1. The isolation media were supplemented with Benomyl (50 mg/ml) and Nalidixic acid (20µg/ml) to inhibit the growth of fungi and bacteria, respectively.

2.2.1.3 Medium for purification and maintenance

The actinobacterial isolates had to be purified before they were stored as glycerol stocks. International *Streptomyces* Project 2 (ISP2) medium was used to purify the isolated actinobacteria. The pure cultures were then kept in cryovials containing 50% glycerol and put in a freezer (-80^{0}C) after first keeping the glycerol vials at -20^{o}C for 1 day.

2.2.1.4 Wood sample preparation

The tree trunk 20-30 cm in diameter was cut horizontally to make a round wood piece with 6 cm thickness. It was then cut through the middle along the diameter using 70% ethanol sterilized saw to make a plank. The plank surfaces (top, bottom, and the 2 sides) were removed to obtain clean surfaces. The wood plank was then cut into smaller pieces of 1 cm thickness. Each piece was put in a sterile bag and each bag was numbered according to the position of the pieces (number 1 for the outer piece, 2 for the next one and so on). These pieces of wood were used to produce wood shavings and wood powder.

2.2.1.4.1 Wood shavings

Each piece wood of 1 cm thickness was wiped with 70% ethanol using sterile tissue paper and then sliced with a sterile sharp knife to make thin wood shavings (the knife was autoclaved if possible or at least wiped with 70% ethanol). The wood shavings were collected on sterile aluminium foil or sterile petri dishes.

2.2.1.4.2 Wood powder

Two methods were used to make wood powder. First, the 1 cm wood pieces were sawed aseptically. The resulting wood powder was collected in a sterile aluminium foil sheet. Second, the tree trunk, 20-30 cm in diameter, was cut horizontally to make a round wood piece with 6 cm thickness. The wood bark was removed before use and then it was drilled aseptically using a 70% ethanol sterilized drill along the diameter to make holes with 3 cm between holes. The drilling points were numbered before drilling to record the origin of the samples. The powder from the first 1 cm depth was discarded and the powder from the subsequent holes was collected in a sterile aluminium foil sheet.

2.2.2 Isolation, purification, and maintenance of endophytic actinobacteria

In this study, the isolation of actinobacteria was run three times. In the first isolation, wood powder and wood shavings of Sydney Blue gum were used as the source of isolation. The wood shavings and the wood powder were obtained from the inner part (close to the centre) and outer part (about 4 cm from the bark) of the wood. Ten wood shavings approximately, were put onto 5 plates

containing humic acid vitamin agar (HVA) and 5 plates of tap water yeast extract agar (TWYE). The wood powder was spread out evenly onto the medium with 5 replicates for each media.

The second isolation was conducted using wood shavings from tree trunks of Sydney blue gum and Lemon scented gum. The wood shavings were collected from the centre, between the centre and the outer, and the outer part of the tree trunks. Four isolation media were used: HVA, VL70-CMC GGCMC), VL70-AA (GGAA), and VL70-sugars (GGXA). Approximately ten pieces of wood shavings from each sample were put on 8 plates of the same isolation medium.

The third isolation was done using wood powder collected by drilling a freshly cut Australian eucalyptus tree trunk as described in section 2.2.1.4.2. The wood powder from five drilling points was put evenly onto HVA, VL70-CMC, VL70-AA, and VL70-sugars with 8 replicates for each medium. Table summarized the three rounds of the endophytic actinobacteria isolation in the present study.

Table 2.1 The isolation of endophytic actinobacteria from wood

Round	Sample sources	Treatment	Media used	No of plates per medium
1	Sydney blue gum	Wood shavings and wood powder	HVA, TWYE	5
2	Sydney blue gum	Wood shavings	HVA, VL70-CMC, VL70-AA, VL70-sugars	8
2	Lemon scented gum	Wood shavings	HVA, VL70-CMC, VL70-AA, VL70-sugars	8
3	Stringy bark eucalyptus	Wood powder	HVA, VL70-CMC, VL70-AA, VL70-sugars	8

The isolation plates were kept in a sealed plastic box with wet tissue paper inside and the plates were incubated at 27ºC for 16 weeks. The tissue paper was wetted with water when it started to dry or changed with new tissue paper to prevent contamination. The isolation plates were observed every week to see any new colonies of actinobacteria that grow on isolation media. Actinobacteria that grew on the isolation media were transferred to mannitol soy or ISP2 media. The day when the actinobacterial colonies emerged onto isolation media was recorded.

The isolated actinobacteria were purified using the single colony streak method. A single colony that appeared on the purification plate was transferred to a new ISP2, or mannitol soy agar media. As soon as the culture grew well, the spores or mycelia were transferred and kept in glycerol stocks and stored in -80ºC.

2.2.3 The assessment of the ability of the actinobacterial isolates to grow on different isolation media

Each isolated actinobacterium was streaked onto all the isolation media: HVA, VL70-CMC, VL70-AA, and VL70-sugars. The plates were then kept in a sealed plastic box with wet tissue paper inside and incubated at 27ºC. The growth of each isolate on each medium was observed and recorded over 7 -10 days incubation. Once slow growing actinobacteria were transferred from isolation media to new growth media they can grow in 1-2 weeks. Thus, the observation for the assessment of the actnobacteria to grow on different isolation media was done over 7-10 days, even though an actinobacteria were isolated after week 2.

2.2.4 Identification of endophytic actinobacteria

2.2.4.1 DNA isolation

Tris-EDTA pH 7.4 was put in a sterile 1.5 ml eppendorf tube at the volume of 500 µl followed by the addition of 10 µl lysozyme 15 mg/ml. Two loopfuls of actinobacterial cells were then added into the tube. The mixture was homogenized using a vortex and it was spun followed by incubation at 37ºC for 60 min. After incubation, 10 µl of 1% proteinase K and 32.5 µl of 10% SDS were added. The mixture was flick mixed and spun quickly followed by incubation at 55ºC for 60 min. Subsequently, 100 µl of 5 M NaCl and 65 µl of CTAB/NaCl were added and the mixture was incubated at 55ºC for 10 min. Then, 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the tube was left at room temperature for 30 min with intermittent shaking every 10 min. The tube was then centrifuged at 12,000 rcf for 15 min. The supernatant was transferred to a new sterile 1.5 ml eppendorf tube followed by the addition of 500 µl chloroform and inverse mixing every 7-8 min for 15 min. The mixture was then centrifuged at 12,000 rcf for 15 min. The supernatant was transferred to a new sterile 1.5 ml eppendorf tube and 20 µl of 10 mg/ml of RNAse was added afterwards. The

tube was then incubated 37ºC for 60 min. After incubation, 500 µl chloroform was added to the tube and it was inverse mixed every 7-8 min for 15 min. The mixture was then centrifuged at 12,000 rcf for 15 min. The supernatant was transferred to a new sterile 1.5 ml eppendorf tube followed by the addition of 0.1x volume of 3 M Na Acetate (50 µl) and 3x volume of 100% ethanol (1 ml). The tube was left at -20ºC overnight. The mixture was then centrifuged at max speed 16,000 rcf for 15 min. The supernatant was removed carefully to avoid the disruption of the DNA pellet. The pellet was washed twice with 70% ethanol then it was dried by placing tubes in the heating block at 55ºC with the lids open for approximately 10 min or until the pellet dried. Subsequently, the pellet was resuspend in 50 µl or less of nuclease free sterile H_2O (depending on the size of the pellet).

2.2.4.2 Polymerase Chain Reaction

The DNA templates for the Polymerase Chain reaction (PCR) were prepared as described in 2.2.4.1. The PCR reaction was done in 50 µl system containing 40 µl PCR master mix and 10 µl DNA template. Universal 16S rRNA primers were used for PCR amplification: forward primer 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and reverse primer 765r (5'-GTAGCGGTGAAATGCGTAGA-3') for partial sequencing or reverse primer 1492r (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3') for full sequencing. One reaction of PCR master mix contained nuclease free water 11.8 µl, 10X Standard Taq reaction buffer 5 µl (1X Taq reaction buffer: 10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 0.125 µl of 10 mM dNTPs, Taq DNA polymerase 0.25 µl, 2 µl of 10 µM forward primer 27f, and 2 µl of 10 µM reverse primer 765r for partial sequencing or 1492r for full sequencing. The tubes containing a mixture of 10 µl DNA and 40 µl PCR master mix were then put in the PCR machine by using the following program: 94ºC for 2 minutes (initial denaturation), 40 cycles at 94ºC for 60 seconds, 45-55ºC for 60 seconds, and 72ºC for 2 minutes, followed by 72ºC for 10 minutes (final extension) and the PCR was held at 20ºC. The PCR products were purified using MoBio DNA purification kit and the protocols were followed as described by the manufacturer.

2.2.4.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted to confirm the presence of whole genome DNA after DNA extraction and the PCR products. The agarose powder was mixed with 0.5X TBE to make the concentrations of 0.8% and 1% for the whole genome and PCR product respectively. The mixture was then put in the microwave until the agarose was completely melted. After the mixture was cooled down to approximately 50°C, 10% of gel red was added and the gel was poured into a casting tray attached with a sample comb. The gel was allowed to solidify at room temperature and as the agarose set, it was submerged with 0.5 X TBE. A mixture of 5 µl DNA samples or PCR products and 1 µl of 6x loading dye were then loaded into the wells and the electrophoresis was run. The gel was then subjected to a UV trans illuminator to visualise the DNA.

2.2.4.4 Genes sequence analysis

The purified PCR products were sent to Macrogen, Korea for sequencing. The sequence results were then compared to an online database using BLAST at EzTaxon website (http://www.eztaxon.org/)

2.3 Results and Discussion

2.3.1 The Abundance of wood-associated bacteria

Almost all plant species harbor one or more endophytes (Strobel and Daisy, 2003). Nowadays, a culture-independent based method is carried out to analyse the endophyte communities including its population in their habitats (Ding and Melcher, 2016, Ding *et al.*, 2013). This gives the information on population of both unculturable and culturable endophytes. However, this method, which is usually based on the PCR amplification of the 16S rRNA gene has limitations due to its biased primer design and generally only the target organisms are identified (Hong *et al.*, 2009, Pinto and Raskin, 2012). On the other hand, culture dependent methods do not cover unculturable endophytes, hence the data does not represent the whole community. However, it is important to isolate pure cultures to study the molecular analysis of endophytes that can only be done using culture-dependent methods. Therefore, several studies applied culture dependent and culture independent approaches

to gain more comprehensive data on the endophytic community (Turner *et al.*, 2013, Tian *et al.*, 2007)

This study was conducted based on culture-dependent methods with a rational approach to improve the isolation of endophytic actinobacteria. Some believe that the number of isolated endophytes in culture-dependent methods do not represent the true number of endophyte in the sample. This is because mainly fast growing microbes and microbes that are in direct contact with the isolation medium will be isolated (Dees and Ghiorse, 2001, GroBkopf *et al.*, 1998). This was reasonable as commonly, in a study involving microbial isolation, the isolation plates were incubated for up to 4 weeks which did not allow the slow growing or low abundant bacteria to develop. Actinobacteria take longer to develop from a single cell to a single colony so that they can be isolated. This is faster for more common actinobacteria. This limitation can be improved by extending the incubation time up to 16 weeks, using low nutrient isolation media, and extensive plating developed by Kaewkla and Franco (2013). Applying this approach, they isolated 576 isolates from roots, stems and leaves of 4 Australian trees. It is worth noting that most *Streptomyces* appeared within 3 weeks incubation and more than half of non-*Streptomyces* spp. emerged at or after 6 weeks. Therefore, in this study the isolation plates were incubated for 16 weeks and the isolation media that gave the greatest number of isolates and diversity were chosen based on the study by Kaewkla and Franco (2013) to improve the isolation of target endophytic actinobacteria.

The first isolation was conducted as an initial isolation to observe if endophytic actinobacteria can be isolated from the outer and the inner parts (close to the centre) of the wood from an Australian eucalyptus tree trunk. Few studies have shown that actinobacteria can be isolated from wood and most of them used wood collected from 5-10 depth of the tree trunk. Endophytic actinobacteria have been isolated from the sapwood of small trees belonging to the family of Celastraceae (Pullen *et al.*, 2002). Another work also successfully isolated endophytic actinobacteria from the stems of Australian trees (native pine, Red gum, eucalyptus, and native apricot). The stem samples were obtained from wood 10 cm deep after removing the bark (Kaewkla and Franco, 2013). A research study by Blanchette (1981) identified three actinobacteria isolated from the discoloured wood of 40-

50 years old living silver maple. Shigo (1976) detected actinobacteria accounting for 18 % of the total microbes from the wood chip samples of a two year old red maple tree.

Some actinobacteria were initially isolated from the first run of isolation. All of them were obtained from HVA medium and most of the actinobacteria were isolated from wood shavings samples obtained from wood pieces which from close to the centre of trunk. Only one isolate was obtained from wood powder taken from the outer part of the trunk (close to the bark). Some of the isolates showed similar morphology on ISP2, ISP3, and ISP 5, so finally 5 actinobacteria were obtained from the first isolation.

One of the factors that influence the isolation of actinobacteria is the composition of the isolation medium and the conditions under which it is incubated. Various isolation media either general or selective have been used to isolate actinobacteria. As actinobacteria live with other microbes in the natural habitats, it is important to choose media that facilitate the isolation of actinobacteria and minimize the growth of others. It is difficult to separate actinobacteria from other bacteria because of of actinobacteria are slower growing (El-Nakeeb and Lechevalier, 1962, Hirsch and Christensen, 1983). Thus, it is important to use media that either allows the growth of actinobacteria or supresses the growth of other bacteria.

Two isolation media, tap water yeast extract agar (TWYE) and humic acid vitamin agar (HVA) that should support the growth of actinobacteria and restrict the growth of eubacteria were used in the first isolation in this study. Some proposed that actinobacteria have the ability to survive and grow in nutritionally poor media such as TWYE or even tap water agar (Goodfellow, 2010). These media also impede the growth of eubacteria and fungi effectively because of its low organic carbon content, thus allow the slower growing actinobacteria to appear on the isolation media. Eight different low organic carbon media were used to isolate soil actinobacteria and almost 50% among 217 of isolated actinobacteria were obtained from TWYE (Crawford *et al.*, 1993). In line with these results, El-Shatoury *et al.* (2006) found that TWYE was the most effective among the 4 isolation media that were used to isolate endophytic actinobacteria from medicinal plants based on the number of

isolated actinobacteria. More recently, by putting crushed samples of medicinal plants on 11 isolation media, Qin *et al.* (2009a) also isolated endophytic actinobacteria more effectively in terms of their frequency from TWYE compared to other media with a high nutrient content. In addition, at least two novel actinobacteria, identified as *Pseudonocardia endophytica* sp., nov. (Chen *et al.*, 2009) and *Pseudonocardia antimicrobica*, sp. nov (Zhao *et al.*, 2012) were also successfully isolated from *Lobelia clavata* and *Artemisia annua*, respectively using TWYE.

HV (humic acid vitamin) agar was another isolation medium chosen in the first isolation. Hayakawa and Nonomura (1987) used humic acid as a sole carbon and nitrogen source in HV medium. They found that this medium gave significantly higher numbers of actinobacterial colonies compared to 7 other media generally used for the isolation of actinobacteria such as starch-casein –nitrate agar, colloidal chitin agar, and soil extract agar. A greater number of genera other than *Streptomyces* that usually dominate the common isolation media could also be recovered using HV medium including *Microbispora, Streptosporangium, Dactylosporangium, Microtetraspora,* and *Thermomonospora*. Kaewkla and Franco (2013) also isolated a high diversity of non-*Streptomyces* from Australian native trees with nine different genera on HV agar, including two unique genera, *Gordonia* and *Nonomuraea* that were not isolated from 9 other media used in the isolation. Therefore, HV has been used widely to isolate actinobacteria especially from plants and terrestrial habitats (Otoguro *et al.*, 2001, Kim *et al.*, 2011, Meklat *et al.*, 2011, Huang, 2012, Taechowisan *et al.*, 2003).

HV was superior compared to other isolation media as it supported the growth of actinobacteria while retarding the growth of non-filamentous bacteria. HV contains complex carbon and nitrogen sources. This type of medium has a high carbon to nitrogen ratio that allows the actinobacteria to grow over the general bacteria. It is usually difficult for bacteria to grow on media containing complex carbon and nitrogen sources. They are also unable to use high molecular weight, resistant organic polymers (Goodfellow, 2010). Furthermore, research showed that actinobacteria are able to utilize or degrade humic substances (Filip and Tesarova, 2005). The effectiveness of HV for actinobacteria isolation is also due to the property of humic acid that triggers germination of spores (Hayakawa and Nonomura, 1987).

Therefore, the isolation media should not be the main reason for the low number of actinobacteria (5 isolates) obtained in the first run of isolations. This was possibly due to the low abundance of endophytic actinobacteria residing in the wood of the tree trunk. Numerous endophytic actinobacteria have been isolated from roots, stems, and leaves so it was known that these endophytes are able to colonize roots, stems, and leaves. Most research found that roots harbor the highest number of endophytic actinobacteria followed by stems and leaves. However, little is known about wood-associated endophytic actinobacterial communities. Bulgarelli *et al.* (2012) revealed that endophytic bacteria are able to colonize the wooden compartment which is basically dead or dying cells. They are also found in xylem (Turner *et al.*, 2013)

As only a few actinobacteria were obtained, the second isolation was conducted as described in section 2.2.2 to explore further on the wood-associated endophytic actinobacteria. The wood samples were obtained from Sydney Blue Gum and Lemon scented gum tree trunks. The wood of Sydney blue gum tree trunk was used in the first isolation, but for the second isolation the samples were taken from different positions which were numbers 4, 9, and 14 while the samples for first isolation were obtained from pieces numbered 17 and 25 as illustrated in figure 2.1. The whole wood sample of the Lemon scented gum tree and the position of wood pieces used for the isolation are illustrated in figure 2.2. After 16 weeks of incubation, 3 actinobacteria were obtained. All of the actinobacteria were isolated from wood samples close to the centre of the tree trunk. Two of them were isolated from Sydney blue Gum wood on HVA plates. The other one was isolated from Lemon scented gum tree on VL70-sugars medium.

In order to increase the chances of isolating a higher number of actinobacteria in the second isolation, the larger sample was plated out onto isolation media by using 4 isolation media and increasing the number of isolation plates per medium up to 8 plates. This means that the sample was plated out to 40 plates. HV was used in the second isolation as most actinobacteria were isolated from this medium in the first isolation. In the second isolation three VL70 based isolation media were used instead of TWYE because no actinobacterium was isolated from TWYE. Kaewkla and Franco (2013) successfully isolated endophytic actinobacteria in terms of their number and diversity using

VL70 supplemented with 17 amino acids, sugars, and CMC. Even though larger samples were plated out, still only few actinobacteria were obtained from the second isolation. Two actinobacteria were isolated from Sydney Blue Gum and 1 actinobacterium from Lemon scented gum. This number was lower compared to that of actinobacteria isolated from the previous isolation. These results could be due to the low density of culturable actinobacteria from the wooden compartments of both gum trees.

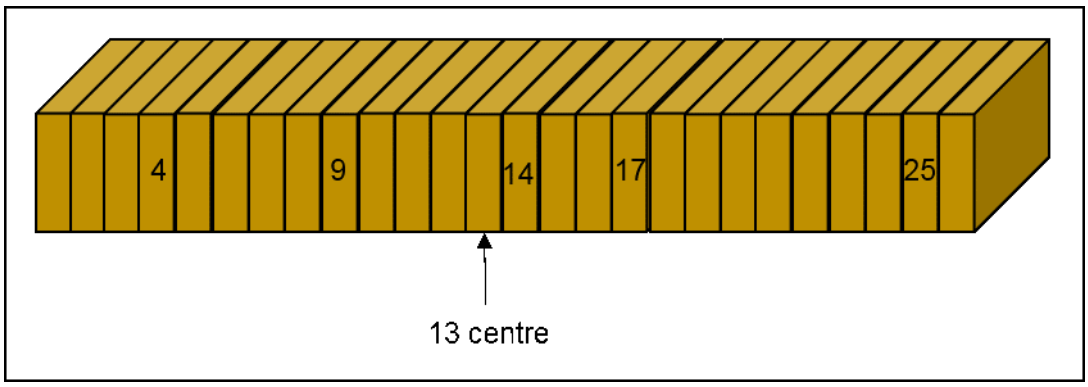


Figure 2.1 The illustration of wood samples from the plank of Sydney Blue gum tree

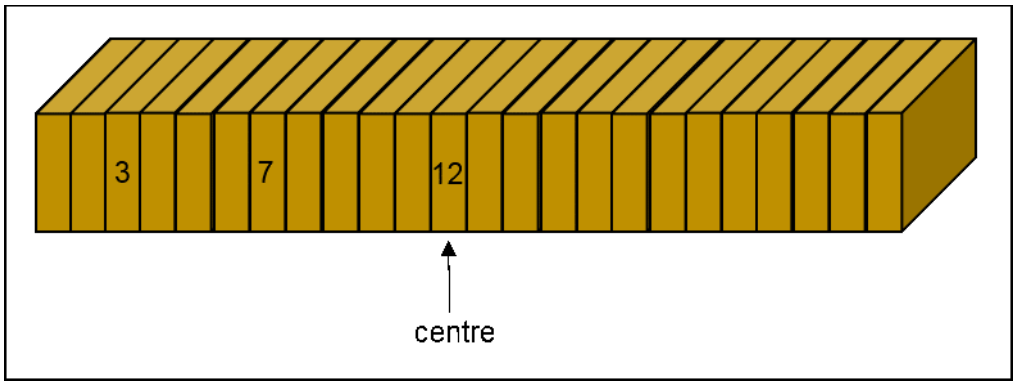


Figure 2.2 The illustration of wood samples from the plank of Lemon scented gum tree trunk

Therefore, another attempt was made to isolate actinobacteria from wood. Wood powder was used as the isolation source and was collected by drilling the wood of a freshly cut tree as described in sections 2.2.4.1.2 and 2.2.2. A total of 51 endophytic actinobacteria were isolated using this method. Most of them were obtained from HVA, followed by GGAA (gellan gum based VL70-AA), and VL70-CMC (gellan gum based VL70-CMC). No actinobacteria were isolated from VL70-XA (gellan gum VL70-sugars based) medium. The total number of actinobacteria isolated from each medium is shown in figure 2.3.

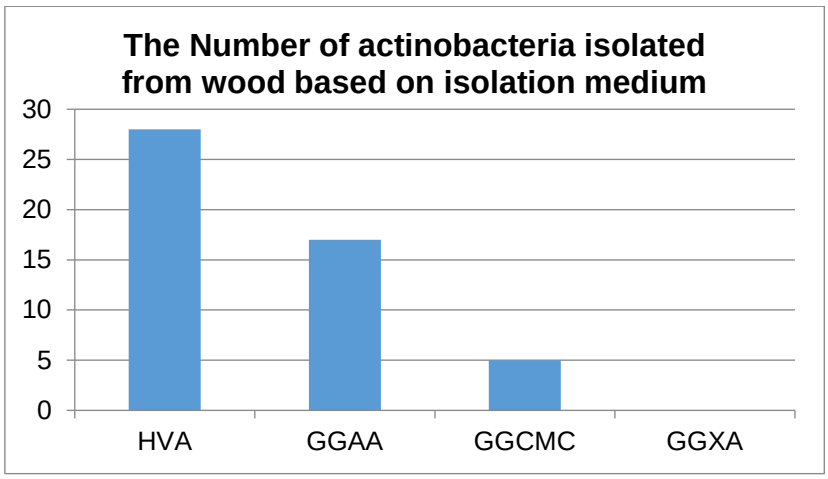


Figure 2.3 The number of isolated actinobacteria (from the third isolation) based on the isolation media: HVA and 3 gellan gum (GG) based media (GGAA, GGCMC, GGXA)

In the third isolation, actinobacteria were isolated from all isolation media except GGXA. The results showed that HV was the most effective medium to isolate actinobacteria. Among the total of 51 isolates, about 50% (28) were isolated from HVA. In the first and second isolation, all actinobacteria was also isolated on HV medium, except one which is isolated from VL70 supplemented with sugars (GGXA) in the second isolation. The effectiveness of HV to isolate actinobacteria has been discussed before in this section.

Amino acids are likely to support the growth of actinobacteria as 17 isolates were obtained from GGAA. This finding was in line with the results of Qin *et al.* (2009a) and Kaewkla and Franco (2013). They isolated higher numbers of endophytic actinobacteria on isolation media containing amino acids. Sapwood and heartwood contain nitrogen and the most important nitrogen compounds are amino acids (Pallardy, 2010). Seventeen amino acids were found in the xylem sap of 60 tree species in North Carolina. The majority are arginine, asparagine, aspartic acid, citruline, glutamic acid and glutamine (Barnes, 1963). Additionally, Escher *et al.* (2004) detected other amino acids such as proline and arginine in the xylem sap of *Populus* spp. and *Abies alba*. Further study is required to understand how of amino acids affect the colonization of endophytes (Qin *et al.*, 2009a)

The addition of carboxymethylcellulose (CMC) as a growth substrate to VL70 medium was less effective compared to amino acids as growth substrate. The number of actinobacteria isolated from VL70-CMC was 3 times higher than that of actinobacteria isolated from VL70-amino acids. This

result was different from the results of Kaewkla and Franco (2013) who isolated most endophytic actinobacteria from media supplemented with CMC. This suggested that not many actinobacteria inhabiting the wood in this study could utilize cellulose which is the main component of the cell walls of woody plants (Pallardy, 2010). Several studies revealed that actinobacteria are able to degrade lignocellulose. It has been found that a number of *Streptomyces* species showed cellulolytic activity (Fathallh Eida *et al.*, 2012, Abdulla and El-Shatoury, 2007). Separately, Sutherland *et al.* (1979) and Antai and Crawford (1981) reported the decomposition of softwood and hardwood lignocellulose by different species belonging to the *Streptomyces* genus. Some other genera including *Nocardiopsis, Micromonospora, Nocardioides* (El-Shatoury *et al.*, 2006) and *Microbispora* (Fathallh Eida *et al.*, 2012) also exhibited cellulolytic activity.

No actinobacteria were isolated from VL70 supplemented with sugars (glucose, galactose, arabinose, and xylose) as growth substrates. This result was obtained when monomers or mixed monomers were used as growth substrate compared to sugar polymers (Schoenborn *et al.*, 2004, Chin *et al.*, 1999). In addition, more endophytic actinobacteria were isolated from VL70 supplemented with CMC compared to these supplemented with a mixture of glucose, galactose, arabinose and xylose (Kaewkla and Franco, 2013). Nutrient shock was observed when the microbes were transferred from low nutrient environments to high nutrient solid media as the cells were likely damaged from osmotic stress or were not able to suddenly utilize a large amount of nutrient (Azevedo *et al.*, 2012). Many simple sugars are commonly present in woody plants including sapwood and heartwood in small amounts and only traces of arabinose and xylose are found free in plants (Pallardy, 2010). Polymers could be better as substrate because the exposure to high concentrations of growth substrate will be reduced using polymers as they need to be hydrolysed initially, thus avoiding nutrient shock (Chin *et al.*, 1999).

The isolation results showed a significant difference between the first two isolations and the third isolation in terms of the number of isolated actinobacteria. Using the same isolation media in the second and third isolations, 51 isolates were obtained from the third isolation, but only 3 isolates were obtained from the second isolation. As the form of the wood samples between the second and

the third isolation was different, i.e. wood shaving and wood powder for the second and third isolation, respectively, it was proposed that the surface area of the samples was the main factor that influenced the rate of isolation of actinobacteria from wood. Drilling the wood generated fine particles of wood powder. Thus, when the wood powder was put on the isolation media a bigger surface area of sample was exposed compared to the wood shavings. This meant wood powder gave higher probability than wood shavings of exposing the bacteria living within the plant tissue onto the isolation media. It was also suggested that drilling assisted in separating the middle lamella cells adjoining two plant cells together. This would release microbes living in association within the plants which mainly reside in the intercellular spaces. Jiao *et al.* (2006) applied enzyme to hydrolyse cell walls to separate the middle lamella cell and release the endophytic microbes.

If the main reason was not the exposure due to the powder then the other reasons are abundance of actinobacteria residing within the wood and the freshness of the samples. The abundance and the diversity of endophytic actinobacteria are shaped by the plant species, type of soils, and other associated environmental conditions (Govindasamy *et al.*, 2014). The wood samples used as source of isolation originated from different tree species and locations so the population and diversity of their endophytic associated actinobacteria may have been different. Conn and Franco (2004) revealed that the population and diversity of wheat endophytic actinobacteria were associated with the soil where the plants grew. The plants that grew in the soil with a higher number of organisms had higher actinobacterial populations and diversity. The abundance of endophytic bacteria was also different between plant species that host the microbial community as observed by Ding and Melcher (2016).

Another factor that was considered to contribute to the low number of actinobacteria obtained from the first isolation and the second isolation was the freshness of the wood sample. The wood sample for the first and second isolation could not be processed as soon as the trees were cut. Thus, it might be argued that the low number of actinobacteria isolated from the first and second isolation was due to the wood samples that were drying out. This was supported by results in the present study showing a significantly higher number of actinobacteria isolated from freshly cut wood tree trunks than those from less fresh wood. Fresh samples, which are treated within 24-48 hours after

collection, are commonly used in the most isolation works involving plant materials. However, it was proposed that endophytic actinobacteria can be isolated from the wood of trees that were not fresh or even old wood as they can survive in the form of spores (Franco, personal communication). This is supported by the work of Baum *et al.* (2003) who isolated a higher number of endophytic fungi from wood samples that were incubated under drying treatment for a period of time compared to that of fresh wood. It was suggested that the decrease in water concentration and the increase in oxygen concentration initiated the activation of latent mycelia (Rayner, 1986). Therefore, the low number of isolates obtained from the first and second isolations is possibly due to the low abundance of actinobacteria in the wood rather than the freshness of the samples.

Based on the position where the wood powder was taken (third isolation), most actinobacteria were isolated from spot 9 which is in the sapwood site. It was unexpected that only 2 actinobacteria were isolated from spot 2 that was also in the sapwood site. No actinobacteria were obtained from spot 5 which at centre of the tree trunk. However, actinobacteria were isolated from spots 4 and 6 which were the closest to spot in the centre. A higher number of actinobacteria was obtained from spot 4. It was almost twice of those from spot 6. Interestingly no actinobacteria were obtained from spot 3. Figure 2.4. illustrated the sampling site of wood powder and graph 2.5. shows the number of actinobacteria isolated at each the sampling site.

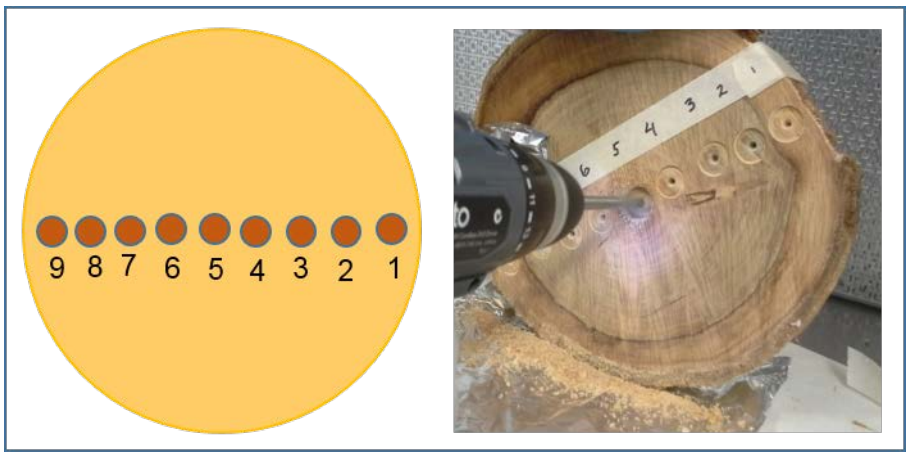


Figure 2.4 Sampling site of the wood powder

Endophytes have been found inhabiting wood of tree trunks (Blanchette, 1981, Shigo, 1976, Oses *et al.*, 2008). It is widely suggested that the endophytes enter their host plants initially through root

cracks at lateral root junction, called ‘crack entry’ (James *et al.*, 2002, James *et al.*, 1998, Monteiro *et al.*, 2012, Gopalaswami *et al.*, 2000). These endophytes are able to move upward, through intercellular spaces (this needs the secretion of active cell wall degrading enzymes), lumen xylem vessels, transpiration stream as reviewed by Compant *et al.* (2010) and translocated from the roots to aerial part of the plants (Turner *et al.*, 2013). In this study, the number of endophytic actinobacteria that were successfully isolated from the wood of tree trunks supported the ability of these microbes to colonize xylem.

Based on the results, there was no pattern of the distribution of actinobacteria in the tree trunk, e.g. the number of actinobacteria were the highest in the outer part of the tree trunk and lower in its inner part (cross section). This suggested that endophytic actinobacteria randomly inhabit the sapwood (young xylem) and heartwood. Endophytes mainly reside in the intercellular spaces within parenchyma tissue (McCully, 2001). Thus, it was not unusual that most of endophytic bacteria in this study were isolated from a niche within the sapwood as it contains the living parenchyma cells. Oses *et al.* (2008) proved the presence of fungal colonization along the xylem parenchyma in tangential and transverse sections of xylem samples of healthy wood. Sapwood also serves as food storage that could be an advantage for actinobacteria that reside in this niche. On the other hand, it was interesting that a number of endophytic actinobacteria were also obtained from heartwood. Heartwood is secondary xylem comprising dead elements formed as woody plants undergo secondary growth and have no further role in physiological processes (Pallardy, 2010). Despite reports of bacteria in the xylem or in sap of stems, McCully (2001) argued that xylem is not the ideal niche for endophytes particularly for the crops or grasses. The reason is that a xylem vessel is a non-functioning vessel, thus bacterial invasion in the xylem vessels bring hazards to the plants (McDonough and Zimmermann 1978, Zimmermann 1983). However, it is not quite relevant for woody plants. Woody plants undergo secondary growth which is lacking in herbaceous plants. A woody plant with decayed heartwood can survive for many years as the heartwood together with the thin shell of sapwood is still able to give mechanical support. The isolation of actinobacteria from heartwood in the present study revealed that secondary xylem is a suitable niche for endophytes.

This result was in accordance with the study of Bulgarelli *et al.* (2012) showing that wood compartment which contains metabolically inactive lignocellulosic matrices is sufficient for the colonization of actinobacteria. In a study using ^{14}C labelled lignocellulose, lignin labelled lignocellulose and ^{14}C labelled wood lignin, Crawford (1978) proved that some streptomycetes were able to degrade lignocellulose. They attacked the cellulose parts and to a lesser degree the lignin components. In addition, a number of wood-inhabiting microfungi from various genera have been isolated from the healthy as well as stained or decayed heartwood (Hutchison, 1999). Figure 2.5 shows the number of actinobacteira based on the sampling sites of the wood powder.

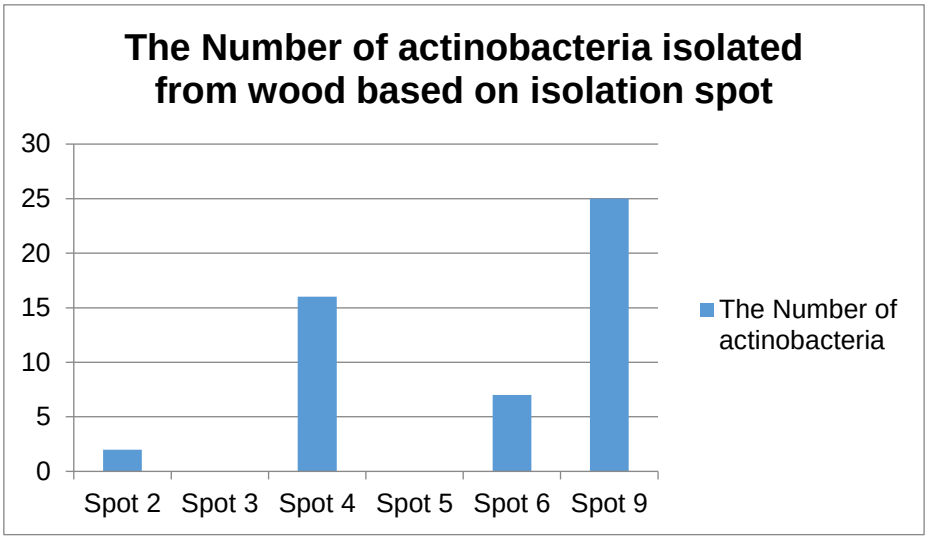


Figure 2.5 The number of isolated actinobacteria based on the sampling site of the wood powder

The emergence of actinobacteria on agar media was also observed weekly. The highest number of actinobacteria were isolated on week two. A decrease in the number of isolated actinobacteria was observed after week two. No actinobacteria were obtained from week 13 and 14 but a few endophytic actinobacteria were isolated on week 16 when the isolation was ended. The actinobacteria isolated each week over 16 weeks is shown in figure 2.6.

Weekly observation was done to remove any actinobacteria for purification as soon as possible after they emerged on the plate. This allowed the slow growing actinobacteria which are potentially novel species to develop on the isolation medium. Applying this method, actinobacteria that grew after week 4 were successfully isolated. It is inadequate to keep the isolation plates for 4 weeks as the slow growing microorganisms will be over run (Vartoukian *et al.*, 2010). The results showed that after

4 weeks incubation, a number of actinobacteria were isolated which would have gone undetected or regarded as unculturable if the incubation time was not extended. Figure 2.7 shows an actinobacterial colony grew on an isolation medium of wood powder sample

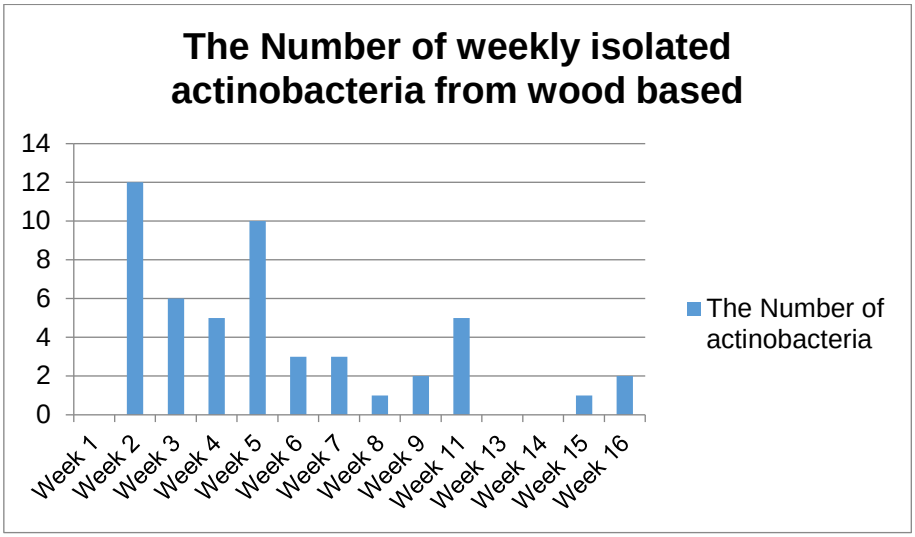


Figure 2.6 The number of isolated actinobacteria weekly

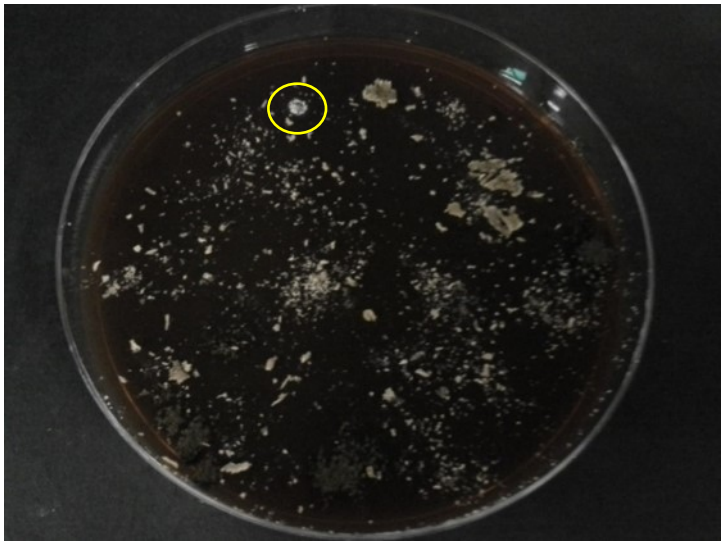


Figure 2.7 The emergence of an actinobacterial white colony from wood powder sample on an isolation medium (circled)

2.3.2 The assessment of actinobacterial growth on different isolation media

All the isolates can grew on all isolation media irrespective of the medium on which they were isolated. Therefore, the type of isolation medium was probably not as important as having sufficient plant material plated out. This can be explained by using soil actinobacteria for an illustration. The

number of soil actinobacteria per gram soil is usually between 10^4 and 10^6 colony forming unit (Goodfellow and Williams, 1983). One g of soil dissolved in 9 ml sterile water will result in between 10^3 and 10^5 CFU per ml. If the highest concentration (10^5 CFU per ml) was taken for the illustration and this was further diluted 10, 100, and 1000 times for plating out, the concentration of actinobacteria in each soil suspension will be 10^4, 10^3 and 10^2 CFU/ml, respectively. If 100 µl was plated out on the isolation media, only between a thousandth and one hundred thousandth of actinobacteria will be on the plate, which means only small numbers of actinobacteria were put on the plate. Hence, having adequate number of plates has to be considered in an isolation procedure as it has a significant impact on the results.

2.3.3 Identification of wood associated actinobacteria

Of the 51 isolates obtained from the third isolation, 21 have been successfully identified based on 16S rRNA sequence as listed in table 2.2. These 21 isolates showed good 16S rRNA sequencing results for identification. One actinobacterium isolated from the first isolation was identifies as having 99.48% similarity with *Actinomadura citrea*^{T}. The result showed that endophytic actinobacteria isolated from wood belong to diverse genera including *Actinomadura* (from the first isolation)*, Streptomyces, Asanoa, Actinoplanes,* and *Actinoallomurus* (from the third isolation). Another interesting result is that 7 isolates are likely to be new species, 6 of them were obtained from the third isolation. The similarities of 16S rRNA gene sequences for these isolates to the closest type strains were less than 99% (4 isolates), 99.25% (1 isolate), 99.4 (1 isolate), and 99.48%, (1 isolate).

It was interesting that rare actinobacteria which are *Actinomadura, Actinoplanes* (Lazzarini *et al.*, 2001), *Actinoallomurus and Asanoa* were isolated from the wood of a tree trunk. These genera are classified as rare actinobacteria as their isolation frequency is much lower than *Streptomyces* (Tiwari and Gupta, 2012). According to the LPSN database, less than 20 species of actinobacteria belonging to the genera of *Actinoallomurus* and *Asanoa* have been isolated so far. The number of the species according to the LPSN database for all the genera of isolated actinobacteria from wood is presented in table 2.3.

This study also demonstrated that rare actinobacteria or *Streptomyces* recovered from the isolation media after week 5 were more likely to be new species. Three of the isolates recovered at week 6 (1 isolate) and week 11 (2 isolates) are potentially new members of the genus *Streptomyces*. However, *Streptomyces* obtained at week 5 or before are common ones, e.g. *Streptomyces canus* and *Streptomyces ciscaucasicus*. They were frequently found as endophytes within legumes (Le *et al.*, 2016). This suggested that there is a greater chance to find novel *Streptomyces* by isolating slow growing ones. Furthermore, prolonging the incubation period in an isolation work gives a higher chance of obtaining novel species.

A number of genera other than *Streptomyces* were isolated from stem samples of woody plants taken up to 10 cm depth after removing the bark. These included the genera of *Micromonospora, Polymorphospora, Nocardiopsis, Promicromonospora, and Pseudonocardia* and six of the isolates were confirmed to be new species (Kaewkla and Franco, 2013). These results indicated that wood is potential resource for either rare or novel species of actinobacteria and even novel genera. An endophytic nocardioform-like strain was isolated from the root of Grey Box, an Australian eucalyptus, and is classified as a new genus, *Flindersiella* gen.nov, (Kaewkla and Franco, 2011).

On terrestrial habitats, wood is a relatively unexplored niche for actinobacteria compared to soil. Only a few studies have been done on the isolation of actinobacteria from wood, especially the heartwood. Pullen *et al.* (2002) isolated actinobacteria from the wood of Celastraceae which is a woody bush with a height up to 4 m. One of the isolates was hitherto found to be a new species and produced new chlorinated antibiotics designated as celastramysins A and B. Even though exploration of endophytic actinobacteria from plants has been done frequently, this has been from roots, leaves, and stems.

Table 2.2 Identified endophytic actinobacteria isolates based on 16S rRNA gene sequencing

No	Isolation week	Actino codes	Isolation spots*	Identification based on 16 S rRNA	Similarity (%)	Sequence Lenghts (bp)
1	2	HV9(a)2	9	*Streptomyces canus*	100	1,351
				Streptomyces ciscaucasicus		
2		HV6(g)1	6	*Streptomyces niveus*	98.11	1,326
3	3	AA9(b)2	9	*Streptomyces niveus*	98	1,350
4		HV4(a)3	4	*Streptomyces canus*	100	1,338
5	4	AA9(a)6	9	*Streptomyces pseudovenezuelae*	98.96	672
6		AA9(c)3	9	*Streptomyces fulvissimus*	100	680
7		AA9(c)4.3	9	*Streptomyces griseolus*	99.85	674
8		HV9(a)3	9	*Streptomyces canus*	99.55	670
				Streptomyces ciscaucasicus	99.55	670
				Streptomyces caerulatus	99.55	670
9	5	HV9(a)6	9	*Streptomyces canus*	100	675
				Streptomyces ciscaucasicus	100	675
				Streptomyces caerulatus	100	675
10		HV4(e)6	4	*Actinoplanes ferrugineus*	97.64	1,360
11		HV4(f)7	4	*Streptomyces glomeroauranthicus*	99.33	1,344
12		HV4(f)7.1	4	*Streptomyces rishiriensis*	99.7	1,346
13	6	HV4(a)8	4	*Streptomyces canus*	100	671
				Streptomyces ciscaucasicus	100	671
				Streptomyces caerulatus	100	671
14		HV9(b)10	9	*Streptomyces cocklensis*	98.66	1,340
15	7	HV6(g)4	6	*Asanoa ishikariensis*	99.4	1,326
16	11	AA9(a)10	9	*Streptomyces alboniger*	99.25	1,340
17		AA9(b)11	9	*Actinoallomurus coprocola*	98.76	1,375
18		CMC6(a)2	6	*Streptomyces cinerochromogenes*	98.66	674
19	13	HV9-13	9	*Actinoallomurus bryophytorum*	99.25	1,328
20	14	HV4(f)20	4	*Streptomyces glomeroauranthicus*	99.54	647
21	16	HV6(d)6	6	*Actinoallomurus bryophytorum*	99.85	1,334

Table 2.3 The number of species and subspecies that belong to the genera identifies in the present study

Genera	Number of species	Number of subspecies
Actinomadura	79	2
Actinoallomurus	17	0
Actinoplanes	44	0
Asanoa	6	0
Streptomyces	788	38

On the basis of 16S rRNA gene sequence analysis comparison using EzTaxon database, one of the isolates from wood designated as DG1 exhibited a close relationship with members of the genus *Actinomadura*. It shared the highest 16S rRNA gene sequence similarity with *A. maheshkaliensis* (99.4%), *A. citrea*^{T} (99.48%), *A. mexicana*^{T} (99.4%). However, the neighbour-joining and maximum likelihood trees indicated that strain DG1 fell in a lineage with *A. citrea*^{T} and *A. mexicana*^{T}.

Furthermore, the trees also showed that strain DG1 and *A. mexicana*^{T} do not have the same most recent common ancestor. Strain DG1 and *A. citrea*^{T} had the same most recent common ancestor and their 16S rRNA genes shared 99.48% similarity.

This value is higher than the determined threshold (98.7%) to discriminate two strains at the species level (Stackebrandt and Goebel, 1994). However, they were possibly distinct strains because many of the bacteria with validly published names do not follow this cut-off value and has ≥ 98.7% 16S rRNA gene similarity. Many genera in the phylum Actinobacteria respect the two cut-off values of 95% and 98.7% to differentiate bacterial isolates at genera and species levels, respectively. Among 33 of studied genera, 24 (72.2%) were more likely to have >50% inter species 16S rRNA gene sequence similarities of between 95% and 98.7%. This suggested that some of the genera do not or poorly respect both thresholds; one of them is the genus *Actinomyces* (Rossi-Tamisier *et al.*, 2015). There is no report so far on how well the genus *Actinomadura* complies with the cut-off values. An example is *A. mexicana*^{T} and *A. citrea*^{T} which exhibit 99% similarity (Quintana *et al.*, 2003). Therefore, strain DG1 that shares 99.48% similarity with *A. citrea*^{T} might belong to new species. The ability of strain DG1 to produce active compounds in this case against resistant *S. aureus* could be one of the properties that differentiate strain DG1 and *A. citrea*^{T}. There is no report so far on the activity of *A. citrea*^{T}. Morphological, physiological and biochemical analysis have to be done to evaluate the phenotypic features of strain DG 1 and the closest type strains to justify if strain DG 1 belongs to a new species.

The isolation of *Actinomadura* in the present study indicated that wood could provide a potential niche for the isolation of the genus *Actinomadura* from plants. The isolation of *Actinomadura cellulosylitica* (Jiao *et al.*, 2015) that hydrolysed cellulose suggested the ability of this genus to live inside wood. At the time of writing, only a few actinobacteria species of the genus *Actinomadura* were reported as endophytes. At present, among 77 of validly published names of *Actinomadura* species, three of them were isolated from plants. Qin *et al.* (2009b) isolated endophytic actinobacteria identified as *Actinomadura flavalba* from leaves of medicinal plants. Other endophytes were isolated from roots of herbaceous plants and woody plants identified as *Actinomadura*

longicatena (Itoh *et al.*, 1995) and *Actinomadura syzygii* (Rachniyom *et al.*, 2015), respectively. The species of the genus *Actinomadura* were mostly recovered from soil and others have been isolated from various sources (Parte, 1997) such as mangrove sediment (He *et al.*, 2012), hive (Promnuan *et al.*, 2011), compost (Puhl *et al.*, 2009, Mayer, 1979) clinical materials (Hanafy *et al.*, 2006, Mayer, 1979) sewage ditch (Lu *et al.*, 2003), mud hot spring and rock (Lee and Lee, 2010).

Among the rare genera of actinobacteria, *Actinomadura* is one of the most important targets in screening programs for pharmacologically active compounds (Dairi *et al.*, 1999), especially antiinfectives (Lazzarini *et al.*, 2001). More than 250 antibiotics have been produced by *Actinomadura* strains (Lazzarini *et al.*, 2001). Thus, the isolation of novel species belonging to this genus, especially the ones from plants (endophytes) should lead to the discovery of a wide range of novel bioactive compounds. Wood is a promising choice for the source of isolation of the novel *Actinomadura* strains as this part of plants is relatively untapped compared to other parts, such as roots, stems and leaves.

Two isolates obtained from wood in this study designated as HVA6(d)6 and AA9(b)11 belong to the rare genus *Actinoallomurus*. Strain HVA 6(d)6 and AA9(b)11 share the highest 16S rRNA gene similarity with *Actinoallomurus bryophytorum*^{T} (99.85%) and *Actinoallomurus coprocola*^{T} (98.76%), respectively. Despite the very high similarity between strain HVA6(d)6 and its closest type strain, *Actinoallomurus bryophytorum*, the isolation of strain HVA6(d)6 showed that different species of genus *Actinoallumurus* are associated with plants. Considering that the threshold of 16S rRNA gene similarity to differentiate strains at the species level is 98.7%, isolate AA9(b)11 has a high probability of being a new species.

The genus *Actinoallomurus* is one of the promising rare actinobacteria that produce bioactive secondary metabolites. The genus *Actinoallomurus* belongs to the family Thermomonosporaceae together with five other genera including the genus *Actinomadura*. Among the genera in this family, the genus *Actinomadura* is the most prolific producer of secondary metabolites until Pozzi *et al.* (2011) revealed that *Actinoallomurus* strains also have potential to produce secondary metabolites.

They possess several pathways for bioactive secondary metabolites production. A significant number of strains with a reasonable degree of diversity could also be accessed as a discrete level of genotypic diversity was observed in this genus. These factors make the genus *Actinoallomurus* an attractive source to search for novel antibiotics (Pozzi *et al.*, 2011).

Plants are one of the potential sources for the isolation of *Actinoallomurus* strains. This was supported by the isolation of *Actinoallomurus* strains in this study and other works that had successfully isolated *Actinoallomurus* from plants. Seventeen species of *Actinoallomurus* have been isolated so far (LPSN, 2017) and 5 of them were obtained from plants identified as *Actinoallomurus acaciae* (Thamchaipenet *et al.*, 2010), *Actinoallomurus liliacearum* (Koyama *et al.*, 2012), *Actinoallomurus vinaceus* (Koyama *et al.*, 2012), *Actinoallomurus oryzae* (Indananda *et al.*, 2011), and *Actinoallomurus radicium* (Matsumoto *et al.*, 2012).

Most of the *Actinoallomurus* strains isolated from plants originated from roots, so this study showed that another part of plants, the wood, also serves as a niche for *Actinoallomurus* strains. There is a great chance of isolating new diverse *Actinoallomurus* species by exploring wood as isolation sources because at least two reasons. First, wood can be obtained from about 300,000 plant species that exist in the world. They grow in various different and unusual environments that serve as unique habitats for rare actinobacteria (Strobel and Daisy, 2003) including *Actinoallomurus* strains. Second, Pozzi *et al.* (2011) predicted that *Actinoallomurus* strains are spread widely, thus the possibility to isolate actinobacteria from this genus is high.

Strain HV4(e)6 isolated in this study belongs to the genus *Actinoplanes*. Neighbour joining and maximum likelihood based phylogenetic trees showed that the closest type strains to the isolate HV6(e)6 are *Actinoplanes ferrugineus*^{T} (97.64% 16S rRNA gene similarity) and *Actinoplanes tereljensis*^{T} (97.42 16S rRNA gene similarity. Considering that the cut off of 16S rRNA gene similarity to differentiate two strains at the species level is 98.7%, isolate HV6(e)6 has a high chance of being identified as a new strain or species.

This study showed that the genus *Actinoplanes* is able to associate with plants as an endophyte. An endophytic *Actinoplanes* was also isolated before from soybean root, identified as *Actinoplanes hulinensis* (Shen *et al.*, 2013), though this species is not listed in the LPSN database. About 78% of *Actinoplanes* species listed in LPSN database were isolated from soil, and none of them were obtained from plants.

The isolation of novel *Actinoplanes* species could make a significant contribution to the discovery of unknown bioactive secondary metabolites. The genus *Actinoplanes* is one of the main producers of secondary metabolites among the rare genera of actinobacteria. It has been reported that more than 120 antibiotics have been produced by the genus *Actinoplanes* (Lazzarini *et al.*, 2001). Nevertheless, while the focus to tap novel bioactive compounds from the so called minor genera of actinobacteria is increasing, Genilloud *et al.* (2011) observed the rediscovery of known compounds from these group even though the number is much fewer than those found in the *Streptomyces* group. About 21% of known compounds were redetected in *Actinoplanes* spp. Therefore, the search for novel *Actinoplanes* members should centre on unique biotopes or those which are relatively unexplored, such as wood. This niche has the prospect of housing this genus as indicated by the isolation of an *Actinoplanes* strain in the present study. Wood can be taken from a huge number of higher plant species growing in different unusual environmental settings and has high possibility of producing interesting novel species including *Actinoplanes.*

An isolate designated as HV6(g)4 was obtained from a wood sample belonging to the genus *Asanoa*. It shared the highest 16S rRNA gene similarity with *Asanoa ishikariensis*^{T} (99.4%), *Asanoa iriomotensis*^{T} (99.17), and *Asanoa ferruginea*^{T} (99.02%). Neighbour joining and maximum likelihood based phylogenetic trees also showed that strain HV6(g)4 has the same most recent common ancestor with *A. ishikariensis,* but not with two other most similar type strains. The 16S rRNA gene similarity values of strain HV6(g)4 to the closest type cultures were out of the determined threshold value to discriminate two strains at the species level which is 98.7% (Stackebrandt and Ebers, 2006). However, it is possibly a novel species considering that some genera of actinobacteria do not follow this threshold (Rossi-Tamisier *et al.*, 2015) and there is no report so far of it included the genus

Asanoa. At least two *Asanoa* species with validly published name do not respect the 98.7 % threshold. *Asanoa* siamenis shares 99.5 % similarity with its sister group in the phylogenetic tree (Niemhom *et al.*, 2013). *Asanoa endophytica* shares 99.39%, 99.31 % and 99.17% similarities with its three closests type strain in a clade (Niemhom *et al.*, 2016). However, the DNA-DNA relatedness showed that both are new species. At the time of writing only 6 species of the genus *Asanoa* with validly published names were reported. Only one of them was an endophyte isolated from the rhizome of *Boesenbergia rotunda;*this increases the probability for the isolate HVA 6(g)4 being a novel species.

There is no report so far on the bioactive secondary metabolites produced by *Asanoa* species. The genus *Asanoa* belongs to the family Micromonosporaceae together with 16 other genera. Only three genera of this family have been reported to produce antibiotics: the genera *Micromonospora* and *Actinoplanes* are the main ones and the genus *Salinispora* which was just discovered about a decade ago. Nevertheless, it is not impossible that the genus *Asanoa* has potential to possess diverse pathways in secondary metabolism and of rendering novel compounds. The production of secondary metabolites that might arise from different pathways were detected among a number of *Actinoallomurus* strains. It was not known before that this genus has the ability to produce active compounds (Pozzi *et al.*, 2011). Further studies are needed to evaluate the ability of *Asanoa* species to produce bioactive compounds. These can be done by (1) examining type I polyketide synthethase (PKS) and nonribosomal peptide synthase (NRPS) which are involved in the major secondary metabolite-synthetic pathways in actinobacteria (Komaki *et al.*, 2015), (2) analyse the genotypic diversity among *Asanoa* species that indicates their degree of diversity. Genotypic diversity determines whether a genus is a valid source for bioactive compounds (Pozzi *et al.*, 2011).

Among 6 species that belong to the genus *Asanoa*, only one of them was reported to be an endophyte and the rest were isolated from soils. *Asanoa* species is one of the uncommon genera of actinobacteria that are rarely obtained in an isolation work. Woods could be potential niches for other members of *Asanoa* as indicated by the isolation of a strain identified as *Asanoa* and considering that wood of trees is still an unexplored niche for the isolation of this rare genus.

Wood samples used in this study also generated two isolates identified as *Streptomyces* species, which are highly likely to be new species. Their 16S rRNA gene similarity with the closest type strains was less than 98.7%, which is the threshold value to differentiate two species. The first isolate is AA9(b)2 which has the closest similarity with *Streptomyces niveus*^{T} (98.11%), *Streptomyces intermedius*^{T} (97.59%), *Streptomyces pulveraceus*^{T} (97.59%), and *Streptomyces drozdowiczii*^{T} (97.59%). Another isolate, HV6.1, showed similar morphological characteristics as AA9(b)2 and both strains have the same closest type strains, but HV6.1 was isolated from different isolation media and a different site of wood. The second isolate is AA9(b)10 that has the closest similarity with *Streptomyces cocklensis*^{T} (98.66%), *Streptomyces rubidus*^{T} (98.36%), and *Streptomyces yeochonensis*^{T} (98.08%). It is noteworthy that strain AA9(b)10 was isolated at week 6 and considered to be slow a growing *Streptomyces*.

An isolate designated as AA9(a)10 was also identified as *Streptomyces* species. It has 99.25% similarity with its closest type strain, *Streptomyces alboniger*^{T}. It was isolated at week 9, thus this strain is a slow growing *Streptomyces* and this makes it possible to be a new one. Further polyphasic based identification is required to verify its novelty.

This result indicated that woods are prospective sources for the isolation of potentially new *Streptomyces* species. In addition, extending the incubation time allowed the slow growing *Streptomyces* in the wood to appear. Having high quality of biological materials is one of the prerequisites in drug discovery programmes to obtain novel natural products. This can be achieved by exploring new or neglected habitats (Goodfellow and Fiedler, 2010). Compared to other parts of plant, e.g. root, stem and leaves, wood is relatively untapped as an isolation source for endophytic actinobacteria. Therefore, wood can be used as a choice of niche to discover new *Streptomyces* members.

It is noteworthy that continued efforts to harness the genus *Streptomyces* in the screening of secondary metabolites will lead to the discovery of novel bioactive compounds. The genus *Streptomyces* is unsurpassed as the most prolific producer of bioactive secondary metabolites

(Berdy, 2005). Nevertheless, after the number of antibiotics discovered from this genus increased almost exponentially for 17 years (since 1947), it reached a peak in 1970s and declined rapidly in the late 1980s and 1990s. It was proposed that the major cause for stagnation in antibiotic discovery was the decline in the screening effort rather than the exhaustion of compounds produced by this genus. At least 150,000 compounds were estimated to be present in this genus and only around 3% of compounds has been reported so far, leaving a large number of antibiotics to be discovered from the genus *Streptomyces* (Watve *et al.*, 2001).

CHAPTER 3

POLYPHASIC TAXONOMY OF THREE SELECTED ACTINOBACTERIA

3.1 Introduction

Seven endophytic actinobacteria strains isolated from wood in this study were proposed to be new species, to date, based on 16S rRNA gene sequencing as discussed in section 2.3.3. One of them designated as DG1 was obtained from the inner part close to the centre of wood sample taken from a Sydney Blue Gum tree. The other 6 isolates were recovered from the inner part (close to the centre) and outer part (close to the bark) of wood from Australian eucalyptus. A polyphasic taxonomy was conducted for selected isolates to endorse their novelty.

The analysis of the 16S rRNA gene is an approach to achieve the paramount goal in taxonomy which reflects the relationship between organisms that reflect an “order in nature” (Kampfer and Glaeser, 2012). The rRNA genes are the most valuable molecular tools to measure the phylogenetic relationship. In bacteria, the 16S rRNA genes are the most conserved compared to the other two ribosomal RNA genes, 23S and 5S rRNA (Rajendhran and Gunasekaran, 2011). Thus, it was proposed to be a valid chronometer to measure the rate of change in evolution (Woese, 1987). The 16S rRNA gene has become a practical molecular marker for the establishment of a hierarchical taxonomic system. Taxonomy based on 16S rRNA as a foundation in prokaryote taxonomy at the genus level and above has been endorsed by other molecular markers and total or partial genome comparisons (Kampfer and Glaeser, 2012).

A comprehensive characterization should be instituted to a strain or set of strains that appear to be new taxa (Tindall *et al.*, 2010, Moore *et al.*, 2010). The aim is to identify all traits that determine the novelty of strains, and thus facilitate the established characterization and identification (Moore *et al.*, 2010). Nowadays, an integrated characterization called polyphasic taxonomy has been considered as the most suitable basis for classification (Young, 2001). Polyphasic taxonomy include the characterization of phenotypic and genotypic traits. The properties that can be observed resulting

from gene expression of an organism are classified into phenotypic traits whereas the properties within the genetic material are categorized into genotypic traits (Moore *et al.*, 2010).

Phenotypic characterisation is a conventional method to describe prokaryotes. However, it is important for characterization because a biochemical function of a gene alone generally cannot be used to predict the phenotypes. Some genes might only control one phenotype, but most of them affect many phenotypes. Some phenotypes are also under the control of more than one gene. Therefore, phenotypic characterization supports the genomic annotation in a way that phenotypes describe biological activity of the genes (Bochner, 2003). Phenotypic characterization is sometimes beneficial to distinguish taxa at a higher level, such as phylum, class, order, family and lower levels including species and subspecies (Moore *et al.*, 2010). The features of phenotypic characterization are morphology, physiology and biochemical properties. A number of features such as cell shape and size, motility, and spore formation are examined for morphological characterization. Physiological and biochemical characterization includes information on the ability to grow at different temperatures, pH, and salt concentrations, the ability to metabolize compounds, and the presence of a wide range of enzymes (Vandamme *et al.*, 1996). A part of phenotypic characterization is chemotaxonomy to evaluate the chemical constituents of cells including the outer layer of the cells such as peptidoglycan, teichoic acid, mycolic acid, the cell membrane for example fatty acids, polar lipids, respiratory lipoquinones, pigments, and cytoplasmic elements (Tindall *et al.*, 2010).

Nowadays, the introduction of molecular data in the systematic studies of prokaryotes is unquestioned. It is not always an obligatory, depending on the level of taxon. RNA and chromosomal DNA composition do not change by the environmental changes and the nucleic acids are generally distributed. Therefore, genomic information are outstanding instruments as standards for various comparisons, e.g. DNA base ratio (mol% G+C; G+C content; G+C%) which is useful to separate groups in taxonomy, DNA-DNA similarity, and rRNA analysis that has revolutionized prokaryotic taxonomy. The composition of G+C (% mol G+C) content is the first genetic feature that was used in prokaryote characterization. A significantly different G+C content value indicates the different species of bacteria (Rossello-Mora and Amann, 2001). Despite its limited sensitivity for

determination purposes, G+C content is useful parameter to characterize the whole nature of genomic DNA. An organism is classified into different genera whose the G+C content is more than 10% (Moore *et al.*, 2010). As genetic methods develop, DNA-DNA hybridization was established as a gold standard technique to compare prokaryotes. The DNA-DNA hybridization value of 70% was suggested as the cut-off value to differentiate prokaryotes at species levels. Prokaryotes are classified into the same species if the DNA-DNA hybridization value is higher than 70% (Wayne *et al.*, 1987). However, Rossello-Mora and Amann (2001) indicated that DNA-DNA hybridization should not be used alone and that it should be followed by phenotypic determinations that describe a DNA similarity. This technique is laborious and can generate some errors. Various methods of exploring the DNA have been developed to give more reliable results to discriminate prokaryotes, such as DNA fingerprinting, e.g. amplified fragment length polymorphism (AFLP), PCR fingerprinting techniques (REP-,BOX-, ERIC-PCR, RAPD), and Amplified Ribosomal DNA Restriction Analysis (ADRA) (Kampfer and Glaeser, 2012).

3.2 Methodology

3.2.1 Morphological analysis of 3 potentially novel actinobacteria strains

Morphological characterization of the strains encoded as DG1, AA9(a)10, and AA9(b)2 were performed according to Shirling and Gottlieb (1966). Actinobacteria were grown on ISP2 overlayed with sterile cellophane until a good growth was achieved. The spores or mycelia were transferred to 3-5 ml sterile distilled water to prepare a turbid suspension of inoculum. The suspension of spores or mycelia was then inoculated onto media recommended by the International *Streptomyces* Project (ISP): yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol asparagine agar (ISP 5), and tyrosine agar (ISP 7). Three other media were also included for the morphological characterization: Bennet's, half Potato Dextrose Agar (HPDA), and Nutrient Agar (NA). A hatch streak method was applied for the inoculation which was done in duplicate for each medium. The plates were incubated at 27°C for 14 days or until the cultures grew well. The growth and the colour of substrate mycelia and aerial mycelia were recorded. The colour was described based on Methuen Handbook of Colour (Kornerup *et al.*, 1978).

3.2.2 Physiological and chemical analyses

3.2.2.1 Growth at different temperature

The growth of actinobacteria at different temperatures was tested according to the method of Kurup and Schmitt (1973). The actinobacteria were cultured on ISP 2 agar plates by making three streak lines and incubated at 15°C, 27°C, 37°C, and 45°C (in duplicate). The growth was observed at 7 and 14 days.

3.2.2.2 pH tolerance

The ability of actinobacteria strains to grow at different pH was examined on ISP2 medium. The pH of ISP 2 agar plates were adjusted before autoclaving using 0.1N NaOH or 0.1N HCl at pH 4, 5, 6, 7, 8, 9, and 10. The ISP agar plates were made in duplicates for each pH. The plates were incubated at 27°C and the growth was recorded at 7 and 14 days.

3.2.2.3 Tolerance to NaCl

The ability of actinobacteria to grow in a medium containing high concentrations of salt was tested on ISP 2 agar medium. The actinobacteria isolates were inoculated onto ISP 2 agar media in the presence of 1%, 3%, 5%, 10%, 15%, and 20% NaCl (w/v). The plates were incubated at 27°C and the growth was recorded at 7 and 14 days.

3.2.2.4 Acid production from carbohydrate

The capability of actinobacteria strains to utilize carbohydrate was performed based on the acid production from carbohydrates described by Gordon *et al.* (1974). Eighteen carbohydrates were used: L-arabinose, D-cellobiose, D-fructose, D-galactose, D-maltose, D-mannose, D-melezitose, methyl α-D-glucoside, L-rhamnose, sucrose, D-trehalose, D-xylose, adonitol, meso-erythritol, myo-inositol, D-mannitol, and D-sorbitol. A basal inorganic medium containing $(NH4)_2PO_4$ (1 g/l), KCl (0.2 g/l), $MgSO_4.7H_2O$ (0.2 g/l), and agar (15 g/l) was prepared. The pH was adjusted at 7.4 before adding 0.04% bromocresol purple 15ml/L basal medium. After autoclaving, each filter sterilized carbohydrate was added at 1% final concentration to each basal medium. Normally, 10% carbohydrate was made for stock solution. The medium was subsequently poured into tubes to make agar slants. A basal agar medium without carbohydrate was used as control. The actinobacteria

strains were inoculated to the agar slant containing each carbohydrate including control medium (in duplicate) and incubated at 27°C. The result was recorded as positive if the colour of the medium changed to yellow.

3.2.2.5 Decomposition of adenine, L-tyrosine, hypoxanthine and xanthine

Adenine (0.5 g), L-tyrosine (0.5 g), hypoxanthine (0.5 g), and xanthine (0.4) g were suspended separately in 10 ml of distilled water and autoclaved. Each suspension was then mixed with nutrient agar medium (100 ml) which was autoclaved separately and poured into sterile petri dishes. The actinobacteria strains were streaked (cross streak) to the media (in duplicate) and incubated at 27°C for 14 days. The result was recorded as positive if the crystals underneath and around the growth disappeared (Gordon *et al.*, 1974).

3.2.2.6 Decomposition of casein

Five grams of skim milk powder was suspended in 50 ml distilled water. Separately, 1 g of agar was also suspended in 50 ml distilled water. After autoclaving, both suspensions were cooled to 45°C. The two suspensions were then mixed thoroughly and poured into sterile petri dishes. Each strain was streaked across the plate and incubated at 27°C for 14 days. The positive result was observed by the formation of clear zone underneath and around the culture.

3.2.2.7 Decomposition of urea

Urease broth was prepared followed Gordon *et al.* (1974) by adding KH_2PO_4 (10 g), Na_2HPO_4 (9.5 g), yeast extract (1 g), 0.04% solution of phenol red (20 ml) to 1 L of distilled water. The pH was adjusted to 6.7 and autoclaved. Ten mililiters of 15% urea solution was filter sterilized and added to 75 ml of urease broth. A volume of 2.5 ml of the mixture was pipetted aseptically into sterile test tubes and inoculated with actinobacteria strains (in duplicate). The tubes were then shaken for 14 days at 27°C. An alkaline reaction indicated the presence of urease and was observed by the colour change of media to pink compared to control media without urea which was red.

3.2.2.8 Hydrolysis of starch

1.5 g of soluble starch was dissolved in 10 ml cold distilled water. It was subsequently mixed with 100 ml of nutrient agar which had been adjusted to pH 7.4. The mixture was autoclaved and poured into sterile petri dishes. The actinobacteria were streaked across the plate and incubated at 27°C. After 14 days, the plates were flooded with Lugol's Iodine solution (0.1% w/v iodine and 0.2% w/v KI). The test was done in duplicate. The plates were incubated at room temperature for 15-30 minutes. The formation of clear zone around the culture demonstrated the hydrolysis of starch.

3.2.2.9 Use of organic acid

The use of organic acid was examined according to Kurup and Schmitt (1973). A basal medium was prepared by adding NaCl (1g), MgSO4.7H2O (0.2 g), (NH4)2PO4 (0.1 g), KH2PO4 (0.5 g), 0.04% phenol red (20 ml) and agar (15 g) to 1 L distilled water. The pH was adjusted at 6.8. Two grams of organic acid in sodium salt (acetate, benzoate, citrate, lactate, malate, propionate, and tartrate) was added separately to the basal medium. A basal medium without the addition of organic acid was used as a control. The medium was autoclaved and poured into sterile tubes to make agar slants. The actinobacteria were streaked onto the medium and incubated for 14-21 days at 27°C. The formation of purple colour indicated a positive result.

3.2.3 Chemotaxonomic analysis

3.2.3.1 Diaminopimelic acid (DAP) Cell wall analysis

3.2.3.1.1 Preparation of DAP cell wall hydrolysate

The actinobacteria were cultured on ISP 2 medium lined with sterile cellophane. After a good growth was achieved, a loop full of cells or spores were scrapped and transferred to 10 cm glass Pyrex test tube. One ml of 6 N HCl was added to the test tube followed by autoclaving at 121°C for 15 minutes. The suspension was cooled down to room temperature and 2 ml of distilled water added followed by mixing the suspension thoroughly. The mixture was filtered by gravity through Whatman filter paper no. 1 and it was subsequently evaporated in a boiling water bath until dry after which 1 ml of distilled water was added. This step was repeated twice followed by the addition of 40 µl of distilled water to

the final dried residue. The cellular hydrolysate was kept at -20°C until the TLC analysis could be conducted.

3.2.3.1.2 Thin Layer Chromatography for DAP cell wall analysis

TLC for cell wall DAP analysis was performed on a TLC cellulose coated aluminium plate. The plate was preheated at 110°C for about 60 minutes before use. The cell hydrolysates were thawed and then diluted 1 in 10 with sterile distilled water. The diluted cell hydrolysate (2.5-20 µl) was applied to TLC plate in a 1 cm lane and dried using a hair dryer. A standard mixture containing both –meso and –LL DAP (0.1M DAP in 0.2 M NaOH) was also applied to the plate. The plate was placed into a solvent system containing pyridine:6 M HCl: water:methanol (10:4:26:80) that was equilibrated in a TLC chamber before developing the TLC plate and it was run for approximately 3 hours. The solvent system could be reused up to three times.

3.2.3.1.3 Colour development of DAP analysis

The plate was removed from the TLC chamber when the solvent system was about 1.5 cm from the top of the TLC plate. The plate was allowed to dry in the fume hood and was subsequently sprayed with a 0.2% ninhydrin in acetone spray reagent. The dry plate was heated in the oven at 100°C for 5-10 minutes to develop the colour for DAP detection. The DAP were observed as olive green fading to yellow bands. The LL-isomer moved further than the meso-isomer.

3.2.3.2 Sugar cell wall analysis

3.2.3.2.1 Preparation of sugar cell wall hydrolysate

Sugar cell wall analysis was performed according to Hasegawa *et al.* (1983). The actinobacteria were cultured on ISP 2 medium lined with sterile cellophane. After a good growth was achieved, a loop full of cells or spores were scrapped off and transferred to 10 cm glass Pyrex test tubes containing 200 µl 0.25 N HCl. A lid was placed on each tube and heated at 121°C for 15 min (standard autoclave run). The hydrolysate was allowed to cool and centrifuged at 12,000 g. The supernatant was taken out and filtered with Whatman paper No.1 to remove the cell debris. The cell hydrolysate was then stored at -20°C until TLC could be done.

3.2.3.2.2 Thin Layer Chromatography for sugar cell wall analysis

Two microlitres of the hydrolysate was spotted onto a cellulose coated aluminium TLC sheet (Merck 5552). A 1 µl aliquot of a standard solution 1% (w/v) (arabinose, galactose, glucose, mannose, rhamnose, ribose and xylose) was also applied to the TLC plate. The plate was then run using a solvent system consisting of n-butanol: water: pyridine: toluene (10:6:6:1) which had been equilibrated in the chromatography chamber for up to 1 hr. This biphasic solvent system could be used only 2 times. The sugar cell wall hydrolysate was developed in an ascending chromatography system for 3 hours.

3.2.3.2.3 Colour development for sugar cell wall detection

The TLC plate was air dried and sprayed with the aniline phthalic acid reagent that was prepared by dissolving aniline (2 ml) and phthalic acid (3.3 g) in water saturated n-butanol (100 ml). The TLC plate was heated in the oven at 100◦C for 10 min to develop colour for sugars identification.

3.2.3.3 Lipid cell wall analysis

The composition of actinobacteria lipid cell wall was conducted followed the method of Minnikin *et al.* (1984)

3.2.3.3.1 Preparation of dried cells for menaquinone and phospholipid analysis

Actinobacteria isolates were grown on ISP2 plates lined with sterile cellophane for 7-10 days or until good growth was achieved. Two loops of cells or spores were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of liquid ISP2 medium. The flasks were put in the shaker incubator at 28°C, 150 rpm for 7-10 days to achieve a good growth. The biomass was then collected by centrifugation at 3000g followed by washing three times with sterile distilled water. The washed biomass was then freeze dried and kept at -20°C for further analysis.

3.2.3.3.2 Menaquinone analysis

About 50 mg of dried biomass prepared in section 3.2.3.3.1 was put into a 8.5 ml polytetrafluoroethene capped tube. Two mililiters mixture of methanol-0.3% aqueous sodium

chloride solution (100:10) and 2 ml of petroleum ether were added to the tube. The tube was subsequently placed on an end by end shaker for 15 minutes mixing. The mixture was allowed to separate and the upper layer was transferred to a clean tube. Petroleum ether was then added (1 ml) and the tube was put again on an end by and shaker for another 15 minutes mixing. The mixture was allowed to separate and the upper layer was taken out to combine with the previous upper layer. This upper layer fraction was evaporated using a vacuum evaporator and the resultant extract was further purified for menaquinone analysis.

The extract was resuspended in 50 µl acetone and applied on 10X10 cm pre-coated silica TLC plate (a 60 F_{254} plastic backed plate). Standard vitamin K (1%) was also applied on the TLC plate. The plate was run using a solvent system containing hexane:diethyl ether (85:15) which had been equilibrated for an hour. The TLC plate was taken out from the TLC chamber as soon as the solvent reached about 1.5 cm from the top of the plate. The plate was air dried in the fume hood followed by identification of the presence of menaquinone as a dark brown band and a green fluorescent background under 254 nm UV light. The Rf value of the menaquinone band should be the same as standard vitamin K.

The menaquinone band was scraped from the TLC plate and extracted with 1 ml of acetone. It was vortexed followed by shaking in a shaker for 30 minutes and then centrifuged at 8000 g for 5 minutes. The supernatant was transferred to a clean tube and dried using a vacuum evaporator. The dried sample was resuspended with 50 µl acetone and transferred to a small vial. Reverse phase LC/MS employing UV detection and electrospray mass spectrometry (ESI) was run to analyse the purified menaquinone. The column was ZORBAX Eclipse XBD-C18, 4.6 x 150 mm, 5 µm and the solvent was isopropanol:methanol (1:1) at a flow rate of 1.0 ml/min.

3.2.3.3.3 Phospholipid analysis

The lower layer after methanol-aqueous sodium chloride-petroleum ether extraction (section 3.2.3.3.2) was heated in a boiling water bath for 5 minutes followed by cooling in a 37°C water bath for 5 minutes. Two point three mililiters of chloroform:methanol:water (90:100:30) mixture was added

to the tube. It was then put on an end by end mixing shaker for 60 minutes followed by centrifugation at 3000 rpm for 10 minutes and the supernatant was transferred to a clean tube. A 0.75 ml mixture of chloroform:methanol:water (50:100:40) was added to the tube containing the residue after the supernatant was taken out. It was then mixed on an end by end shaker for 30 minutes, followed by centrifugation at 3000 rpm for 10 minutes and the supernatant was taken out and combined with the previous one. Another 0.75 ml was added to the tube containing the residue after the supernatant was taken out, followed by mixing on an end by end shaker for 30 minutes and centrifugationat 3000 rpm for 10 minutes. The supernatant was taken out and combined with the previous one. Chloroform (1.3 ml) and 0.3% aqueous sodium chloride solution (1.3 ml) were subsequently added to the combined supernatant. It was centrifuged at 3000 rpm for 10 minutes after mixing thoroughly. The upper layer was discarded and the lower layer was evaporated using a vacuum evaporator until it dried.

The polar lipid extract was dissolved in 60 µl of chloroform-methanol (2:1) and 10 µl each was applied to the corner of 5 aluminium backed silica TLC plates with 6.6 x 6.6 cm dimensions. Two dimension thin layer chromatography was performed to identify the composition of cell wall polar lipids. The plate was developed in a solvent system containing chloroform:methanol:water (65:25:4) for the first direction followed by running it in chloroform:acetic acid:methanol:water (40:7.5:6:2) for the second direction (the TLC plate was rotated 90° towards the position where the sample was spotted).

The TLC plates were sprayed using 5 spray reagents to detect the presence of phospholipids. The first plate was sprayed with 5% ethanolic molybdophosphoric acid, followed by heating at 180°C for 15 minutes to reveal the presence of all lipids. The second plate was sprayed with ninhydrin spray reagent followed by heating at 110°C for 5 minutes. Polar lipids that have free amino groups, for example phosphatidylethanolamine and phosphatidylserine, give positive reaction with ninhydrin reagent as pink spots. If he pink spots appeared, they were marked lightly with pencil. After the plate cold, it was then sprayed with molybdenum blue reagent to reveal the presence of phospholipids such as phosphatidic acid, phosphatidylethanolamine, phosphatidylserine as blue spots. The third TLC plate was sprayed with alpha napthol sulphuric acid reagent and then heated at 110°C for 15

minutes to detect the presence of glycolipids as brown spots. The fourth plate was sprayed with periodate-Schiff reagent for the identification of alpha glycols. Phosphatidylglycerol presents as a rapidly developing purple colour, phosphatidylinositol appears as yellow colour after 40 minutes or more, while glycolipids including phosphatidylinositolmannosides shows up as slowly developing blue colour. The last plate was sprayed with Dragendorff reagent. Phosphatidylcholine and its lyso derivatives and sphingomyelin give a positive reaction as orange spots.

3.2.3.3.4 Fatty Acid Methyl Esters (FAMEs)

Actinobacteria were grown on ISP2 plate lined with sterile cellophane. When a good growth was achieved two loops of cells/spores were inoculated into 250 ml flasks containing 50 ml of Tryptic Soy Broth (TSB) or ISP2 broth to obtain good growth. The flasks were put on the shaker at 150 rpm, at 28°C for 7-10 days to obtain a good growth. The biomass was harvested by centrifugation at 3000 g followed by washing with sterile distilled water 3 times.

About 100-300 mg of wet cells was put in a clean 13x100 mm glass test tube and subjected to sequential reactions as follow:

1. Saponification.

   The cells were treated with a strong methanolic base at high temperature to expose fatty acids from the cell lipids and transformed to their sodium salts. One mililiter of 3.75 M NaOH in methanol (reagent 1) was added to wet cells in the tubes. The tubes were vortexed briefly and heated in a boiling water bath at 87°C for 5 minutes. The tubes were further vortexed vigorously for 5-10 seconds and returned to the water bath for 25 minutes to complete the 30 minutes heating.

2. Methylation

   This step converts the fatty acids (as sodium salts) to fatty acid methyl esters which increase the volatility of fatty acids for Gas Chromatography analysis. Three point two mililiters of 3.2 N HCl

in methanol (reagent 2) was added to each tube and vortexed briefly. The tubes were subsequently put in a water bath at 80 ±1°C for 10 ± 1 minute.

3. Extraction

   Liquid-liquid extraction was done to remove fatty acid methyl esters from the acidic aqueous phase. A mixture of 1 : 1 hexane:tert-butyl methyl ether (1.25 ml) was added to the cooled tubes which were then mixed on an end by end mixing shaker for approximately 10 minutes. The mixture was allowed to separate and the aqueous (lower) layer was pipetted out and discarded.

4. Base wash.

   This step was done to reduce contamination of the injection port liner, the column and the detector. Three ml of 0.3 M NaOH was added to each tube and then mixed for 5 min on a shaker. About two third of the organic phase was pipetted out, passed through a clean Pasteur pipette that has been filled with anhydrous sodium sulphate (cotton was put at the lower end of the pasteur pipette) and the organic phase was collected in a GC vial capped and ready for analysis.

The samples were subjected to Gas Chromatography and the fatty acid composition was identified using the MIDI Hewlett-Packard Microbial Identification (MIS) as described in the Microbial Identification Inc (MIDI) protocol.

3.2.3.4 DNA sequence analysis

3.2.3.4.1 16S rRNA gene sequence determination

The procedure for 16S rRNA gene determination has been described in chapter 2 section 2.2.2

3.2.3.4.2 Phylogenetic analysis

The 16S rRNA gene sequence of each of the isolates chosen for polyphasic taxonomy was multiply aligned with the 16S rRNA gene sequence of the most closely representative species available in the EzTaxon database using the CLUSTAL W. Phylogenetic trees were constructed based on the neighbour-joining and maximum likelihood methods using the software package MEGA version 6. A

Kimura two parameter model was used to calculate pairwise distances for the neighbour-joining algorithm.

3.3 Results and Discussion

3.3.1 The selection of 3 actinobacteria species for polyphasic taxonomy

Among 7 isolates that were predicted to be novel, three of them were selected for polyphasic taxonomy to confirm their novelty: DG1, AA9(a)10, and AA9(b)2. Based on 16S rRNA gene sequencing, strain DG1 belongs to the genus *Actinomadura* and has the closest relationship with *Actinomadura* citrea^T. Strain DG1 showed activity against multi resistant *S. aureus* overexpressing *NorA* efflux pump, but not A. *citrea*^{T}. Despite the high 16S rRNA similarities between DG1 and the closest type strain (99.4%), they could be different species/strains due to their ability to produce different active compounds. Therefore, it was chosen for polyphasic taxonomy.

Two other isolates were identified as *Streptomyces* species. Strain AA9(B)2 has 98.11% similarity with *Streptomyces niveus*^{T} based on 16S rRNA gene sequencing. This isolate has a greater chance to be a new species as the recommended threshold for 16S rRNA similarity value is 98.7% to discriminate at species level (Stackebrandt and Ebers, 2006). Thus, this isolate was subjected to polyphasic taxonomy to confirm its novelty. Another isolate was designated as AA9(a)10. It has 99.25% 16S rRNA gene sequence with the closest strain, *Streptomyces alboniger*^{T}. Isolate AA9(a)10 is a slow growing actinobacterium, as it was recovered from the isolation plate at week 11. It is most likely that slow growing actinobacteria would be a new species as the probability for this type of isolate to be picked up previously is lower than the faster growing ones. Thus, despite the high 16S rRNA gene similarity between isolate AA9(a)10 and the closest type strain, it was chosen for polyphasic taxonomy characterization. It was shown that many *Streptomyces* species have over 99% 16S rRNA similarity with their phylogenetically closest neighbours. *Streptomyces atroaurantiacus* has more than 99% similarities to other *Streptomyces* species. *Streptomyces durmitorensis* shares 99.6% similarity and 99.2% with *Streptomyces aureus* and *Streptomyces*

kanamyceticus, respectively. However, the DNA similarities are less than 70% (Goodfellow *et al.*, 2012).

3.3.2 Endophytic actinobacterium strain DG1

3.3.2.1 Morphological and cultural analyses

The morphological and cultural characteristics of strain DG1 were compared to the three closest type cultures: *Actinomadura citrea*, *Actinomadura mexicana* and *Actinomadura maheshkaliensis.* Strain DG1 grows well on almost all media used for morphological characterization. More abundant growth was observed on yeast extract-malt extract agar than oatmeal agar. Most Actinomadura strains grow very well on oatmeal agar, but some grow better on yeast extract-malt extract agar such as *Actinomadura kijaniata* and *Actinomadura macra*. Isolate DG1 forms substrate mycelia and aerial mycelia which differentiate into straight chain of warty ornamentation spores (figure 3.1), except on Bennet's and half strength Potato Dextrose agar media. Pale yellow to greenish yellow aerial mycelia were observed and it becomes turquoise at maturity. Powdery aerial mycelia were formed after cultivation for 10-14 days.

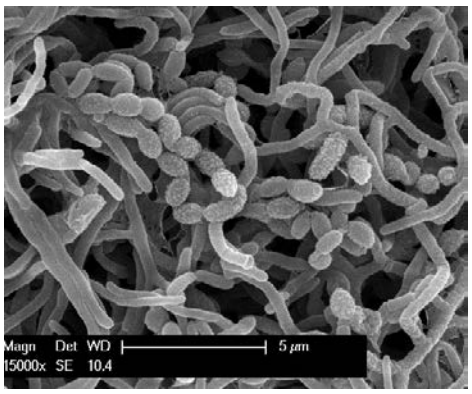


Figure 3.1. Scanning electron microscopy of strain DG1 showing straight chains of warty ornamented spores after culture on ISP2 for 15 days at 28° C. Bar, 2 µm

The morphological and cultural properties of DG1 are consistent with *Actinomadura* strains which characteristically form substrate mycelia and spore bearing, powdery aerial mycelium on media such as inorganic salts-starch, oatmeal, and yeast extract-malt extract agars after about one to two weeks cultivation. Mature aerial mycelia can be blue, cream, grey, green, pink, yellow or white which may make this genus superficially different from *Streptomyces*. However, spores have not been detected

in some strains such as *Actinomadura fibrosa* and *Actinomadura latina*, and some strains lack aerial mycelium, especially the ones that originated from clinical isolates (Goodfellow *et al.*, 2012). The cultural characteristics of strain DG1 on different media are shown in Table **3.1**.

3.3.2.2 Physiological and biochemical characteristics

Physiological and biochemical properties of strain DG1 were analysed and compared directly with those of the closest related actinobacteria, *A. citrea*^{T}, *A. mexicana*^{T} and *A. maheshkhaliensis*. *Actinomadura* strains are able to utilize a wide range of sugars as carbon sources to grow. Strain DG1 metabolized most carbons used in this study as sole carbon source, except melezitose, galactose, mannose, methyl D-glucopyranoside, meso erythritol and sorbitol. It decomposed L-tyrosine, but not adenine, xanthine and hypoxanthine. Hydrolysis of starch and casein by DG1 strain was observed, but urea was not hydrolysed. Species belonging to the genus *Actinomadura* mostly hydrolyse casein, but the ability to hydrolyse starch and to produce urease varies between species. Strain DG1 has the ability to assimilate organic acids including acetate, citrate, lactate and propionate, weakly assimilates malate, but is unable to assimilate tartrate. It grows at temperatures between 27 and 37°C and between pH 6-10. No growth was observed at pH 4 and 5. The maximum concentration of NaCl tolerated by this strain is 3%.

Table 3.1 Cultural characteristic of strain DG1

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract malt extract agar (ISP2)	Good	Good pale yellow spores	yellowish white
Oatmeal agar (ISP3)	Good	Good pale turquoise spores	yellowish white
Inorganic salt starch agar (ISP4)	Moderate	Moderate light turquoise at the edge,pale yellow spores in the middle	yellowish white
Glycerol asparagine agar (ISP5)	Good	Good pinkhish white spores at the edge and turquoise white spores in the middle	yellowish white
Tyrosin agar (ISP7)	Moderate no melanin pigment	Moderate yellowish white spores	pale yellow
Bennett's agar	Good	good, greenish yellow,no spore	greenish yellow
Half strength potato dextrose agar	Good	good, greenish yellow, no spore	greenish yellow
Nutrient agar	Moderate	moderate, ivory with white spores in some area	yellowish white

Several physiological and biochemical properties differentiate *Actinomadura* strain DG1 from phylogenetically related reference strains mentioned above. Strain DG1 is dissimilar from *A. citrea*^{T}

as it does not grow at pH 5 and grows poorly at 15°C, while *A. citrea*^{T} grows poorly at pH 5, but grows well at 15°C. Also, *A. citrea*^{T} assimilates benzoate and weakly assimilates lactate, whereas strain DG1 assimilates lactate, but not benzoate. The ability to grow at pH 5 and at 15°C, to decompose adenine and hypoxanthine, to hydrolyse starch, to assimilate citrate and benzoate, to utilize fructose and mannitol differentiate strain DG1 from *A. mexicana*^{T}. Strain DG1 is different from *A. maheshkhaliensis* in its ability to grow at 15 °C, tolerate salt, decompose hypoxanthine, hydrolyse starch and urea, and to assimilate propionate. The physiological and biochemical properties that distinguish strain DG1 from the phylogenetically closest type strains are summarized in table 3.2. Different results was obtained on the utilization of galactose by *A. mexicana*^{T} and the utilization of fructose by *A. maheshkhaliensis* In this study compared to a literature. In this study, it is showed that *A. mexicana*^{T} was able to utilize galactose, but it was not as mentioned in Bergey's Manual of Systematic Bacteriology (Goodfellow *et al.*, 2012). *A. maheshkhaliensis* utilized fructose, but it was not as observed by Ara *et al.* (2008). These differences could happen as all methods to characterize phenotypic traits develop different degrees of reproducibility and comparability of results between different laboratories, even though the trends in phenotypic analysis for substrate utilization are generally stable and reliable (Moore *et al.*, 2010) .

Table 3.2 Phenotypic characteristics that differentiate strain *Actinomadura* DG1 from other *Actinomadura* related reference strains

Strains: 1. DG1, 2. *A. citrea* NRRL B16121^{T}, 3. *A. mexicana* JCM 13236^{T}, 4. A. *maheshkhaliensis* JCM 13934. All data were obtained in this study: (+) indicated the positive activity or growth occurred, (-) indicated no activity or no growth (w) indicated week activity, (p) indicated poor growth

Characteristics	1	2	3	4		Characteristics	1	2	3	4
Decomposition of:						Carbon utilization of:				
Hypoxanthine	–	–	w	+		Fructose	+	+	–	+
						Galactose	–	–	+	–
Hydrolyis of:						Mannitol	+	+	–	+
Starch	w	w	–	+						
Urea	–	–	–	+		Salt tolerance				
						5%	–	–	–	+
Acid assimilation of:										
Benzoate	–	+	+	NG		Growth at				
Citrate	+	+	w	+		15°	p	+	+	+
Lactate	+	w	+	+						
Malate	w	w	w	†		Growth at pH				
Propionate	+	+	+	–		5	–	p	p	–

3.3.2.3 Chemotaxonomic analyses

Chemotaxonomic characters of strain DG1 were compared to those of *A. citrea*^{T}, *A. mexicana*^{T}, and *A. maheshkhaliensis*, which are the type strains most closely related to strain DG1 based on 16S rRNA gene sequencing. The cell wall of strain DG1 contained meso-diaminopimelic acid (meso-DAP). Galactose, glucose, and mannose were detected as sugar constituents. The genus *Actinomadura* commonly has meso-DAP and galactose, glucose, and mannose as the cell wall sugars. Some species may also contain other sugars, for example ribose. Thus, the types of diaminopimelic acid and sugars in the cell wall of strain DG1 are in accordance with those of the genus *Actinomadura*, and support the classification of this strain to this genus.

Strain DG1 was found to contain predominant proportions of hexahydrogenated menaquinones with nine isoprene units, MK-9(H_6), followed by the corresponding octahydrogenated component, MK-9(H_8) and minor proportions of the corresponding tetrahydrogenated component, MK-9(H_4). Dihydrogenated menaquinone with nine isoprene units, MK-9(H_2), was also detected at concentrations less than 5%. This was consistent with the composition of menaquinones for most of *Actinomadura* strains, even though nine isoprene units of octahydrogenated menaquinones are usually present in small amounts and the corresponding dehydrogenated component is not mentioned as a common menaquinone constituent. However, it was detected in *A. citrea*^{T} at very low concentrations (1%) (Kroppenstedt *et al.*, 1990).

Polar lipids analysis of strain DG1 showed the presence of DPG, PG, and PI. Most *Actinomadura* species contain diphosphatidylglycerol (DPG), phosphatidylinositol (PI), and phosphatidylinositol mannosides (PIms) (Lechevalier *et al.*, 1977). However, the presence of these polar lipids varies between species and PG was also detected in some species (Kroppenstedt *et al.*, 1990, Ara *et al.*, 2008). The presence of DPG, PG, and PI supported the classification of strain DG1 into the genus *Actinomadura*.

Hexadecanoic (16:0) and 10-methyl octadecanoic (tuberculostearic acid) are the predominant fatty acid components of strain DG1 accounting for 44.67% and 15.68%, respectively. This is in

accordance with the characteristic of most *Actinomadurae* strains that contain a complex mixture of fatty acids dominated with hexadecanoic (16:0), 14-methylpentadecanoic (iso-16:0), and 10-methyloctadecanoic (Kroppenstedt *et al.*, 1990). These further supports the classification of strain DG1 into the genus *Actinomadura*. The third highest fatty acid constituent is cis9-18:1 that is present in comparable proportions with tuberculostearic acid. *Iso*-16:0, another major fatty acid present in most *Actinomadura*e strains, was also detected in strain DG1, but at less than 5%. The fatty acid composition that was detected in strain DG1 and all closely related type strains is summarized in table 3.3. Only fatty acids that were present at a concentration of 1% or more are included in the table.

Table 3.3 The fatty acid composition (%) of strain DG1 and its phylogenetically closest related reference strains

Fatty acids	Species			
	DG 1	*A. citrea* ^{T}	*A. maheshkhaliensis*	*A. mexicana* ^{T}
14:0	4.96	2.86	5.29	2.45
15:0	0.83	0.99	0.45	5.94
Iso-16:0	4.47	4.27	1.81	10.42
Cis9 16:1	5.83	9.44	11.7	5.34
10 methyl 16:0	0.57	1.06	0.27	1.28
16:0	44.67	47.16	37.24	37.98
Cis9 17:1	0.24	0.38	0.29	2.05
17:0	0.67	0.72	0.54	3.59
10 methyl 17:0	0.27	0.38	0.17	2.13
Cis9 18:1	14.37	10.83	19.44	11.32
18:0	3.7	1.22	4.97	1.38
TBSA 10 methyl 18:0	15.68	18.93	12.96	13.57

3.3.2.4 Genetic analyses

3.3.2.4.1 DNA sequence analyses

Based on the 16S rRNA gene sequence, strain DG1 has the highest similarity with *Actinomadura citrea*^{T}, *Actinomadura mexicana*^{T}, and *A. maheshkhaliensis*. The similarity values between strain DG1 and *A. citrea* is 99.48% and 99.4% between strain DG1 and both *A. mexicana*^{T} and *A. maheshkhaliensis*.

3.3.2.4.2 Phylogenetic trees for strain DG1

The 16S rRNA gene sequence of strain DG1 was subjected to phylogenetic analysis. Validly published *Actinomadura* species, except for *A. maheshkhaliensis*, were used for 16S rRNA gene sequence comparison with strain DG1, as shown in figure 3.2. The phylogenetic tree indicated that

strain DG1 is a member of the genus *Actinomadura* in the family of Thermonomosporaceae. Strain DG1 falls in the same clade with two of its closest neighbours, *Actinomadura citrea*^{T} and *Actinomadura mexicana*^{T}. The affiliation between strain DG1 and the three closest type strains was supported by both neighbour-joining and maximum likelihood algorithms. Figure 3.2 shows the neighbour-joining tree based algorithm of strain DG1 and its closest type strains.

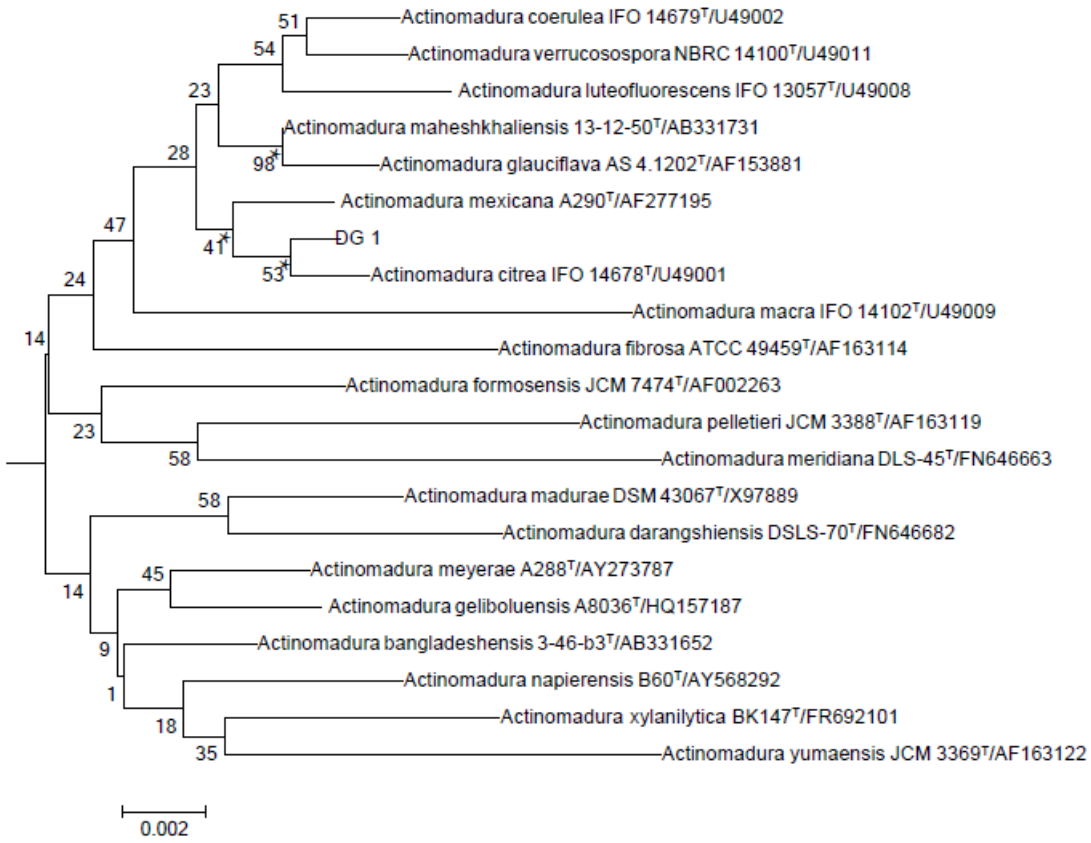


Figure 3.2 Neighbour-joining tree demonstrating the relationship between strain DG1 and representative related members of the genus *Actinomadura.* Asterisk indicates branches of the tree that were also recovered using maximum-likelihood treeing algorithms

3.3.2.5 Differentiation of strain DG1 and the closest type strains

Based on the phenotypic properties, a number of differences were observed between strain DG 1 and its phylogenetically closest type strains *Actinomadura citrea*^{T}. Strain DG1 and *A. citrea*^{T} have warty ornamented spores, but the spore chains of strain DG1 are straight whereas *A. citrea*^{T} forms hooked or curly spore chains (Goodfellow *et al.*, 2012). The physiological and biochemical characteristics of strain DG1 are different from *Actinomadura citrea*^{T}. Strain DG1 is not able to assimilate benzoate, and weakly assimilate lactate whereas *A. citrea*^{T} can to assimilate both organic

acids. Strain DG1 grew poorly at 15°C and not at all at pH 5, while *A. citrea*^{T} showed good growth at 15°C and grow at pH 5 even though it was poor.

Chemotaxonomy data showed that the major menaquinone of *A. citrea*^{T} is MK-9(H6), accounting for 80%, followed by small proportion (15%) of MK-9(H8) (Kroppenstedt *et al.*, 1990). This composition is common for *Actinomadura* species. Strain DG1 also contains of MK-9(H6) as the highest menaquinone followed by MK-9(H8), but there was only about 10% difference between MK-9(H8) and MK-9(H6). Fatty acid composition of both strains is differentiated only by the presence of palmitoleic acid (9-*cis*-hexadecanoic acid) which is two times higher in *A. citrea*^{T} and stearic acid (octadecanoic acid) which is four times higher in strain DG1. The composition of other detected fatty acids of strain DG1 and *A. citrea*^{T} are similar (table 3.3)

Strain DG1 and *Actinomadura mexicana*^{T} share 99.4% similarity, which is almost the same as the similarity value between strain DG1 and *Actinomadura citrea*^{T}. These species are also in the same clade. However, more differences between strain DG 1 and *Actinomadura mexicana*^{T} were observed based on phenotypic characteristics that distinguished both strains. Hooked spore chains were formed in *A. mexicana*^{T} (Quintana *et al.*, 2003), but *A. citrea* formed straight chains of spores (Goodfellow *et al.*, 2012). Their morphological characteristics, including growth, the formation of spores and the colour of aerial mycelium on 8 medium are different. They had different profiles for the decomposition of xanthine, benzoate and citrate assimilation, and the utilization of fructose, galactose and mannitol as presented in table 3.2. Both strains also have different compositions of menaquinone and fatty acids. Menaquinone MK-9(H8) was not present in *Actinomadura mexicana*^{T}, but a considerable amount of this menaquinone was detected in strain DG1. Menaquinone MK-9(H2) was also not detected in *Actinomadura mexicana*^{T}, but it was present in small amounts in strain DG1. Among 12 fatty acids that were present at ≥ 2% in either *Actinomadura mexicana*^{T} or strain DG1, 6 fatty acids were found to show different proportions ranging from 2-6 times, as shown in table 3.3.

The similarity value of 16S rRNA gene sequence of strain DG1 and *Actinomadura maheshkhaliensis* was also 99.4%. However, both strains were present in different clusters. This indicated that strain

DG1 is different from *A. maheshkhaliensis*. This is supported by differences between their physiological and biochemical properties, including the ability to decompose hypoxanthine, hydrolyse starch and urea, and assimilate malate and propionate. *A. maheshkhaliensis* grow well at 15°C and in medium containing up to 5% NaCl. Strain DG1, on the other hand, grew poorly at 15°C and only tolerated up to 3% NaCl in the growth medium.

A number of differences based on chemotaxonomic, physiological and biochemical characteristics were observed between strain DG1 and its closest type strain, *A. citrea*, as discussed above. However, they are not strong enough to conclude that strain DG 1 is a novel species. Apart from the finding that the similarity between strain DG1 and *A. citrea* outweighs their dissimilarity, the ability to produce bioactives compound should not be overlooked. Strain DG1 produces compounds active against resistant *S. aureus,* overexpressing the efflux pump (see chapter 4 and 5). At the time of writing, there was no report on bioactive compounds produced by *A. citrea*. Thus, strain DG1 could be a subspecies of *A. citrea* as in the case of the identification of *A. cremea* subspecies *rifamycini*. There are no characteristic differences between this strain and *A. cremea* except for the ability of *A. cremea* subspecies *rifamycini* to produce rifamycin (Goodfellow *et al.*, 2012)

3.3.2.6 Description of strain DG1

Strain DG1 forms yellowish white substrate mycelium and aerial mycelium which differentiates into straight chains of spores. The spores are warty ornamented. Abundant pale yellow spores form on glucose-yeast-malt extract agar and become turquoise at maturity. No spores are formed on Bennet's medium and half strength potato dextrose agar and the aerial mycelia were greenish yellow. It grew well between 27° and 37°C, pH 6-10, and is able to tolerate up to 3% NaCl in the medium. Strain DG1 degrades L-tyrosine and casein, weakly degrades starch, but not urea. It assimilates organic acids including acetate, citrate, lactate, propionate and weakly assimilates malate. It is able to utilize a wide range of sugars, except melezitose, galactose, methyl-D-glucopyranoside, meso-erythritol and sorbitol. The isomer of DAP is *meso* whereas galactose and glucose occur in the whole cell hydrolysate. Hexadecanoic (16:0) and 10-methyl octadecanoic are the predominant fatty acid components of strain DG1. The major menaquinone present in strain DG1

are MK-9(H6) and MK-9(H8). Strain DG 1 has activity against resistant *Staphylococcus* overexpressing Nor A efflux pump (evident for this statement refer to chapter 5).

3.3.3 Endophytic actinobacterium strain AA9(a)10

3.3.3.1 Morphological and cultural analysis

The morphological and cultural characteristics of strain AA9(a)10 was compared to the three closest type culture strains, *Streptomyces alboniger*^{T}*, Streptomyces phaeolutegriseus*^{T}*, and Streptomyces galilaeus*^{T}. Moderate to good growth of AA9(a)10 was observed on all media used for morphological characterization, except for nutrient agar. However, it poorly produces spores on almost all media used for morphological characterization, including on oatmeal agar normally induces sporulation. This is likely due to different cultivation conditions that can influence spore formation. Good sporulation was only observed on inorganic salt-starch agar medium (ISP4), possibly due to the high carbon:nitrogen content in ISP4. Spore production is commonly prolific on this type of media. The formation of spores is controlled by many different genes.

The aerial mycelium, offer features for detailed microscopic characterization. *Streptomyces* species produces chains of three to up to more than 50 spores (Goodfellow *et al.*, 2012). Strain AA9(a)10 formed long chains of cylindrical spores with smooth surface as showed in figure 3.3. This supports the classification of AA9(a)10 into the genus *Streptomyces*.

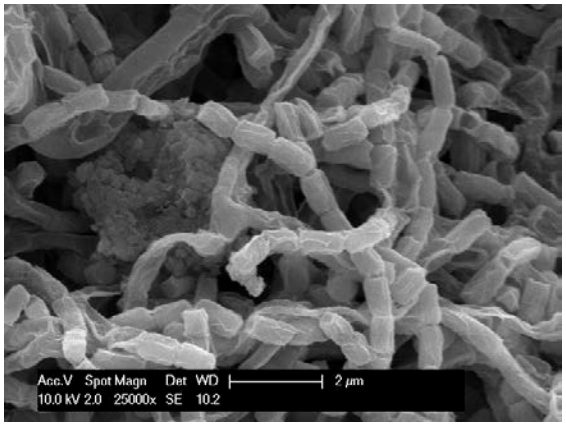


Figure 3.3 The scanning electron microscopy of strain AA9(a)10 showing the formation of spores chains

Strain AA9(a)10 produced light brown pigment on tyrosine agar medium (ISP 7), indicating the production of melanin. Brown diffusible pigment was observed on oat meal agar. Some

Streptomyces species are known to form coloured diffusible pigments in some media (Goodfellow *et al.*, 2012). The cultural characteristics of strain AA9(a)10 on different media are shown in table 3.4.

Table 3.4 Cultural characteristics of strain AA9(a)10

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract malt extract agar (ISP2)	Good	Good, dull yellow, no spore	Dull yellow
Oatmeal agar (ISP3)	Moderate	Moderate, bamboo light with very scarce white spores	Bamboo light
Inorganic salt starch agar (ISP4)	Good	Good, dull yellow, with pearl white spores on some areas	greyish yellow
Glycerol asparagine agar (ISP5)	Moderate	Moderate, dull yellow , no spore	yellowish grey
Tyrosin agar (ISP7)	Good, light brown pigment (camel)	Good, Brown (somalis), no spore	Light brown
Bennett's agar	Moderate	Moderate,yellowish grey, no spore	yellowish grey
Half strength potato dextrose agar	Moderate	Moderate, wax yellow, no spore	yellowish white
Nutrient agar	Poor	Poor, yellowish white, no spore	yellowish white

3.3.3.2 Physiological and biochemical characteristics

Physiological and biochemical properties of strain AA9(a)10 were analysed and compared directly with those of the closest related actinobacteria, *S. alboniger, S. phaeoluteigriseus, and S. galilaeus*. As for other species that belong to the genus *Streptomyces*, strain AA9(a)10 widely used carbon sources for energy and growth, such as cellobiose, glucose, mannose, trehalose. This strain was also able to metabolize other sugars as sole carbon source including arabinose, galactose, maltose, rhamnose, and xylose. The vast majority of *Streptomyces* species degrade polymeric substrates, such as adenine, hypoxanthine, casein, L-tyrosine and starch. Some *Streptomyces* species have been found to produce amylase that supports their ability to degrade starch (Chakraborty *et al.*, 2009, Syed *et al.*, 2009). It was observed that strain AA9(a)10 decomposes adenine, hypoxanthine, and weakly degrades xanthine but not L-tyrosine. Some members of the genus *Streptomyces* degrade xanthine, such as *Streptomyces ferralitis* (Saintpierre-Bonnacio *et al.*, 2004), *Streptomyces capillispiralis* (Mertz and Higgens, 1982), and *Streptomyces avermectinius* (Takahashi *et al.*, 2002) and some do not. Starch, casein, and urea are hydrolysed by strain AA9(a)10. Strain AA9(a)10 has the ability to assimilate acetate, lactate and propionate, but not benzoate, citrate, malate and tartrate.

It grows at temperatures between 15 and 27°C and between pH 4-10. The pH growth of most *Streptomyces* ranges from 5.0 to 9.0, with an optimum pH close to 7 (6.5-8.0). However, a number of species in this genus have been reported to be either acidotolerant or acidophilic and grow at pH 3.5 to 6.5 with optimum pH between 4.5 and 5.5. No growth was observed on media containing more than 3% NaCl.

A number of different physiological and biochemical properties are present between strain AA9(a)10 and phylogenetically related reference strains mentioned above. Strain AA9(a)10 is dissimilar from *S. alboniger* as it does not grow at 37°C, it can grow at pH 4 and unable to tolerate 5% or more NaCl. In contrast, *S. alboniger*^{T} can grow at 37°C, is unable to grow at pH 5 but can grow on medium containing 5% NaCl although the growth is poor. Strain AA9(a)10 can assimilate lactate, but not citrate or malate. Inversely, *S. alboniger*^{T} assimilates citrate and malate, but not lactate. Differences between AA9(a)10 and *S. alboniger* to grow on the media containing fructose, mannitol, adonitol, and sucrose were also observed. Strain AA9(a)10 degrade casein more than *S. alboniger*^{T}. The ability to grow at pH 4, to grow at 37°C, to decompose L-tyrosine, to hydrolyse urea, to assimilate citrate and malate, to utilize 3 out of 17 sugar types, differentiate strain AA9(a)10 from *S. galilaeus*^{T}. Strain AA9(a)10 differed from *S. phaeoluteigriseus*^{T} in its capability to grow at pH 4 and 5, to grow at 37°C, to tolerate salt, decompose xanthine, to assimilate citrate and malate and to utilize 4 among 17 sugars used in this study. The physiological and biochemical properties that distinguish strain AA9(a)10 from phylogenetically closest type strains are summarized in table 3.5.

Table 3.5 Different physiological and biochemical characteristics between strain AA9(a)10 and its phylogenetically closest type strains

Strains: 1. AA9(a)10, 2. *S. alboniger* NRRL B1832^T, 3. *S. galilaeus* NRRL 2772^T, 4. *S. phaeoluteigriseus* NRRL ISP 5182^T. All data were obtained in this study: (+) indicated the positive activity or growth occurred, (-) indicated no activity or no growth, (w) indicated week activity, (p) indicated poor growth, (?) it was hard to observe the results

Characteristics	1	2	3	4	Characteristics	1	2	3	4
Decomposition of:					Carbon utilization of:				
Xanthine	w	w	w	+	Fructose	-	+	+	+
L-tyrosine	-	-	w	?	Myo-inositol	-	-	+	+
					Mannitol	-	+	-	+
Hydrolyis of:					Methyl-D-glucopyranoside	-	-	+	±
Casein	+	w	+	+	Adonitol	-	+	-	-
Urea	+	+	-	+	Sucrose	-	+	+	-
Acid assimilation of:					Growth at				
Citrate	-	+	+	+	37°	-	+	+	+
Lactate	+	-	+	+					
Malate	-	+	+	+	Growth at pH				
					4	+	-	-	-
Salt tolerance					5	+	+	-	+
3%	+	p	+	+					
5%	-	p	-	p					

3.3.3.3 Chemotaxonomic analysis

Chemotaxonomic characters of strain AA9(a)10 were compared to those of *S. alboniger*, *S. galilaeus*, and *S. phaeoluteigriseus*., which are the most closely related type strains to strain AA9(a)10 based on 16S rRNA gene sequencing.

The cell wall peptidoglycan of strain AA9(a)10 is LL-diaminopimelic acid. This is consistent with that of *Streptomyces* species even though in some cases a small amount of meso-DAP could be found. Galactose, glucose, and rhamnose were detected as sugar constituents. It has been reported that *Streptomyces* occasionally harbour diagnostic sugars found in actinobacteria (Goodfellow *et al.*, 2012). There is no characteristic pattern of sugars for species belonging to this genus (Lechevalier and Lechevalier, 1970). Mannose, ribose, glucose, galactose, and xylose were detected as whole cell sugars constituent of *Streptomyces bohaiensis* (Pan *et al.*, 2015), while glucose and ribose have been identified in *Streptomyces camponoticapitis* (Li *et al.*, 2016) as well as in *Streptomyces rhizosphaerihabitans* (Lee and Whang, 2016). Sometimes only one type of sugar is found in the cell wall hydrolysate of *Streptomyces* species, for example xylose (She *et al.*, 2016) and glucose (Lee and Whang, 2016).

Phosphatidylglycerol (PG) and some unidentified glycolipids were detected in the cell wall of strain AA9(a)10. The presence of PG is one of the indicators of the *Streptomyces* species, but not the glycolipids. The presence of glycolipids is inconsistent and their composition depends on the culture conditions (Goodfellow *et al.*, 2012)

Strain AA9(a)10 contained MK-7(H0) and MK-7(H2) as major cell wall menaquinones, followed by similar proportions of MK-7(H6), MK-9(H6), MK-9(H8). About 2% of MK-7(H4) and MK-9(H4) were also detected. The main menaquinone component of the genus *Streptomyces* is typically dominated by either MK-9(H6) or MK(H8), or dominated by MK-9(H6) with the present of MK-9(H4) and MK-9(H8) in comparable proportions. Thus, the menaquinone composition of strain AA9(a)10 needed to be reconfirmed. However, It is worth noting that significant amounts of MK-7(H4), MK-7(H6) and MK-7(H8) have been detected in *Streptomyces somaliensis* (Collins *et al.*, 1977).

Anteiso pentadecanoic acid (anteiso-15:0) and 14-methylpentadecanoic acid (iso-16:0) are the major fatty acid components found in the AA9(a)10 cell wall hydrolysate accounting for 33.79% and 23.9% respectively. A number of other fatty acids were present at smaller amounts from 3% to 14%. This is in agreement with the typical fatty acid constituents of the *Streptomyces* members that is dominated by saturated, iso and anteiso-fatty acids. The presence of pairs of iso- and anteiso-15:0 and iso-and anteiso-17 also support the classification of strain AA9(a)10 into the genus *Streptomyces*. In this genus iso-and antesio-branched fatty acids appear in pairs only for the ones with odd carbon atoms. The fatty acid composition detected in strain AA9(a)10 and all its closely related type strains is summarized in table 3.6. Only fatty acids that present at 1% or more are included in the table, unless they are found at 1% or more in other strains.

Table 3.6 Fatty acids composition of strain AA9(a)10 and its phylogenetically closest strains

Fatty acids	Species			
	AA9(a)10	*S. alboniger*	*S. phaeoluteigriseus*	*S. galialeus*
Iso-14:0	4.89	1.83	5.59	5.07
Iso-15:0	6.48	4.27	5.58	5.51
Anteiso-15:0	33.79	29.31	20.28	21.86
15:0	0.43	0.34	0.34	5.51
IsoH-16:1	0.98	2.08	2.86	0.98
Iso-16:0	23.9	15.04	22.11	16.39
Cis9-16:1	0.6	1.33	6.7	5.81
16:0	8.42	4.76	3.45	11.01
9?methyl-16:0	0.07	3.07	4.46	1.89
AnteisoC-17:1	0.44	3.95	4.18	2.34
Iso-17:0	3.21	6.13	5.11	5.94
Anteiso-17:0	14.06	18.65	12.08	15.14
Cyclo-17:0	1.13	2.05	0.31	1.07
17:0	0.22	0.17	0.11	1.07
Iso-19:0	–	2.39	4.12	–

3.3.3.4 Genetic analysis

3.3.3.4.1 DNA sequence analysis

Based on the 16S rRNA gene sequence, strain AA9(a)10 has the highest similarity with *Streptomyces alboniger*^{T}*, Streptomyces phaeolutegriseus*^{T}, and *Streptomyces galilaeus*^{T}. The similarity values between strain AA9(a)10 and the closest type strains is 99.25%, 99.02%, and 98.95, respectively.

3.3.3.4.2 Phylogenetic tree for strain AA9(a)10

The 16S rRNA gene sequence of strain AA9(a)10 was subjected to phylogenetic analysis. Validly published *Streptomyces* species were used for 16S rRNA gene sequence comparison with strain AA9(a)10, as shown as phylogenetic dendogram figure 3.4. The phylogenetic tree indicated that strain AA9(a)10 is a member of the genus *Streptomyces* in the family of *Streptomycetaceae*. Strain AA9(a)10 falls in the same clade and shares the same most common recent ancestor with *S. alboniger*^{T}*.* This makes *S. alboniger*^{T} the closest type strain to AA9(a)10. The next closest strains based on the tree (figure 3.4) and the 16S rRNA gene sequences are *S. phaeoluteigriseus*^{T} and *S. galilaeus*^{T}, respectively. *S. phaeoluteigriseus*^{T} and *S. galilaeus*^{T} together with some other type cultures positioned in the clade nearest to the clade of strain AA9(a)10 and *S. alboniger*^{T}. However, both clades are clustered together. The affiliation between strain AA9(a)10 and the three closest

type strains was supported by both neighbour-joining and maximum likelihood algorithms. Figure 3.4 showed the neighbour-joining tree based algorithm of strain AA9(a)10 and its closest type strains.

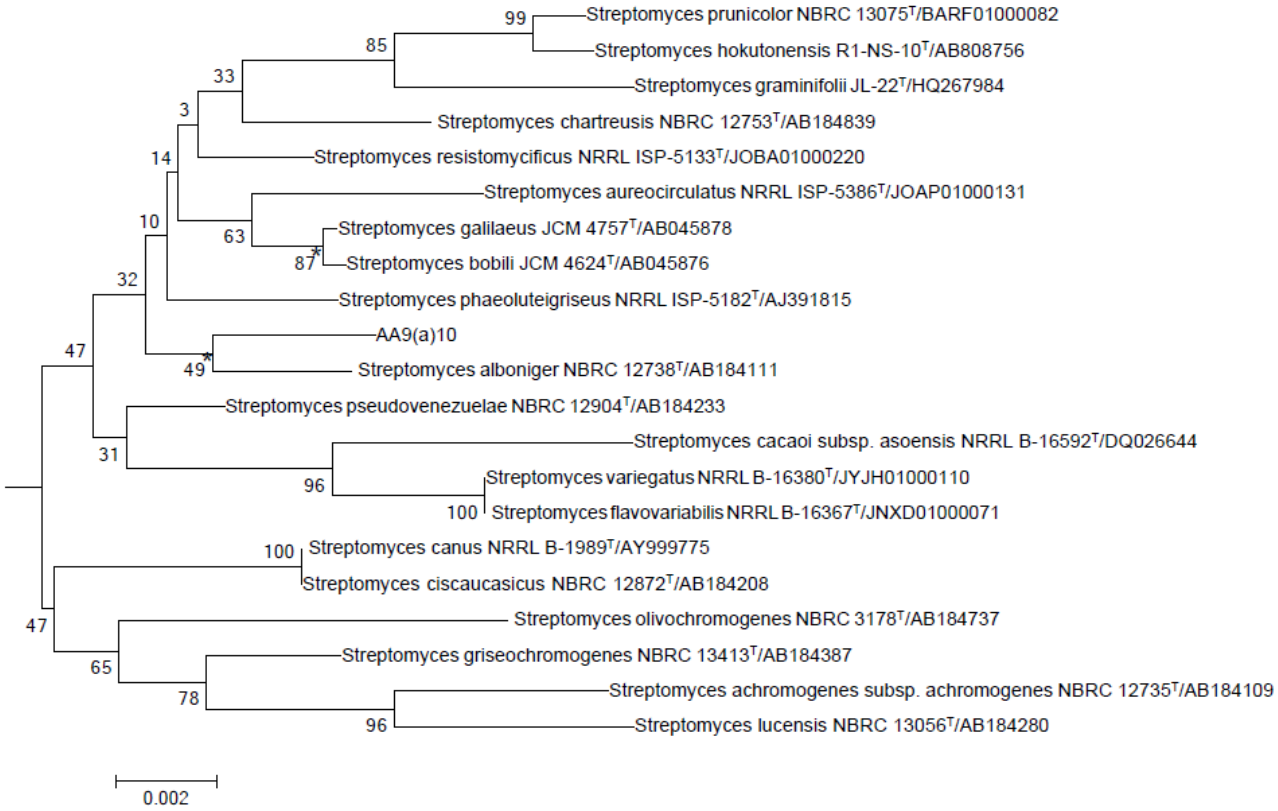


Figure 3.4 Neighbour-joining tree demonstrating the relationship between strain AA9(a)10 and representatives of related members of the genus *Streptomyces*. Asterisks indicates branches of the tree that were also recovered using maximum-likelihood treeing algorithms.

3.3.3.5 Differentiation of strain AA9(a)10 with the closest related strains

Based on the phenotypic properties, a number of differences were observed between strain AA9(a)10 and its phylogenetically closest type strains. Strain AA9(a)10 was culturally dissimilar form *S. alboniger*^{T}. *S. phaeoluteigriseus*^{T}, and *S. galilaeus*^{T} in spore formation, aerial spore mass colour, and the production of diffusible pigments in the media. Strain AA 9(a)10 did not produce spores on any media except ISP4. Inversely, *S. alboniger*^{T} formed white spores on almost all media used in this study, except half strength PDA (spores were observed at the edge of the culture only). *S. phaeoluteigriseus*^{T} produced pale grey spores on most media while *S. galilaeus* formed pale grey spores in most of media even though they were not abundant. The aerial spore mass colour was used to describe *Streptomyces* species in the ISP (Goodfellow *et al.*, 2012). Strain AA9(a)10 generated brown soluble pigment on ISP3 and light brown on ISP7. This is different from *S.*

alboniger^{T} which produced black pigment in ISP2, ISP4, and ISP5 and olive green pigment in half strength PDA. This colour of soluble pigment can be used to differentiate strain AA9(a)10 from *S. alboniger*^{T}. The remarkable colour of soluble pigment such as blue, dark green, red, and violet has a high value in the characterization of the genus *Streptomyces* (Goodfellow *et al.*, 2012). *S. phaeoluteigriseus*^{T} produced dark grey pigment on ISP7 only and *S. galilaeus*^{T} did not produce striking soluble pigment, so this feature could not be used to differentiate both strains from AA9(a)10. However, the light orange to orange colour (depending on the media) of substrate mycelia of *S. galilaeus*^{T}, made it dissimilar from AA9(a)10 which formed yellowish white substrate mycelia on the same media.

There are a number of physiological and biochemical characteristics that differentiate the strain AA9(a)10 from its closest type strains chosen in this study for comparison. All the type strains can grow at 37°C, but not AA9(a)10, and only AA9(a)10 that can grow at pH 4. Some dissimilarities were also observed in the ability to hydrolyse casein and urea, to assimilate citrate, lactate, and to utilize various sugars as shown in table 3.5. The differentiation between AA9(a)10 and *S. alboniger*^{T} based on physiological and biochemical parameters has been as discussed in detail in section 3.3.3.2.

Chemotaxonomy data showed that the major menaquinone of AA9(a)10 are MK-7(H0) and MK-7(H2), followed by similar proportions of MK-7(H6), MK-9(H6), MK(H8). About 2% of MK-7(H4) and MK-9(H4) were also detected. There is no report on the menaquinone content of *S. alboniger*^{T}, *S. phaeoluteigriseus*^{T}, and *S. galilaeus*^{T}. However, most *Streptomyces* species possess MK-9(H6) or MK 9(H8) as the major menaquinone and sometimes MK-9(H8) and MK-9(H4) is present in similar amounts. Among 14 fatty acids that were present at ≥ 2% in either AA9(a)10 or in *S. alboniger*^{T}, *S. phaeoluteigriseus*^{T}, and *S. galilaeus*^{T}, 7 fatty acids showed different proportions, ranging from 0.5-6 times as shown in table 3.6. One of these fatty acids is iso-19:0 which does not occur in AA9(a)10, but is present in *S. alboniger*^{T} (about 2.4%) and *S. phaeoluteigriseus*^{T} (about 4%).

As discussed before, strain AA9(a)10 was positioned in a different clade from *S. phaeoluteigriseus*^{T} *and S. galilaeus*^{T} which were located together with some other *Streptomyces* type strains. The

number of dissimilarities on the phenotypic characteristics indicated that AA9(a)10 was different from both type strains.

Based on the cultural characteristics that are of high value and still used in *Streptomyces* characterization, strain AA9(a)10 differs from its closest type strain *S. albiniger*^{T}. A number of physiological/biochemical and chemotaxonomic features were also different between strains, for example growth at 37°C, growth at pH 4, the assimilation of 3 organic acids, and the utilization of various sugars. Furthermore, their menaquinone and fatty acid compositions support their differences. Nevertheless, considering the high value of similarity between both strains (99.25) and their close position in the phylogenetic tree (figure 3.4), complementary genetic data, e.g. DNA-DNA hybridization is needed to verify its novelty which unfortunately has not been done in this study. However, there are indications that strain AA9(a)10 could be a novel species based on strong phenotypic differences including cultural, physiological/biochemical, and chemotaxonomic characteristics. It is worth noting that AA9(a)10 is a slow growing endophytic actinobacteria which was recovered from the isolation medium at week 11. It is proposed that slow growing actinobacteria are likely to have not been isolated before, as isolation procedure commonly was done up to 4 weeks.

3.3.3.6 Description of strain AA9(a)10

Strain AA9(a)10 grows well on ISP2, ISP4, and ISP7. Spores are formed abundantly only on ISP4. The spores are cylindrical and structured in long chains. It grows well between 15 and 27°C, pH 4-10, and is able to tolerate up to 3% NaCl in the medium. It decomposes adenine, hypoxanthine, weakly degrades xanthine, but not L-tyrosine. Starch, casein and urea were hydrolysed. Strain AA9(a)10 assimilates organic acids including acetate, acetate, lactate, and propionate. It is able to utilize arabinose, cellobiose, galactose, glucose, maltose, mannose, rhamnose, trehalose, and xylose. The diagnostic amino acid of peptidoglycan is LL-DAP whereas galactose, glucose and rhamnose occur in the whole cell hydrolysate. The major menaquinones are MK-7(H0) followed by MK-7(H2). Similar proportions of MK-9(H6), MK(H8) and of MK-7(H6) were present, accounting for 7-8% approximately. About 2% of MK-7(H4) and MK-9(H4) were also detected. Anteiso pentadecanoic acid (anteiso-15:0) and 14-methylpentadecanoic acid (iso-16:0) are the major fatty

acid components. Strain AA9(a)10 is a slow growing endophytic actinobacteria isolated from wood of an Australian native tree.

3.3.4 Endophytic actinobacterium strain AA9(b)2

3.3.4.1 Morphological and cultural analysis

The morphological and cultural characteristics of strain AA9(b)2 were compared to two closest type strains, *Streptomyces niveus,* and *Streptomyces intermedius.* Moderate to good growth of AA9(b)2 was observed on all media used for morphological characterization, except inorganic salt starch agar and nutrient agar. The spores colour varied depending on the medium from greyish lilac to dull violet. However, no spore was formed on ISP 4 and nutrient agar. It is known that spore formation is regulated by different genes and depends on the cultivation conditions. Strain AA9(b)2 formed long spore chains indicating that it belongs to the genus *Streptomyces* (figure 3.5). The cultural characteristics of strain AA9(b)2 on different media are shown in table 3.7.

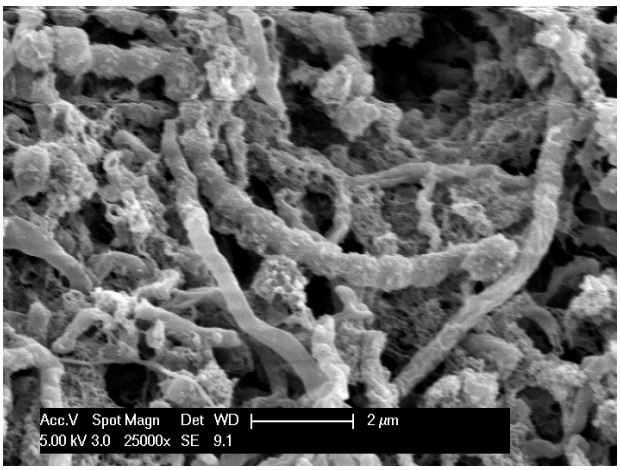


Figure 3.5 The scanning electron microscopy of strain AA9(b)2 after grown on ISP2 for 15 days

Table 3.7 Cultural characteristics of strain AA9(b)2 on various media

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast extract malt extract agar (ISP2)	Good	Good, pale yellow/ivory with greyish lilac spores on some areas	pale yellow/ivory
Oatmeal agar (ISP3)	Moderate	Moderate, greyish lilac spores	poor, can't really see the colour
Inorganic salt starch agar (ISP4)	Poor	poor, ivory	poor, ivory
Glycerol asparagine agar (ISP5)	Good	Good, dull violet spores	dull violet
Tyrosin agar (ISP7)	Good, no melanine pigment	Good, purplish grey spores	ivory/natural
Bennett's agar	Moderate	Moderate, ivory with greyish white spores on a few spot	ivory/natural
Half strength potato dextrose agar	Good	purplish grey spores	purplish grey
Nutrient agar	Poor	poor, ivory/natural	ivory/natural

3.3.4.2 Physiological characteristics

Physiological properties of strain AA9(b)2 were analysed and compared directly with the closest related reference actinobacteria, *S. niveus*^{T} and *S. intermedius*^{T}. As for other species that belong to the genus *Streptomyces*, strain AA9(b)2 used a range of carbon sources for energy and growth, such as cellobiose, glucose, mannose, trehalose. This strain was also able to metabolize other sugars as sole carbon sources including arabinose, fructose, galactose, maltose, mannitol, methyl-D-glucopyranoside, adonitol, rhamnose, and xylose. It was observed that strain AA9(b)2 decomposes hypoxanthine, but not adenine, L-tyrosine, or xanthine. Starch, casein, and urea were hydrolysed by this strain. The vast majority of *Streptomyces* species degrade polymeric substrate, such as adenine, hypoxanthine, casein, L-tyrosine and starch. However, it has been reported that some *Streptomyces* member are not able to degrade adenine or tyrosine. The ability to decompose xanthine varies between *Streptomyces* members. Some *Streptomyces* species have been found to produce amylase that supports their ability to degrade starch (Chakraborty *et al.*, 2009, Syed *et al.*, 2009). Strain AA9(b)2 has the ability to assimilate acetate, citrate, lactate, malate and weakly assimilate propionate. Benzoate and tartrate were not assimilated. It grows at temperatures between 15 and 37°C and between pH 5-10. The pH for most *Streptomyces* to grow ranges from 5.0 to 9.0, with an optimum pH close to 7 (6.5-8.0). No growth was observed on the media containing more than 3% NaCl.

Some physiological properties of strain AA9(b)2 are different from its phylogenetically related type strains. Strain AA9(b)2 grew at 37°C, but not *S. niveus*^{T} and *S. intermedius*^{T}, even though poor growth was observed with *S. intermedius*^{T} at this temperature. Both type strains were able to grow at pH4, and tolerate up to 5% NaCl, but not strain AA9(b)2 (poor growth was observed with strain AA9(b)2 in medium containing 3% NaCl). Strain AA9(b)2 did not degrade adenine, xanthine, and tyrosine. In contrast, *S. intermedius*^{T} degraded all of them. There was no significant difference between AA9(b)2 and *S. niveus*^{T} in the ability to degrade adenine, xanthine, and tyrosine. *S. niveus*^{T} and *S. intemedius*^{T} hydrolyse starch, as do the vast majority of *Streptomyces* species, and urea, but strain AA9(b)2 only weakly hydrolysed urea. A significant difference on the utilization of sugars was only observed between AA9(b)2 and *S. intermedius*. The physiological and biochemical properties that distinguish strain AA9(B)2 from its phylogenetically closest type strains are summarized in table 3.8.

Table 3.8 Different Physiological characteristics between strain AA9(b)2 and its phylogenetically closest type strains

Strains: 1. AA9(b)2, 2. *S. niveus* NRRL 2466^{T}, 3. *S. intermedius* NRRL-1327^{T}. All data were obtained in this study: (+) indicated the positive activity or growth occurred, (-) indicated no activity or growth occur, (w) indicated week activity or growth occurred

Characteristics	1	2	3	Characteristics	1	2	3
Decomposition of:				Carbon utilization of:			
Adenine	-	-	+	Glucose	+	w	+
Xanthine	-	w	+	Myo-inositol	-	w	-
L-tyrosine	-	-	+	Methyl-D-glucopyranoside	+	+	-
				Adonitol	+	+	-
Hydrolyis of:				Rhamnose	+	+	-
Starch	-	+	+	Sucrose	-	w	w
Urea	w	+	+				
				Growth at			
Acid assimilation of:				37°	+	-	p
Propionate	w	+	+				
				Growth at pH			
Salt tolerance				4	-	+	+
3%	p	+	+				
5%	-	+	+				

3.3.4.3 Chemotaxonomy analysis

Chemotaxonomic characters of strain AA9(b)2 were compared to those of *S. niveus*^{T} and *S. intermedius*^{T} which are the most closely related type strains to strain AA9(b)2 based on 16S rRNA gene sequencing.

The cell wall peptidoglycan of strain AA9(b)2 is LL-diaminopimelic acid. This is consistent with that of *Streptomyces* species even though in some cases a small amount of meso-DAP can be found. Galactose, glucose, and xylose (trace) were detected as sugar constituents. It has been reported that *Streptomyces* occasionally harbour diagnostic sugars found in actinobacteria (Goodfellow *et al.*, 2012). Some *Streptomyces* have been reported to contain sugars (Saricaoglu *et al.*, 2014, Xu *et al.*, 2012, Zheng *et al.*, 2014, Zhu *et al.*, 2007). However, there is no characteristic sugar pattern for species belonging to this genus (Lechevalier and Lechevalier, 1970).

Except for unknown phospholipids and unknown glycolipids, the polar lipids detected in strain AA9(b)2 were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine. The last two ones are the major phospholipids found in *Streptomyces* species (Goodfellow *et al.*, 2012). Thus, the polar lipid contents of strain AA9(b)2 support the affiliation of this strain into the genus *Streptomyces*.

Strain AA9(b)2 contains MK-9(H6) and MK-9(H8) as major respiratory quinones, accounting for 42.9% and 38.7% respectively. Smaller amounts of menaquinone MK-9(H4) were also present (12.3%). This menaquinone composition is typical for the members of *Streptomyces,* supporting the affiliation of AA9(b)2 into this genus.

Iso pentadecanoic acid (iso-15:0) and 14-methylpentadecanoic acid (iso-16:0) were the major fatty acids component found in the AA9(b)2 cell wall hydrolysate accounting for 17.57% and 13.57% respectively. Other fatty acids were present at smaller amount from 1% to 10%. This typical for fatty acids of the *Streptomyces* species that is dominated by saturated, iso and anteiso-fatty acids. The presence of pairs of iso- and anteiso-15:0 and iso-and anteiso-17 also support the classification of strain AA9(b)2 into the genus *Streptomyces*. In this genus iso-and antesio-branched fatty acids

appear in pairs only for the ones with odd carbon atoms. The fatty acid composition detected in strain AA9(b)2 and all its closely related type strains is summarized in table 3.9. Only fatty acids that present at 1% or more are included in the table, unless they are found at 1% or more in other strains.

Table 3.9 Fatty acid composition of strain AA9(b)2 and its closest related type strains

Fatty acids	Species		
	AA9(b)2	*S. niveus* ^{T}	*S. intermedius* ^{T}
Iso-14:0	2.8	14.57	4.43
Iso-15:0	17.57	1.59	4.86
Anteiso-15:0	10.15	16.78	17.22
15:0	1.32	0.91	0.47
AnteisoA-15:1	–	5.74	–
Anteiso2OH-15:0	0.42	–	6.04
Iso-16:0	13.57	32.68	16.96
16:0	10.12	9.17	5.38
Iso2OH-15:0/t9-16:1	0.74	–	1.35
IsoH -16:1	–	1.75	3.34
Cis9-16:1	–	3.73	2.81
Anteiso-16:0	–	–	1.3
9?methyl-16:0	–	0.37	2.3
AnteisoC-17:1	0.97	1.27	4.86
Iso-17:0	4.87	0.59	3.02
Anteiso-17:0	1.89	5.22	8.9
Cyclo-17:0	4.67	1.07	6.8
Cis11/t9/t6-18:1/	26.97	0.05	0.12
Iso-19:0	–	–	–
Unknown 17.595 SM	0.31	0.09	2.28
Unknown 17.493 SM	1.33	–	0.65

3.3.4.4 Genetic analysis

3.3.4.4.1 DNA sequence analysis

Based on the 16S rRNA gene sequence, strain AA9(b)2 has the highest similarity with *Streptomyces niveus,* and *Streptomyces intermedius*. The similarity values between strain AA9(b)2 and the closest type strains was 98.00% and 97.56%, respectively.

3.3.4.4.2 Phylogenetic tree for strain AA9(b)2

The 16S rRNA gene sequence of strain AA9(b)2 was subjected to phylogenetic analysis. Validly published *Streptomyces* species were used for 16S rRNA gene sequence comparison with strain AA9(b)2, as shown in the phylogenetic dendogram (figure 3.6). The phylogenetic tree indicated that strain AA9(b)2 is a member of the genus *Streptomyces* in the family of Streptomycetaceae. Based on neighbour joining treeing algorithm, strain AA9(b)2 formed an independent clade, separated from *S. niveus*^{T} and *S. intermedius*^{T}, supported by a high bootstrap value of 100%. The maximum

likelihood treeing algorithm also demonstrated that strain AA9(b)2 is loosely associated with the type strain *S. niveus*^{T} and *S. intermedius*^{T}, supported by a high bootstrap value of 94%. Figure 3.6 shows the neighbour-joining tree based algorithm of strain AA9(b)2 and its closest type strains.

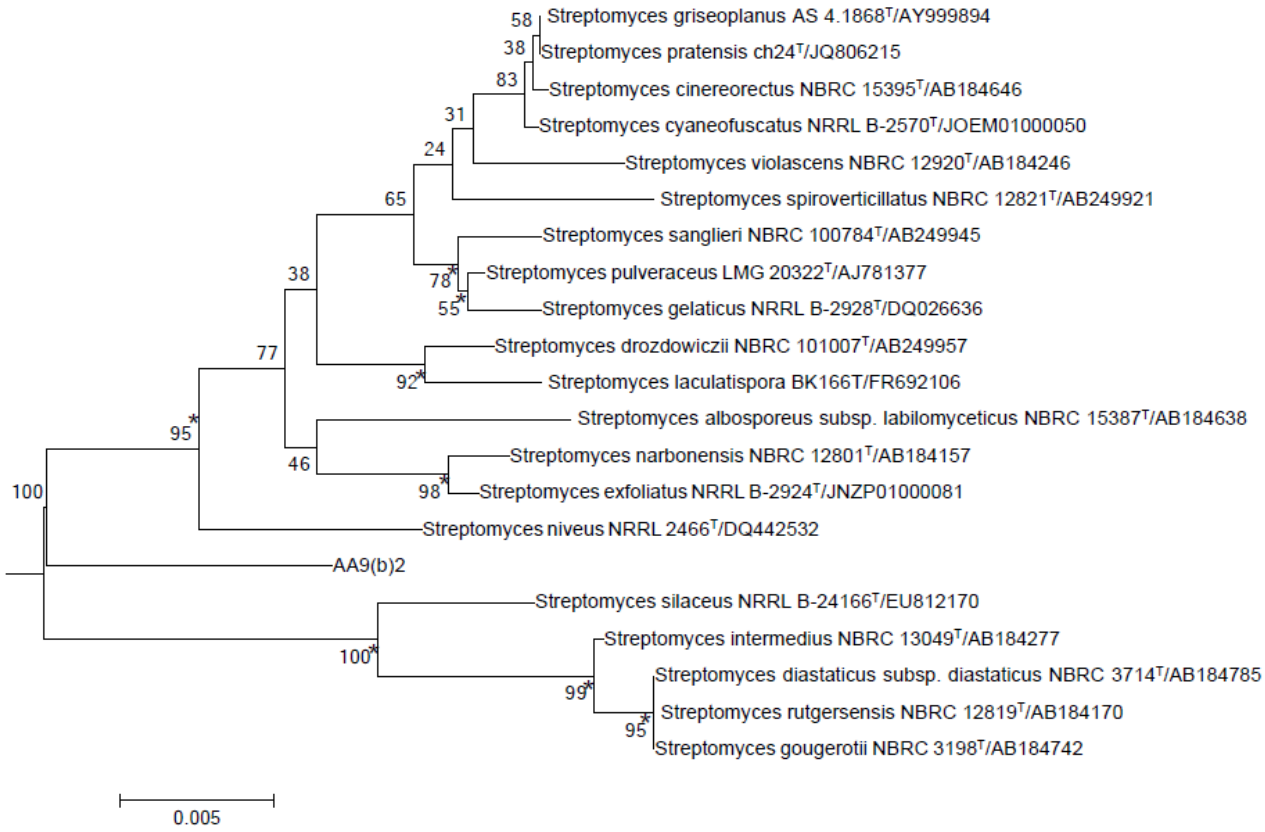


Figure 3.6 Neighbour joining based phylogenetic tree of strain AA9(b)2 and its related representative member of the genus *Streptomyces*. Asterisk indicated branches that were also recovered using maximum likelihood treeing algorithm

3.3.4.5 Differentiation of strain AA9(b)2 with its closest type strains

Based on the phenotypic properties, a number of differences were observed between strain AA9(b)2 and its phylogenetically closest type strains. Strain AA9(b)2 was culturally dissimilar from *S. niveus*^{T} and *S. intermedius*^{T} in spore formation, aerial spore mass colour, and the production of diffusible pigment in the growth media. The aerial mycelium of strain AA9(b)2 produced greyish lilac to dull violet spores on ISP2, ISP3, ISP5, ISP7, and half strength PDA, while *S. niveus*^{T} formed satin white to greyish yellow spores on the same media. In contrast, *S. intermedius*^{T} only formed scarce white to yellowish white spores on ISP2, ISP3, half strength PDA, and Nutrient agar, but no spores were observed on ISP5 and ISP7. However, the production of sporulating aerial mycelium varies between

laboratories with this strain, e.g. it forms spores on ISP3, ISP4, and ISP5 (Goodfellow *et al.*, 2012). Strain AA9(b)2 formed rough spore chains, whereas *S. niveus* as well as *S. intermedius* both generated smooth spore chains. The ornamentation of spore surface is a stable feature and has been used for characterization in taxonomy (Goodfellow *et al.*, 2012).

Strain AA9(b)2 can be distinguished from the closest type strains by a range of physiological characteristics. This strain differs from *S. niveus*^{T} and *S. intermedius*^{T} in its growth at 37°C, at pH5, tolerance to NaCl, ability to degrade adenine, xanthine, and tyrosine, and to hydrolyse starch and urea. The usage of sugars as sole carbon sources swas also different between strain AA9 (b)2 and both type strains. These have been as discussed in detail in section 3.3.4.2.

Chemotaxonomy data showed that there are differences on the major menaquinone composition between AA9(b)2 and *S. intermedius*. Strain AA9(b)2 contains MK-9(H6) and MK-9(H8) at comparable proportions (42.9% and 38.7%, respectively), whereas the major menaquinones of *S. intermedius*^{T} are MK-9(H6) and MK(H4) at similar proportions (36% and 31%, respectively). Menaquinone MK-9(H4) present in strain AA9(b)2, but it is 2.5 times lower than that of *S. intermedius*^{T}. Inversely, a higher proportion (2X) of MK-9(H8) occurred in AA9(b)2, compared to *S. intermedius*^{T}. In addition MK-7(H0) was detected in both strains, but it was 2X higher in *S. intermedius* than AA9(b)2. Minor amounts of MK-9(H2) were detected in AA9(b)2 (3.4%), but there was none in *S. intermedius*^{T}. The menaquinone analysis for *S. niveus*^{T} was not done and there is no report on the menaquinone composition for this type strain. However, most *Streptomyces* species possess MK-9(H6) or MK 9(H8) as the major menaquinone and sometimes MK-9(H8) and MK-9(H4) are present in similar amounts.

A range of differences were found on the fatty acid composition between AA9(b)2 and its closest type strain, *S. niveus*. The major fatty acids of strain AA9(b)2 were iso-15:0 and iso-16-:0 which were present in similar proportions, while the fatty acids of *S. niveus* were dominated by anteiso15:0 and iso16:0. Nonetheless, iso-16:0 was present 2X higher in *S. niveus*^{T} compared to AA9(b)2. *S. niveus*^{T} contained 15% iso-14:0 which was only 3% in AA9(b)2. *S. niveus*^{T} also had considerable

amounts of anteisoA-15:1, Cis9-16:1, but not strain AA9(b)2. Inversely, strain AA9(b)2 contained iso-17 and cyclo-17:0 in amounts 4X higher than *S. niveus*^{T}. It is worth noting that a significant proportion of unseparated bands (27%) were detected in strain AA9(b)2, but they were not found in either *S. niveus*^{T} and *S. intermedius*^{T}. These bands could have been Cis11/t9/t6-18:1.

Several dissimilarities were observed on the fatty acid composition of Strain AA9(b)2 and *S. intermedius*, its next closest type strain acid. Some fatty acids of strain AA9(b)2 were present in either higher or lower percentages than *S. intermedius*, including iso-14:0, iso-15:0, Anteiso2OH-15:0, iso-16:0, anteiso-17:1, anteiso-17:0. Some other fatty acids were not found in strain AA9(B)2, but they were present in *S. intermedius*^{T} between 2% to 3%.

It is evident from genotypic and phenotypic analysis that strain AA9(b)2 can be distinguished from the type strains of the phylogenetically related species mentioned above. The 16S rRNA gene sequence similarity value between strain AA9(b)2 and the most closely related type strains (98% with *S. niveus*^{T} and 97.56% *S. intermedius*^{T}) are below 98.7% which is the threshold value to differentiate strains at the species level. This is supported by the positon of strain AA9(b)2 in the phylogenetic tree where it forms an independent clade from *S. niveus*^{T} and *S. intermedius*^{T}. A range of different phenotypic characteristics also supports the high possibility for strain AA9(b)2 to be a novel species of the genus *Streptomyces*.

3.3.4.6 Description of strain AA9 (b) 2

Strain AA9(b)2 showed moderate to good growth on ISP3, Bennet's agar, ISP2, ISP5, ISP7 and half strength PDA. Greyish lilac to dull violet spores were formed on these media. It grows well between 15° and 37°C, pH 5-10, and is able to tolerate up to 3% NaCl in the medium. Hypoxanthine is degraded, but not adenine, xanthine, and tyrosine. AA9(b)2 hydrolysed casein, weakly hydrolysed urea, but it does not hydrolyse starch. Strain AA9(b)2 assimilates several organic acids including acetate, citrate, lactate, malate, and weakly assimilates propionate. Benzoate and tartrate are not assimilated. It is able to utilize a wide range of sugars as sole carbon sources such as arabinose, cellobiose, galactose, glucose, maltose, mannose, mannitol, methyl-D-glucopyranoside, adonitol,

rhamnose, trehalose, and xylose. Melezitose, myo-inositol, meso-erythritol, sorbitol and sucrose are not utilized. The diagnostic amino acid of peptidoglycan is LL-DAP whereas galactose, glucose and trace of xylose were detected in the whole cell hydrolysate. The major menaquinone are MK-9(H6) and MK-9(H8). Smaller amount of menaquinone MK-9(H4), MK-9(H2), and MK-7(H0) are also present. The fatty acids components are dominated by iso-15:0 and iso-16:0. Strain AA9(b)2 was isolated from the wood of an Australian tree trunk.

CHAPTER 4

SCREENING, FERMENTATION, AND PURIFICATION OF ACTIVE COMPOUNDS

4.1 Introduction

Microbial natural products and whole cell based screening still play an important role in the discovery of antimicrobial compounds (Singh *et al.*, 2011). It is well recognised that microorganisms, such as actinobacteria and fungi, produce a large number of compounds with a diverse range of chemical structures. In order to reduce the problem of dereplication, the focus has been on screening new strains based on the hypothesis they are more likely to possess new biosynthetic pathways leading to new chemical structures.

The emergence of antibiotic resistance further compels the search for new antibiotics, otherwise we may return to the pre-antibiotic era. Nevertheless, it is becoming harder today to discover new antibiotics as perhaps, "the low hanging fruit from the antibiotic tree has been picked" (Payne *et al.*, 2007). Most antibiotics have been discovered from actinobacteria that were present at higher frequencies between 10^{-1}-10^{-7}. Erythromycin, streptothricin, tetracycline, and streptomycin were produced by actinobacteria at frequencies of 10^{-1}-10^{-3} whereas daptomycin, the most recent antibiotic added to the clinical arena was discovered from actinobacteria at a lower frequency of 10^{-7}. New antibiotics are awaiting to be found from actinobacteria that are present at lower frequencies ≤ 10^{-7} (Baltz, 2006). Therefore, a targeted isolation strategy has to be augmented by a unique screening strategy to increase the possibility of discovering new compounds.

4.2 Methodology

4.2.1 Microbial strains used

Actinobacterial cultures. There were two sources of actinobacterial cultures:

1. Cultures from Australian trees (49 isolates) as described in Kaewkla and Franco (2013)
2. Freshly isolated cultures isolated from within the trunks of eucalyptus trees from this study (51 isolates).

The list of actinobacterial cultures and ID can be found in the appendix 2.

Test microorganisms. Resistant and susceptible *Staphylococcus aureus* used in this study. Susceptible *S. aureus* were: *S. aureus* SAK 2107 (*S. aureus* SH1000 originated from 8324-4 with rsbU mutation corrected), SAK2124 (Δ*NorA*), and SAK2703 (SAK 2124 containing plasmid pALC2073), whereas resistant *S. aureus* was *S. aureus* SAK3756 (SAK 2124 containing plasmid pALC2073 harbouring *NorA*),

4.2.2 Production of antimicrobial secondary metabolites

One hundred endophytic actinobacteria were screened for their activity against resistant *Staphylococcus aureus* overexpressing *NorA* efflux pump. Solid state fermentation was carried out to obtain actinobacterial extract for screening. Larger solid state and submerged fermentation was further performed to produce potentially new active compounds that showed activity against the target bacteria, for purification and identification of active compounds.

4.2.2.1 Production on agar-solid state fermentation

The actinobacterial isolates were cultured by streaking on International *Streptomyces* Project 2 (ISP2) agar media at 28°C for about 7-10 days. The culture was then harvested by cutting the whole agar containing the mycelium and spores into smaller pieces. These pieces of agar were put into Erlenmeyer flasks and 30 ml of methanol was added. The flasks were shaken for 4 hours followed by filtration to get the methanol extracts. The methanol extracts were evaporated using rotary evaporator and freeze dried. The dried extracts were kept at -20°C until they were used for bioassays against resistant *Staphylococcus aureus* overexpressing *NorA* efflux pump.

4.2.2.2 Submerged Fermentation

The actinobacterial strains were grown on an ISP 2 agar plate at 28°C for about 7-10 days. Three to five blocks of 0.5 X 0.5 cm agar were cut and used to inoculate 2-3 Erlenmeyer flasks containing seed culture medium (IM22) and shaken at 28*C, 150 rpm for 3-4 days. The seed culture (2.5 ml) was transferred to 250 ml Erlenmeyer flaks containing 50 ml of five production media each: F33, F40, SI, F31, and F26. Each medium was done in triplicate. One millilitre of sample was taken

aseptically every day for 7 days. The samples were centrifuged to separate the mycelia from the supernatant. The mycelia was then extracted with methanol and the methanol extract as well as the supernatant were subjected to bioassay. The production of active compounds against resistant *S. aureus* was examined using the agar-well diffusion assay method. The presence of antibiotic activity was indicated by the formation of zones of inhibition (ZOI) around the wells. The antibiotic activity was measured by the diameter of the ZOI.

4.2.3 Screening for bioactive compounds

4.2.3.1 Minimum Inhibition Concentration (MIC)

Microdilution method was done to evaluate the MIC of sensitive and resistant *S. aureus* overexpressing *NorA* efflux pump used as test microorganisms in this study.

Inoculum preparation. *S. aureus* SAK 3756 and SAK 2703 were cultured on Mueller Hinton Agar (MHA) supplemented with chloramphenicol 10 µg/ml and incubated at 37° for 18-24 hours. A single colony was then inoculated into 2 ml of Mueller Hinton Broth (MHB) supplemented with 10 µg/ml chloramphenicol and 50 ng/ml tetracycline followed by incubation at 37° for 18-24 hours in a shaker incubator. The bacterial suspension was adjusted to an OD of 0.01 that is equivalent to approximately 1 x 10^6 CFU/ml.

Broth microdilution testing. MHB supplemented with 20 µg/ml chloramphenicol and 100 ng/ml tetracycline was prepared and 175 µl was added to 6 wells at first row of 96 well plates. The rest of the rows were filled with MHB supplemented with 100 µl of 20 µg/ml chloramphenicol and 100 ng/ml tetracycline. Norfloxacin and ciprofloxacin stock solutions were then added to the wells in the first row so the concentration of norfloxacin and ciprofloxacin were 31.25 µg/ml and 62.5 µg/ml, respectively, followed by serial dilution until 0.95 µg/ml for ciprofloxacin and 0.5 µg/ml for norfloxacin. No antibiotics were added to the control (positive growth control), and no bacterial suspension was added for the negative control. Bacterial suspension was then added to each well at the volume of 100 µl. The OD was measured after the plates were incubated at 37° for 18-24 hours and the MIC was determined by the lowest concentration of antibiotics that inhibited the growth of the bacteria.

4.2.3.2 Agar-well diffusion assay method for bioactivity

The test microorganisms were prepared by growing resistant *S. aureus* SAK 3756 and susceptible *S. aureus* SAK 2703 in Mueller Hinton broth supplemented with 10 µg/ml chloramphenicol overnight at 37°C. The optical density was then adjusted to 0.2 at 600 nm. A 1% inoculum of each bacterial suspension was then added to separate bottles containing antibiotic agar medium which was prepared according to the manufacturer's directions. The assay medium was sterilized at 121ºC for 35 minutes followed by cooling down to 40ºC approximately, before it was seeded with 1% of the bacterial suspension. Chloramphenicol and tetracycline at a concentration of 10 µg/ml and 50 ng/ml, respectively, were added to the medium to maintain the resistance phenotypes. The medium was poured into plates (20 ml each plate).

Once the medium solidified, wells were made in the Antibiotic agar medium plates using a cork borer to give wells of 6 mm in diameter. The extracts and the standard antibiotics were then put in the wells at a volume of 30 µl. The plates were left slightly open in the laminar airflow for 30 minutes to an hour to dry the surface before they were incubated at 37ºC for 16-24 hours. The formation of Zones of Inhibition (ZOI) around the wells indicated the antibacterial activity, and the diameter of the ZOI were measured.

4.2.4 Submerged Fermentation Scale Up

4.2.4.1 Seed culture and production culture

The medium that gave the highest antibiotic production was selected for further fermentation at a bigger capacity. The actinobacteria were cultured on ISP2 agar plates for 7-10 days. Three to five blocks of 0.5 X 0.5 cm agar culture were added as inoculum to 2-3 Erlenmeyer flasks containing 50 ml IM22 medium. The flasks were shaken at 28°C, 150 rpm. After 3 days, 5 % (v/v) seed culture was then transferred to the selected production medium. This production medium was prepared in 80 250 ml Erlenmeyer flasks containing 50 ml of the medium. The flasks were put on the shaker at 150 rpm, 27^{o} C.

4.2.4.2 Sampling

The production of active compounds was examined by taking a 1 ml sample every day from day 1 initially and then from day 4. Each sample was centrifuged at 3,000 rpm for 15 minutes. The supernatant was transferred to a new tube for bioassay. One milliliter of methanol was added to the mycelium and the mixture was centrifuged at 13,000 rpm for 15 minutes. The methanol extract was transferred to a new tube for bioassay. Bioassay against resistant *S. aureus* SAK 3756 and susceptible *S. aureus* SAK 2703 was performed using the agar-well diffusion assay as describe in section 4.2.2.2.

4.2.5 Extraction

The fermentation broth was harvested and the mycelia was separated from the supernatant by centrifugation or filtration. Ethyl acetate was added to the mycelium at a 1.5:1 in ratio to extract the non-polar active compounds. The mixture was shaken at room temperature for 4 hours. The ethyl acetate extract was then separated from the mycelium and methanol was subsequently added to the mycelium. The mycelia were separated from methanol by filtration. The ethyl acetate and the methanol extract were then evaporated using rotary evaporator. The methanol extract was freeze dried for further analysis.

4.2.6 Bioassay guided purification

4.2.6.1 Silica gel column chromatography

4.2.6.1.1 Column packing

Silica gel was activated by heating at 120°C for 1 hour. The thickness of silica gel in the tray was no more than 1 cm to make sure that it was activated evenly. The silica gel was then submerged in chloroform and the slurry was packed in a glass column that has been plugged with glass wool at the end of the column. As the silica gel slurry settled, the column was tapped to ensure that it had been evenly packed with no air bubbles.

4.2.6.1.2 Sample preparation

The ethyl acetate extract was dissolved completely in a minimum amount chloroform (0.5-2.0 ml). The level of chloroform in the column was raised to about 2 mm above the silica gel bed. The sample was then loaded slowly into the column and the tap at the bottom of the column was opened to bring the sample at about 2 mm above the silica gel bed. This step was repeated until the entire sample was loaded.

For a separate column elution experiment, the dried mycelial methanol extract (1 g) was dissolved in 50 ml methanol. An amount of 1.5 g silica gel was added followed by evaporation in a rotary evaporator to dry the sample. The dried mixture of sample and silica gel was put into the column through a funnel, slowly. A piece of cotton wool was placed on the top of the silica gel column before elution to prevent disturbing the packed column.

4.2.6.1.3 Elution and fraction collection

The column was first eluted with 2 bed volumes of chloroform and subsequently eluted with 2 bed volumes of chloroform-methanol mixture to increase the polarity. The amount of methanol in the mixture was increased slowly to make the ratio of chloroform and methanol as follow: 99:1, 98:2, 97:3, 95:5, 90:10, 85:15, 75:25, 65:35, 55:45, 35:65, 15:85 and then 0:100. About 10 ml of fractions were collected in clean glass tubes. The fractions were evaporated and subjected to bioassay and TLC/bioautography.

4.2.6.2 High Performance Centrifugal Partition Chromatography (HPCPC)

HPCPC is a liquid-liquid chromatographic technique based on partitioning between two immiscible solvents. It is well suited to polar compounds which would normally bind to the silica gel in a column.

HPCPC involved a number of steps as follows:

a. Solvent preparation.

A two phase solvent system was prepared by mixing chloroform:methanol:water (65:35:10) in a separating funnel. The mixture was allowed to equilibrate followed by the separation of upper and lower phases.

b. Filling up of the stationary phase

The rotor was filled up with the upper phase as stationary phase. The pump was set at 5 ml/min and the valve was switched to descending mode. The rotation speed was adjusted to 300 rpm and the stationary phase was set at 20-30% by volume more than of the rotor volume. After filling the rotor with the stationary phase, the solvent flow was stopped and the pump flow speed and the rotation speed were adjusted to 2 ml/min and 11,000 rpm, respectively.

c. Sample injection

The sample was dissolved in the same volume of upper phase and lower phase. It was injected to the coil loop with a micro-syringe. The sample injector was turned to load before injecting and then returned to inject.

d. Elution

The first elution was run in descending mode and the fraction collector was set at 4 minutes. The elution was switched to ascending mode after fraction 50 was collected. While keeping the rotation speed, the mobile phase flow was stopped and the valve was switched to the intermediate position. The upper layer was then used as mobile phase. The valve was switched to ascending mode followed by turning on the pump.

e. Washing the rotor

After completing the separation, the inside of the rotor and rotary joints were washed using methanol or acetone as washing solvents. The pump was adjusted at 10-30 ml/min and the washing solvent was sent to the rotor with a total flow volume of 20-30% larger than the rotor volume.

4.2.6.3 Thin Layer Chromatography (TLC) and Bioautography

Thin layer chromatography was performed using a silica gel 60 F_{254} (Merck). The solvents for the mobile phase were prepared and put into a TLC chamber at about 5 mm depth. A range of mobile phases were used in this study to obtain the ones that were able to separate the active compounds. The mobile phase was obtained from a biphasic system that was prepared by mixing the solvents in

a separating funnel and allowing the system to equilibrate before using the desired phase. Thirty microliters of concentrated samples were applied to a silica gel aluminium plate at 1 cm above the edge. The TLC plate was subsequently placed into the TLC chamber that had been saturated with the mobile phase by putting in a Whatman filter paper for 30-60 minutes. The plate was taken out from the chamber when the mobile phase reached 0.5 cm from the top of the plate. The plate was subsequently air dried in the fume hood followed by detection of the bands under UV light at 254 and 365 nm. The bands presented on the TLC plate were marked using a pencil and the retention factor (Rf) of each band was recorded.

Bioautography was then carried out to examine which bands were the active ones. The TLC plate was cut to fit in a 90 mm petri dish. It was then placed in the bioassay medium so the silica gel side was facing the medium and contact directly with the medium. The TLC plate was taken out from the bioassay media after 1-4 hours and the bioassay medium was incubated at 37°C overnight. The presence of active compounds was indicated by the formation of ZOI with certain bands.

Preparative TLC applies the same principle as TLC, but it is used to purify larger amounts of compounds (Fried and Sherma, 1990). The mobile phase was prepared as described for the TLC method. Fifty milligram of concentrated sample was dissolved in a suitable solvent and applied horizontally onto a glass silica gel plate of 20 X 20 cm. The TLC plate was then developed using a selected mobile phase followed by detecting the bands under 254 and 365 nm UV light. The band that showed activity based on the bioautography was scraped off with a spatula and was transferred to a clean centrifuge tube. One milliliter of [2:8] chloroform:methanol was subsequently added into the tube and mixed by vortexing to elute the compounds from the silica gel. The tube was then centrifuged followed by transferring the supernatant carefully to a new tube for further analysis.

Different solvent systems for TLC were used in this study to obtain the optimum system for the detection of active compounds. The solvent systems are listed in table 4.1, 4.2 and 4.3.

Table 4.1 Solvent system for ethyl acetate of supernatant and methanol extract from submerged fermentaion F40 media (50 ml in 250 ml flasks)

Solvent			Ratio		
Ethyl acetate	:	methanol	7	:	3
Ethyl acetate	:	methanol	4	:	6

Table 4.2 Solvent system for ethyl acetate and methanol mycelial extract (upscale)

Solvent					Ratio					Note	
Chloroform	:	methanol	:	water	65	:	35	:	10	lower phase	Biphasic
Ethyl acetate	:	methanol			7	:	3			one phase	
Ethyl acetate										one phase	

Table 4.3 Solvent system for methanol extract of mycelia

Solvent					Ratio					Note	
Chloroform	:	methanol	:	water	7	:	13	:	8	lower phase	Biphasic
Chloroform	:	methanol	:	water	65	:	35	:	10	lower phase	Biphasic
ethyl acetate	:	methanol	:	water	7	:	13	:	8	upper phase	Biphasic
ethyl acetate	:	methanol	:	water	100	:	14	:	10	upper phase	Biphasic
ethyl acetate	:	toluene			7	:	93			one phase	

4.2.6.4 High performance liquid chromatography (HPLC)

HPLC-photodiode array analysis of the agar culture extract for dereplication of compounds produced by endophytic actinobacteria was conducted by Microbial Screening Technology, Sydney. The methanol extracts of agar cultures were freeze dried and sent for analysis.

Dried samples were dissolved in methanol and subjected to analytical HPLC using C-18 column. A gradient HPLC method was applied using methanol /water as the mobile phases. Two different gradient systems were performed. The first gradient was 10 minutes of 10-40% methanol, 4 minutes of 40%-100% methanol followed by a 2 minute holding time and a 2 minute re-equilibration to 10% methanol. The second gradient was 5 minutes of 5% methanol, 15 minutes of 5%-20% methanol, 10 minutes of 20%-30% methanol followed by a 5 minute holding time at 100% methanol and 5 minutes re-equilibration to 5% methanol. The peaks were measured at 205 nm, 210 nm, 230 nm, 254 nm and 380 nm.

4.2.6.5 The flowcharts for bioassay guided purification

The flowcharts of different approaches for bioassay guided purification carried out in this study is described in figures 4.1 and 4.2.

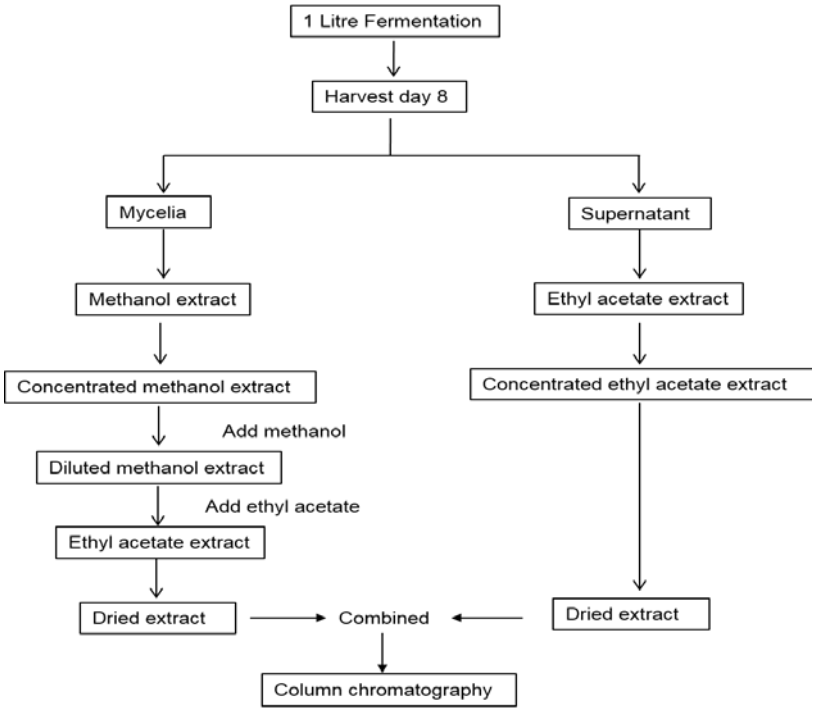


Figure 4.1 The first flowchart of bioassay guided purification approach

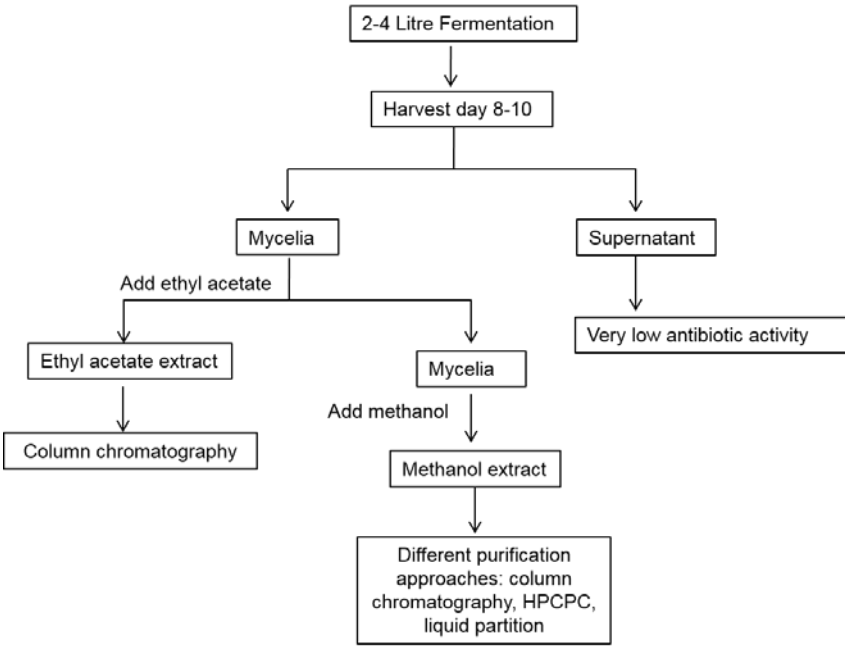


Figure 4.2 The second flowchart of bioassay guided purification appraoch

4.3 Results and Discussion

4.3.1 Screening for antibiotic activity against multi-resistant *S. aureus* SAK 3756 overexpressing *Nor A* gene

4.3.1.1 Minimum Inhibitory Concentration

Minimum inhibitory concentration of standard antibiotic for NorA efflux pump (ciprofloxacin and norfloxacin) was performed to confirm the resistance status of the test strains. The MIC of ciprofloxacin and norfloxacin against susceptible *S. aureus* SAK2703 and resistant *S. aureus* SAK3756 based on the microdilution method are listed in table 4.4 and it was shown that *S. aureus* SAK3756 is resistant to ciprofloxacin and norfloxacin.

Table 4.4 The MIC of ciprofloxacin and norfloxacin against *S. aureus* SAK3756 and 2703

MIC	SAK2703	SAK3756
Ciprofloxacin	0.5 µg/ml	7.8 µg/ml
Norfloxacin	0.95 µg/ml	15.6 µg/ml

4.3.1.2 Testing of compounds from Actinobacteria

Of the 51 methanol extracts derived from endophytic actinobacteria isolated from the wood of Australian plants, 4 extracts of isolates designated as AA4(a)1.2, AA4(a)1.4, DG1 and HVA6(c)3 showed activity against resistant *S. aureus* SAK3756. The antibiotic activity against this resistant *S. aureus* was also produced by 8 extracts of strains designated as CAP69, CAP168, CAP208, CAP 214, EUM63, EUM 187, EUM 199 and EUM244 which were isolated from Australian plants in a separate study (Kaewkla and Franco, 2013). The activity of these extracts against resistant *S. aureus* was higher than or similar to the activity against susceptible *S. aureus* as presented in table 4.5 (only extracts that showed clear ZOI with diameter more than 10 mm were included in the table).

Table 4.5 Actinobacteria isolates showing antibiotic activity against resistant (SAK 3756) and susceptible (SAK 2703) *S. aureus*

No	Actinobacteria codes	Diameter of inhibition	
		SAK 3756	SAK 2703*
1	CAP 69	11	11
2	CAP 168	12	9
3	CAP 208	12	11
4	CAP 214	30	26
5	EUM 63	14	13
6	EUM 187	20	20
7	EUM 199	24	21
8	EUM 244	16	15
9	AA4(a)1.2	13	12
10	AA4(a)1.4	15	12
11	DG1	26	21
12	HVA6(c)3	16	12

The dereplication based on a HPLC/DAD analysis of the extracts done at MST, Sydney, which included matching with Rt and UV-Vis absorbance maxima of the compounds in an extensive database revealed that strains CAP 214 and DG1 belong to the genus *Streptomyces* and *Actinomadura*, respectively, produced unique compounds, whereas other strains produced known compounds. HPLC/DAD analysis indicated the presence of phenazine, platensimycin and desertomycin in the extract of strain EUM 187. Manumycin and asukamycin were detected in the extract of strain EUM 199. A Thiopeptide and anthracycline were detected in the extract of strain AA4.1 and HVA4(f)7, respectively. However, after further purification of the compounds it was revealed that the active compound produced by CAP 214 was nigericin, which is a well-known ionophore antibiotic. This antibiotic was found to be one of the compounds that is frequently rediscovered in *Streptomyces* extracts (Genilloud *et al.*, 2011). Therefore, strain DG1 was selected for further purification and isolation of bioactive compounds.

Another strain isolated from wood designated as CMC4(a)1 also produced unique compounds. The extract of this strain did not show antibiotic activity against resistant *S. aureus*. However, it may possess other active compounds that might be worthy of further exploration.

The bioassay result showed that the methanol extract of the endophytic actinobacteria designated as DG1 produced strong antibiotic activity having a clear zone of 26 mm in diameter. The HPLC/DAD

analysis of the extract also revealed that DG1 produced 4 major compounds (figure 4.3). The retention time and the UV spectra of the peaks (figure 4.3 and 4.4) were compared to the MST extensive database which showed that the compounds are unknown.

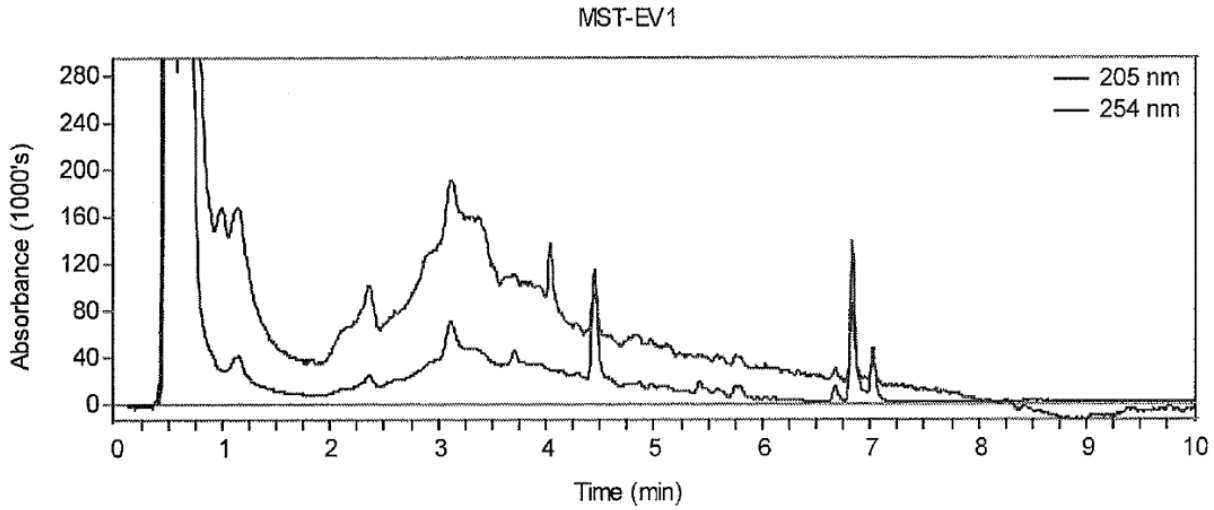


Figure 4.3 HPLC analysis of crude extract DG1

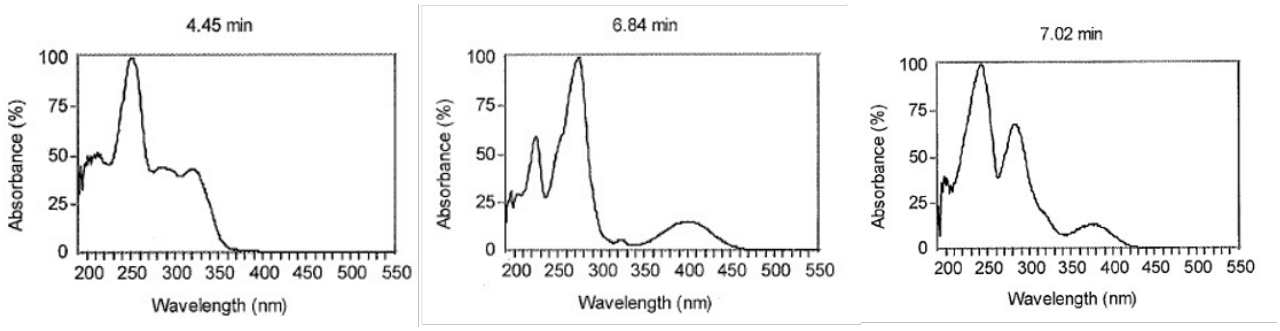


Figure 4.4 UV spectra of the main HPLC peaks

4.3.2 Fermentation and extraction

Strain DG1 which produced antibiotic activity in agar were assessed for production by submerged fermentation using five different media (Lee et al., 2001).

The activity against resistant *S. aureus* was observed in the methanol extract of the mycelium and the corresponding supernatant derived from fermentation using F40 medium. The methanol extract of the mycelium derived from fermentation using F33 medium had no antibiotic activity, but its corresponding supernatant showed activity against susceptible *S. aureus,* though not against the

resistant strain. The antibiotic activity against both susceptible and resistant *S. aureus* was not detected in the extracts of fermentations using F31, F26, and SI media.

The production of antibiotic activity by strain DG1 using F40 medium was detected both in the mycelium and its corresponding supernatant from day four of the fermentation when the first fermentation sample was taken. The antibiotic activity of the supernatant against resistant *S. aureus* increased until day 7 of fermentation before it levelled off until day 10. On the other hand, the antibiotic activity of supernatant against susceptible *S. aureus* was relatively stable from day 4 to day 10 of fermentation. The antibiotic activity of the methanol mycelial extract was relatively constant from day 6 to day 10 of fermentation. The activity of the extract against resistant and susceptible *S. aureus* was similar. However, it is worth noting that its corresponding supernatant had stronger activity against the resistant *S. aureus* compared to the activity against susceptible *S. aureus*. Thus, it is expected that the active compound produced by strain DG 1 has the potential to overcome antibiotic resistance. Based on these results, medium F40 was chosen for scale up fermentation. The antibiotic activity of the supernatant and mycelial methanol extract during submerged fermentation of strain DG1 against resistant and susceptible *S. aureus* is shown in figure 4.5 and 4.6.

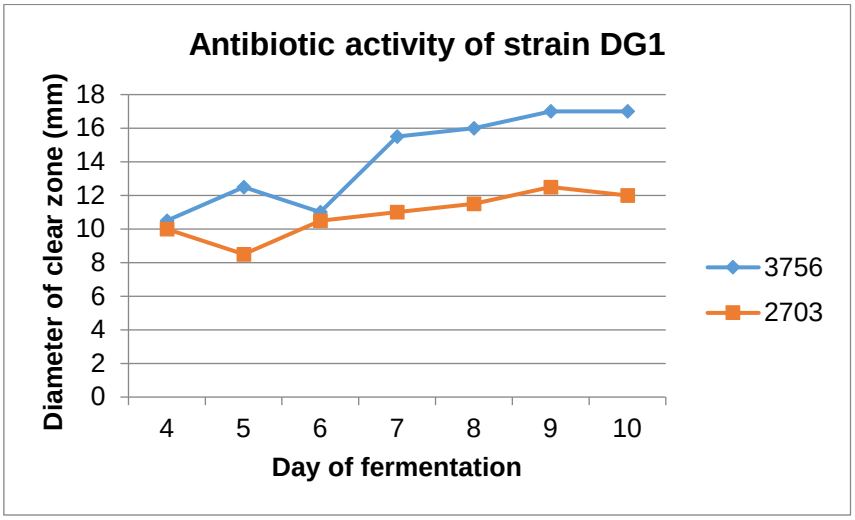


Figure 4.5 Antibiotic activity of the supernatant during submerge fermentation of strain DG1 against resistant strain *S. aureus* SAK 3756 and susceptible strain *S. aureus* SAK 2703

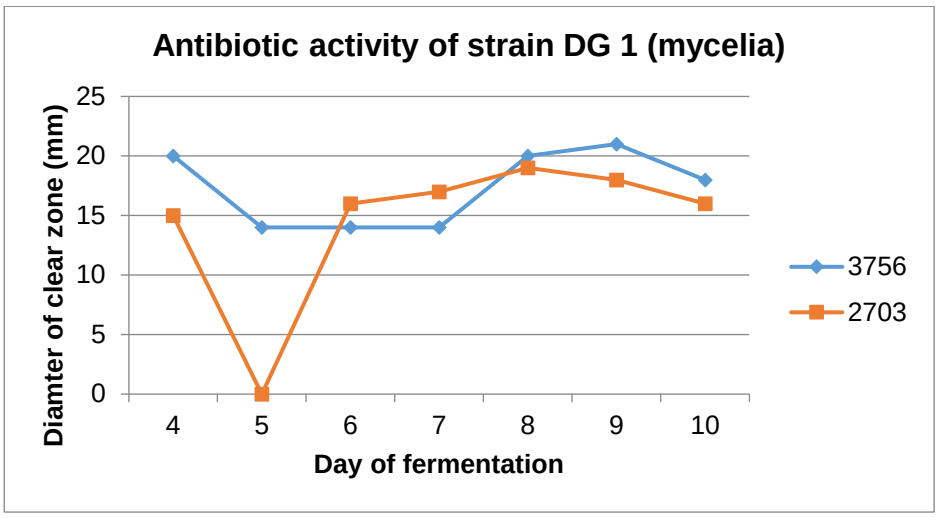


Figure 4.6 Antibiotic activity of the mycelial methanol extract of strain DG1 during submerged fermentation against resistant strain *S. aureus* SAK 3756 and susceptible *S.aureus* SAK 2703

The first small scaled (2 x 50ml) submerged fermentation was run for 8 days and the supernatant separated from the mycelium. The mycelium was extracted with methanol and the corresponding supernatant extracted with ethyl acetate. Both were assessed on thin layer chromatography for initial detection of the active compounds. Using ethyl acetate:methanol (7:3) as mobile phase, bands were detected under 254 nm and 365 nm UV light. After bioautography as shown in figure 4.7, some of the bands showed activity against resistant *S. aureus*. The Rf value of active bands indicated that the active compounds in the ethyl acetate extract are non-polar. The methanol extract contained non polar and polar active compounds as a clear zone was also formed in the initial spot. The active bands formed after bioautography are summarised in table 4.6.

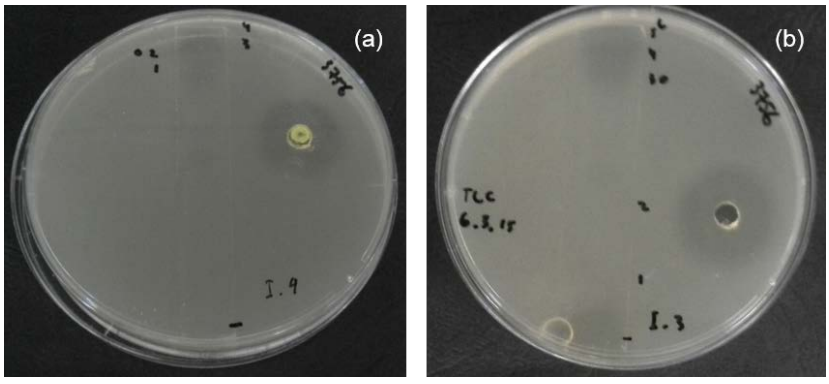


Figure 4.7 Bioautography of the ethyl acetate extract of supernatant (a) and mycelial methanol extract (b), showing antibiotic activity against resistant *S. aureus* strain SAK 3756

Table 4.6 The active bands formed after bioautography of methanol mycelial extract and ethyl acetate extract of the supernatant

Samples	Clear zones	Rf on TLC	UV Detection
Methanol extract of mycelia	Initial spot	0	–
	Band 4	0.85	254 nm
	Band 5	0.88	254 nm
	Between 4 and 5	Between 0.85 and 0.8	Not detected
Ethyl acetate extract of supenatant	Band 1	0.76	254 nm
	Band 3	0.86	365 nm
	Band 4	0.89	254 nm

4.3.3 Bioassay guided purification

4.3.3.1 Silica gel column chromatography of the ethyl acetate extract of the mycelium

The first 1 litre batch was produced by submerged fermentation. This time the extract of mycelia showed a ZOI of 17 mm against resistant *S. aureus*. However, the bioactivity of its corresponding supernatant was low. No clear zone was observed at day 5 and the diameter of clear zone at day 7 was 8 mm. As the antibiotic activity of the methanol extract was quite stable at day 5 and 7 so the fermentation was harvested at day 8. Despite its low activity, the supernatant was extracted with ethyl acetate. The concentrated ethyl acetate extract of the supernatant showed activity against resistant *S. aureus* with 17 mm in diameter of clear zone compared to that of 11 mm against susceptible *S. aureus*. The methanol extract was then extracted with ethyl acetate considering that nonpolar active compounds are easier to further separate, so the focus was put on the nonpolar active compounds. This ethyl acetate extract was subsequently combined with the ethyl acetate extract of supernatant and subjected to column chromatography (figure 4.1).

Among 70 fractions collected from column chromatography, 22 fractions (fraction 15-36) showed clear zones between 8-11 mm in diameter. These were smaller than the clear zones of the crude extract (17 mm). Weak active bands were detected from fractions 15-21 after bioautography. The Rf of the active bands indicated that the compounds were polar which were detected in the mycelial methanol extracts from the 50 ml fermentation. The activity of the compounds reduced significantly after purification using silica gel chromatography. This indicated that some compounds were “lost” during extraction or might have been degraded due to the instability during the process. The activity

of fractions 2-41 after ethyl acetate extract was subjected to column chromatography was shown in table 4.7.

Table 4.7 Bioassay of fractions 2-41 from ethyl acetate extract (first batch of fermentation) after column chromatography, against resistant *S. aureus* SAK 3756

Fractions	Diameter of clear zone (mm)	Fractions	Diameter of clear zone (mm)	Fractions	Diameter of clear zone (mm)	Fractions	Diameter of clear zone (mm)
2	0	12	0	22	10	32	9
3	0	13	0	23	11	33	9
4	0	14	0	24	11	34	8
5	0	15	10	25	12	35	8
6	0	16	11	26	10	36	8
7	0	17	10	27	10	37	0
8	0	18	11	28	9.5	38	0
9	0	19	9	29	9	39	0
10	0	20	10	30	8.5	40	0
11	0	21	9	31	8	41	0

Another 2 litre submerged fermentation batch was then run to confirm results. The methanol extract of the mycelia showed activity but its corresponding supernatant did not have activity. So, only the mycelial extract was used for further purification. A different method was applied for the extraction of the mycelia, which was extracted with ethyl acetate and subsequently extracted with methanol to separate polar and non-polar compounds at the initial stage. After evaporation using rotary evaporator, 86 mg of dried ethyl acetate extract and 1950.3 mg of freeze dried methanol extract were obtained.

Both ethyl acetate and methanol extracts of the mycelium were run on silica gel TLC plates using different mobile systems, ethyl acetate, ethyl acetate:methanol (7:3) and the lower phase of chloroform:methanol:water (65:35:10), as mobile phases followed by bioautography. The methanol extract had good activity with a clear zone of 18 mm. The lower layer of chloroform-methanol-water as mobile phase gave an active band with a Rf of 0.91, indicating non-polar active compounds. Polar active compounds were also present in the extract as clear zones were formed at the base spot.

The ethyl acetate extract of the mycelium was less active (12 mm). Active bands at the Rf of 0.85 and 0.91 were detected after the extract was run with bottom layer of chloroform-methanol-water on

TLC silica gel. The formation of a clear zone just above the initial spot was observed after the extract was run with ethyl acetate indicating the presence of relatively polar active compounds which were still extracted with ethyl acetate.

Based on the results from the first and the second fermentation of DG1, the strain produced polar and non-polar active compounds. Even though non polar active compounds were still detected in the methanol extract of the mycelium and vice versa, but they could be separated on the TLC plate. Figure 4.8 and 4.9 show the bioautogram of ethyl acetate and methanol extracts of the mycelium run with different solvent systems.

The ethyl acetate extract of the mycelium was subjected to silica gel column chromatography and eluted with a chloroform:methanol gradient. The polarity was increased slowly with methanol and then water. Among the 83 fractions collected, 16 fractions (fraction number 20-35) had a low activity with diameters of ZOI between 9-11 mm. These active fractions were subjected to TLC eluted with ethyl acetate. The yellow bands generated by fractions 20 to 21 showed activity after bioautography (figure 4.10), but the inhibition zones are weak. Some fractions having a similar pattern of active bands were pooled for the next analysis.

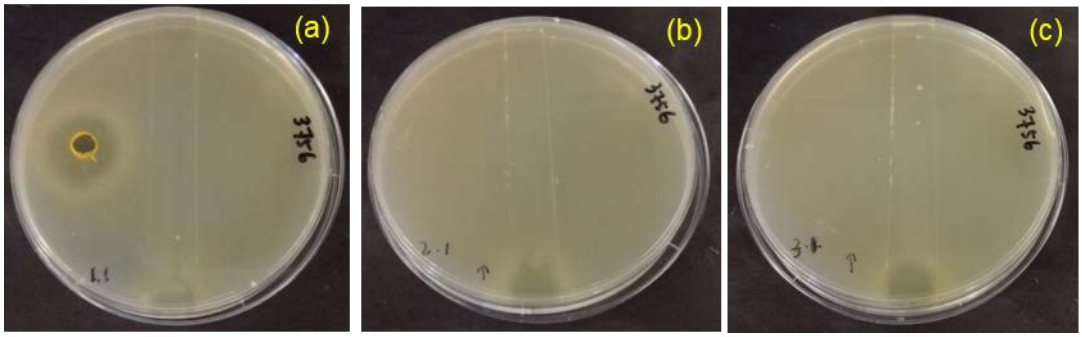


Figure 4.8 Bioautogram of methanol extract developed with lower phase of (a) chloroform:methanol:water (65:35:10, (b) ethyl acetate, (c) ethyl acetate:methanol (7:3)

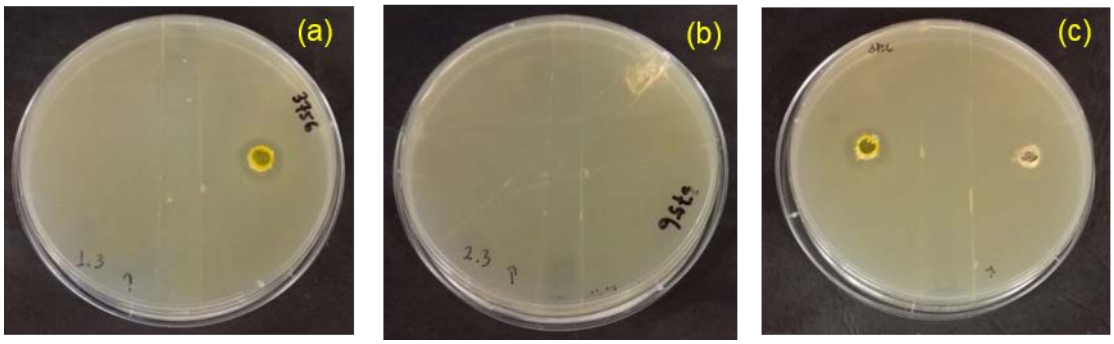


Figure 4.9 Bioautogram of ethyl acetate extract of supernatant developed with (a) lower phase of chloroform:methanol:water (65:35:10), (b) ethyl acetate, (c) ethyl acetate:methanol (7:3)

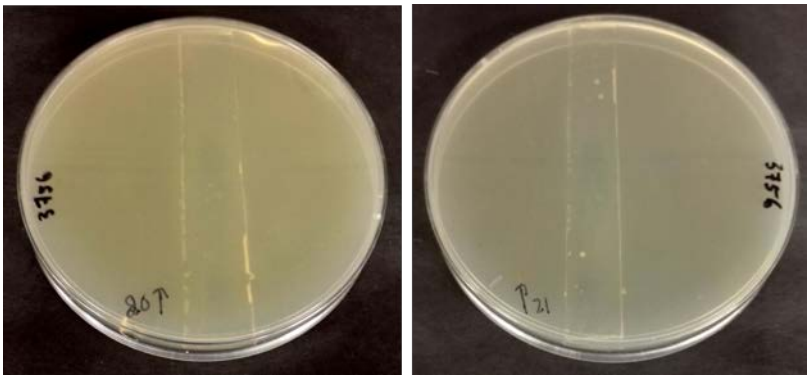


Figure 4.10 Bioautography of active fractions 20 and 21 showing faint ZOI against resistant *S. aureus* SAK 3756

As the activity of ethyl acetate extract was low, the purification of active compounds was attempted using dried methanol mycelial extract which demonstrated better antibiotic activity than the mycelial ethyl acetate extract.

4.3.3.2 High Performance Centrifugal Partition Chromatography

The bioautogram of the methanol extract of the mycelium run on TLC using the lower phase of chloroform:methanol:water system showed a clear zone close to the initial spot (figure 4.8 a). Therefore, some other mobile systems: methanol-ethyl acetate (6:4), ethyl acetate-methanol-water

(7:13:8), ethyl acetate-methanol-water (100:13.5:10), and toluene:ethyl acetate (93:7) were applied to run the extracts to get better separation of polar active compounds. Some bands were separated after developing with methanol-ethyl acetate (6:4) which is a relatively polar system compared to the others, but no clear zone was formed after bioautography. Thus, this system did not separate the active compounds. ZOI were formed at the initial spot and at a Rf of 0.55 after the extract was run with ethyl acetate-methanol-water (7:13:8). A Clear zone was detected at the initial spot after TLC developed the other two mobile systems. These results predicted the presence of relatively non polar and also very polar active compounds in the methanol mycelial extract.

To investigate the solubility of the active compounds in the organic and water phase and to examine the impurities in both phases, the extract was subjected to a liquid-liquid extraction using two different ratios of chloroform-methanol-water, 65:35:10 and 7:13:8. The TLC of the upper and lower layers of both systems revealed that the upper layer formed a clear zone, but not the lower layer. Therefore, the active compound was separated to the upper layer after liquid-liquid extraction. The bioautogram of the upper layer showed a clear zone just above the initial spot for both mobile systems. This indicated that the active compounds were very polar. The chloroform-methanol-water (65:35:10) system gave better clear zone formation and separation of other non active compounds. The TLC, showed that, even though this system still gave a few bands in the upper layer, more bands were observed in the lower layer (figure 4.11 and 4.12). This means more unwanted compounds were separated to the lower layer using chloroform-methanol-water (65:35:10) compared to that of chloroform-methanol-water (7:13:8).

Figure 4.11 TLC of upper phase and lower phase of methanol extract after liquid partition using chloroform:methanol:water (65:35:10) developed with lower phase of this system under 254 and 365 nm UV detections and its bioautography

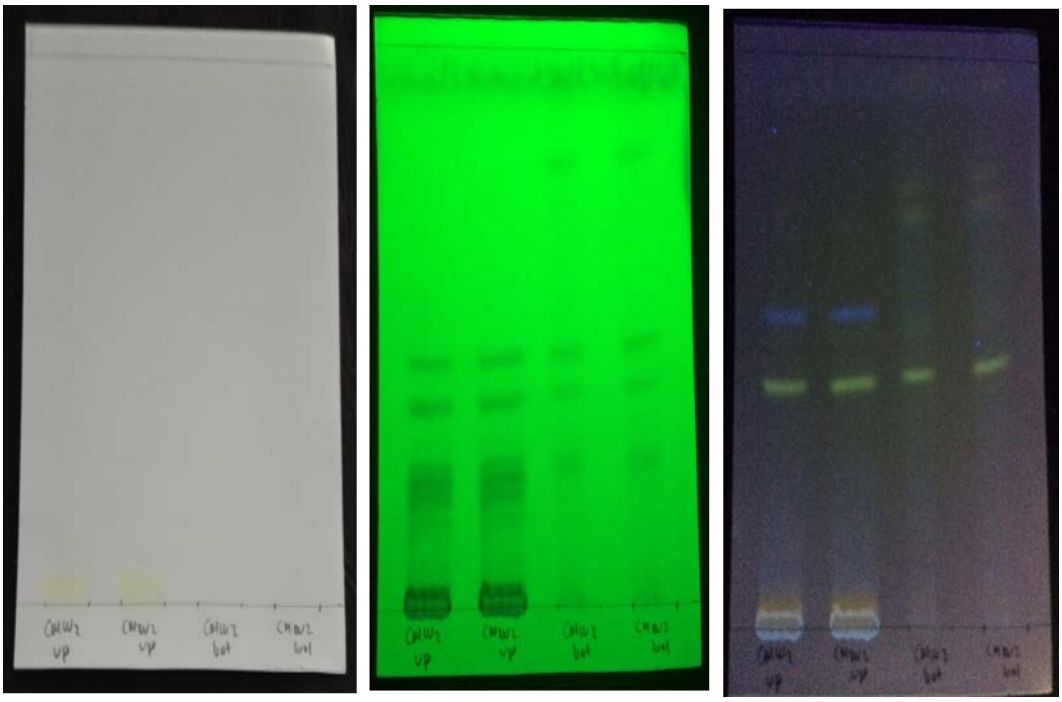


Figure 4.12 TLC of upper phase and lower phase of methanol extract after liquid partition using chloroform:methanol:water (7:13:8) under visual, 254 and 365 nm UV detection

A larger ZOI was formed in a well compared to the clear zone of an active band of the same volume of sample on a TLC plate, indicating that the activity was reduced considerably on the TLC plate. It could be that the active compounds strongly bind to silica gel and did not diffuse to the agar. Another possibility is some compounds work synergistically to inhibit resistant *S. aureus*, thus the activity was reduced when they were separated after TLC.

Based on all these results the dried methanol extracts of the mycelia were subjected to HPCPC to better separate the polar active compounds. One hundred and six fractions were collected after HPCPC. Fractions 1-50 were collected when the lower phase of chloroform:methanol:water (65:35:10) was used as the mobile phase (descending mode). Most fractions formed two phases which were then separated manually. The upper and lower layers of fractions 21-43 showed activity against resistant *S. aureus* with a ZOI between 8-14 mm. However, the activity of the lower layer was in general less than that of the upper layer. Only the lower layer of fractions 32-39 had a ZOI of 10 or 11 mm, compared with 8-9 mm for the rest of the fractions. Fractions 44-50 also had antibiotic activity, but only the upper layer. Figure 4.13 shows the activity of the upper layer fractions 20-50.

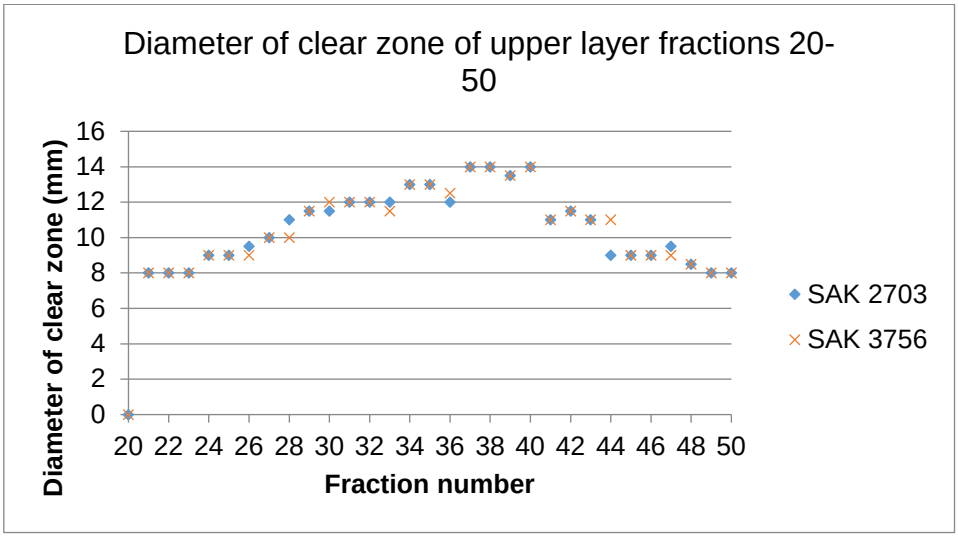


Figure 4.13 The antibiotic activity against resistant (SAK 3756) and susceptible SAK 2703) *S. aureus* of active fractions after HPCPC

Fractions 51-106 were collected under an ascending mode with the upper phase of the solvent system (chloroform:methanol:water=65:35:10) used as the mobile phase. Two layers were also formed for these fractions as happened when the descending mode was applied. No antibiotic activity was detected in the bottom layers of fractions 51-106. However, the upper layer of fractionss 60-65 showed similar antibiotic activity against resistant *S. aureus* SAK 3756 as well as the susceptible *S. aureus* SAK 2703 with the diameter of clear zone between 8-10 mm.

The TLC of the active fractions revealed that the upper layer of fractions 29-50 generated a yellow double band having very close Rf values of 0.21 and 0.25 without any detection aid and under UV365

detection. The TLC plates showing these yellow double bands are presented in figure 4.14. These bands were not detected in the active fractions 21-28. This was possibly due to the low concentration of the active compounds in fractions 21-28 indicated by the smaller diameter of the clear zone compared to, for example, fraction 29 that generated these yellow double bands. Another yellow band appeared at higher a Rf, but it was not active. These bands were also yellow under 365 nm UV detection. Similar yellow bands were also present in the upper layer of active fractions 63-65. Active fractions 60-62 were not well separated. A yellow spot-like band just above the spotting point and a smearing band were observed in fractions 60 and 61, respectively. Fraction 62 showed a broader yellow band on the TLC plate which was brown under UV detection at 365 nm. As well as the upper layer of active fractions 29-50, the lower layer of active fractions 21-43 generated two yellow bands that were visible and appeared as yellow bands under UV detection at 365 nm (these yellow bands are visually faint for fractions 21-26 and 41-43). These two bands have very close Rf values of 0.23 and 0.27. Another yellow band with a higher Rf was detected, but it is appeared as a faint yellow band under UV detection.

The bioautogram showed that one of the yellow double bands with a higher Rf (0.25 and 0.27) had antibiotic activity. The clear zone indicating antibiotic activity was also formed with the yellow band fractions 60 and 62. Based on the TLC pattern of the active fractions 21-50 and 60-65, there might be different active compounds produced by strain DG1.

Some impurities were still present in the active fractions which were detected under UV 254 nm. These impurities were relatively non-polar compare to the active compounds. The upper layer of one active fraction was subjected to UHPLC using gradient a system. Peaks were detected at Rt 2.4, 6.9, and 16.4, but they were too small so it is difficult to conclude whether these were the peaks of active compounds. The bottom layer of one active fraction was subjected to gradient HPLC using methanol-water as mobile phase, but the peaks were also too small to be considered. About 16.9 mg dried of material was obtain from the upper layer active fractions. Preparative HPLC can be a choice to further purify the sample, but it was thought that the quantity of the sample was not enough. Thus, the sample was subjected to NMR, considering that the active compounds appeared as yellow

on the TLC and under UV detection at 365 nm. It was thought they might have structures which lead to the identification of the complete structures. However, the impurities still hindered the ability to analyse the NMR data.

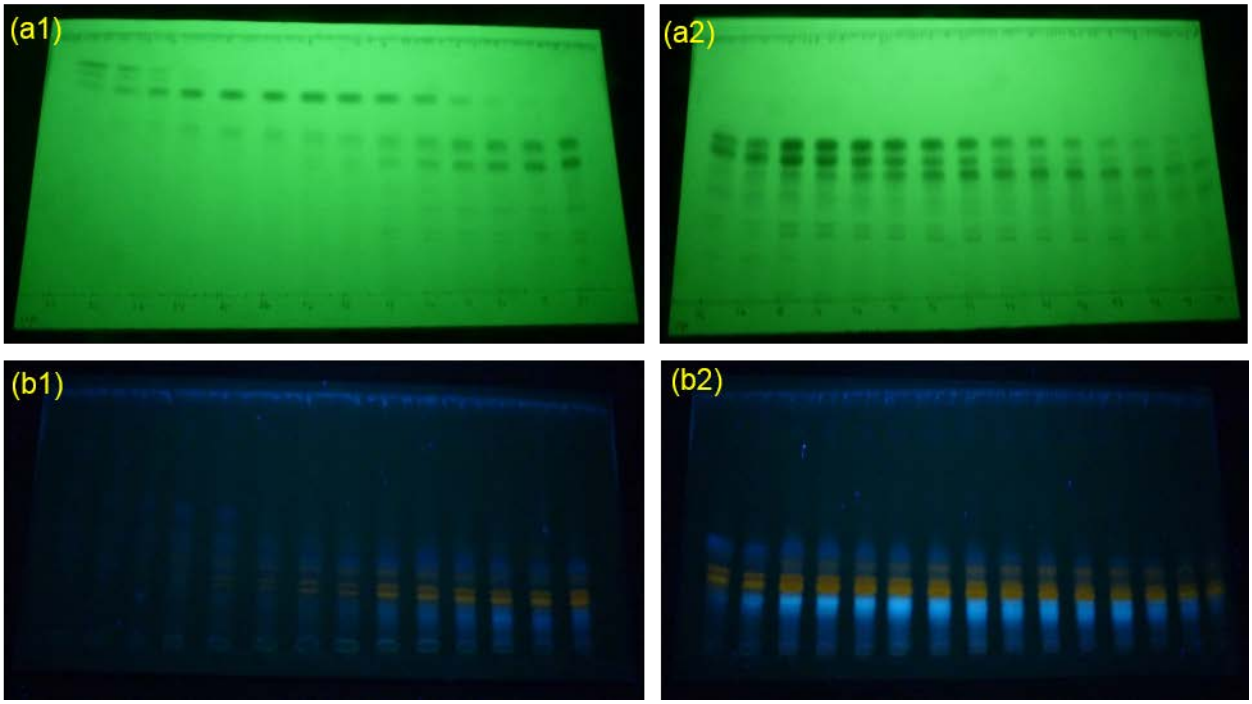


Figure 4.14 TLC of active fractions after HPCPC of methanol mycelial extract: (a1) fractions 21-34 and (a2) fractions 35-50 under 254 nm UV detections, (b1) fractions 21-34 and (b2) fractions 35-50 under 365 nm UV detection showing yellow double bands active against resistant *S. aureus* SAK 3756

4.3.3.3 Liquid-liquid partitioning to purify the active compounds

The previous liquid-liquid extraction using chloroform:methanol:water with the ratio of 65:35:10 and 7:13:8 system demonstrated that active yellow bands were detected in the upper phase. Therefore, a purification based on liquid-liquid extraction was done to isolate the active compounds using a freshly produced dried fermentation extract. Biphasic chloroform:methanol:water systems with the ratio of 65:35:10 and 7:13:8 were applied for liquid-liquid extraction of the crude extract. The upper phase and lower phase after liquid partition, using both systems, had antibiotic activity against resistant *S. aureus* SAK3756 which was higher than the activity against the susceptible *S. aureus* SAK2703. The clear zone of the upper phase was significantly smaller than the lower phase. These results were slightly different from the previous ones (discussed in section 4.4.2), where the activity was observed in upper phases. It is still in question why the results were different.

TLC of the lower layer of both biphasic systems, developed with chloroform:methanol (4:6), formed bands at Rf= 0.89 showing weak antibiotic activity. However, the original extract of the lower phase and the crude extract (dissolved in methanol) in the wells showed better activity with diameters of ZOI of 17-18 mm. Table 4.8 shows the bioautography results of the lower phases after liquid-liquid extraction using two different biphasic systems. Therefore, a simple purification approach was applied to separate the active compounds based on the observation that the active compounds were relatively non polar. By adding water to the lower phase twice, most of the polar compounds were expected to migrate to the water phase and the active compounds stay in the lower phase.

Table 4.8 The bioautography results of the lower layer after liquid-liquid extraction using chloroform:methanol:water witth 65:35:10 in ratio (1), 7:13:8 in ratio (2), and the crude extract (3)

Mobile phase	Samples	Activity against SAK 3756
C:M:W=7:13:8	1	No active band was detected
		The original extract in the well : 17 mm*
	2	A band showing weak activity was detected
	3	No active band was detected
		The original extract in the well : 18 mm*
C:M=4:6	1	A band showing weak activity was detected
		The original extract in the well: 17 mm*
	2	A band showing weak activity was detected
	3	No active band was detected
		The original extract in the well: 17 mm*

*The diameter of clear zone

The presence of active compounds in the lower phase and the possibility of synergy between compounds was subsequently tested using TLC developed with a lower phase of chloroform:methanol:water (7:13:8). ZOI were formed by the extract in the well and on the bioautogram of the lower phase without running on TLC. Three yellow bands (Rf= 0.06, 0.82, and 0.97) having antibiotic activity were detected on the bioautogram of lower phase. However, two yellow bands at Rf 0.82 and 0.97 showed weak antibiotic activity (faint inhibition zones). The yellow band at Rf= 0.82 was detected previously on TLC of the lower phase developed with chlorofom:methanol= 4:6 which confirmed the presence of this active compound in the extract. The bands formed after TLC of the lower extract were divided into 5 sections as shown in figure 4.15 and

scraped. As expected, the methanol extract of scraped TLC bands of section 5 generated clear zone with the diameter of 11.5 mm, because this section contained the active yellow bands. However, no clear zone was formed by the methanol extract of scraped TLC bands from section 1 which included the yellow active band showing a stronger zone of inhibition. Considering the very low Rf of this band, it was proposed that the active compounds bound strongly to the silica gel, so the concentration of the active compounds in the methanol extract was too low to inhibit the test organism. The methanol extract of scraped bands (all bands formed after TLC) was also tested for antibiotic activity and the result revealed that clear zone was formed, but its diameter of inhibition was lower than that of the original. It supported that most of the more polar active compounds in the lower phase were not extracted with 80% methanol. Therefore, the activity of the methanol extract of scraped bands (all bands formed after TLC) reduced significantly compared to the activity of the original lower phase. Table 4.9 shows the bioassay and bioautography after repeated liquid partitioning of the crude extract.

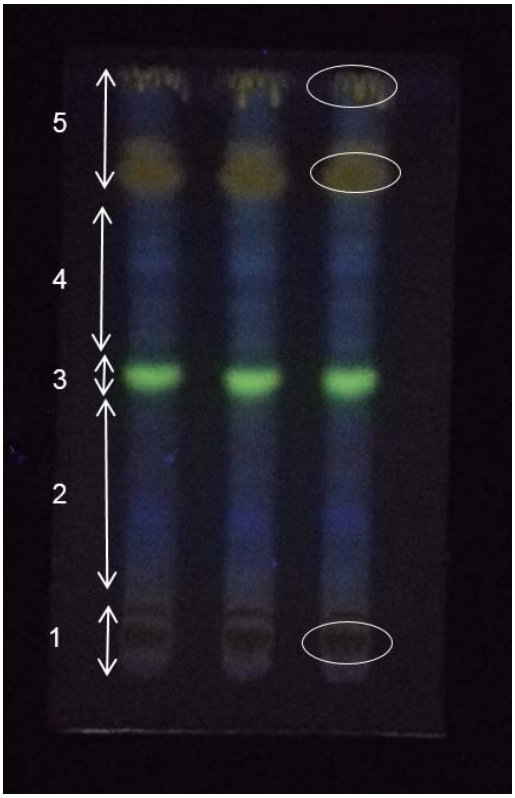


Figure 4.15 The TLC of the lower phase after liquid partition with chloroform:methanol:water (65:35:10) developed lower phase of chloroform:methanol:water (7:13:8) . The first lane was divided into 5 sections, the second lane all the bands were scrapped, the third lane was applied directly to bioautography

It was observed that the size of the ZOI of active bands formed after TLC was not comparable to that of the original extract. Therefore, it was proposed that previously there was a synergy activity between compound which reduced the activity after separation. It was expected that this synergetic activity could be analysed by comparing the activity of themethanol extract of scraped bands (all bands formed after TLC) and the original activity. However, the more polar active compounds were more likely to bind to the silica, so it could not be concluded if there was a synergy between compounds.

Table 4.9 Bioassay and bioautography of the lower layer after repeated liquid partition showing zones of inhibition of resistant *S.aureus* SAK 3756

Plate	Samples	Diameter of inhibition against SAK 3756
1	Upper layer second extraction	0
	Upper layer third extraction	0
	Lower layer second extraction	16.5
	Lower layer third extraction	15
	Original extract	16.5
2	First section of TLC scrapped	0
	Second section of TLC scrapped	0
	Third section of TLC scrapped	0
	Fourth section of TLC scrapped	0
	Fifth section of TLC scrapped	11.5
	Original extract	16
3	Bioautography of original extract without running the TLC	Clear zone was formed on the spot
	Original extract	16
4	Bioautography of original extract after TLC developed with lower layer chloroform:methanol:water (7:13:8)	Active band at the bottom (yellow spot)
		Active band at the top (yellow spot), faint
		Active band at the top (yellow spot), very faint
	Scrapped TLC	11
	Original extract	16.5

TLC of the lower phase after third the extraction formed two yellow bands having weak antibiotic activity. These bands were also detected on TLC and bioautography of the lower phase after the first extraction (figure 4.16). In contrast, the original lower phase after the third extraction showed good antibiotic activity. TLC of the lower phase after the first extraction formed an active yellow band just above the base line, which was also detected in the previous result (figure 4.15). However, this band with a very low Rf was not detected in the lower phase after the third extraction. These results indicated the presence of different active compounds produced by strain DG1. Figure 4.16 presents

the TLC of lower phase after the first and third extraction and figure 4.17 presents the bioautography after the third extraction.

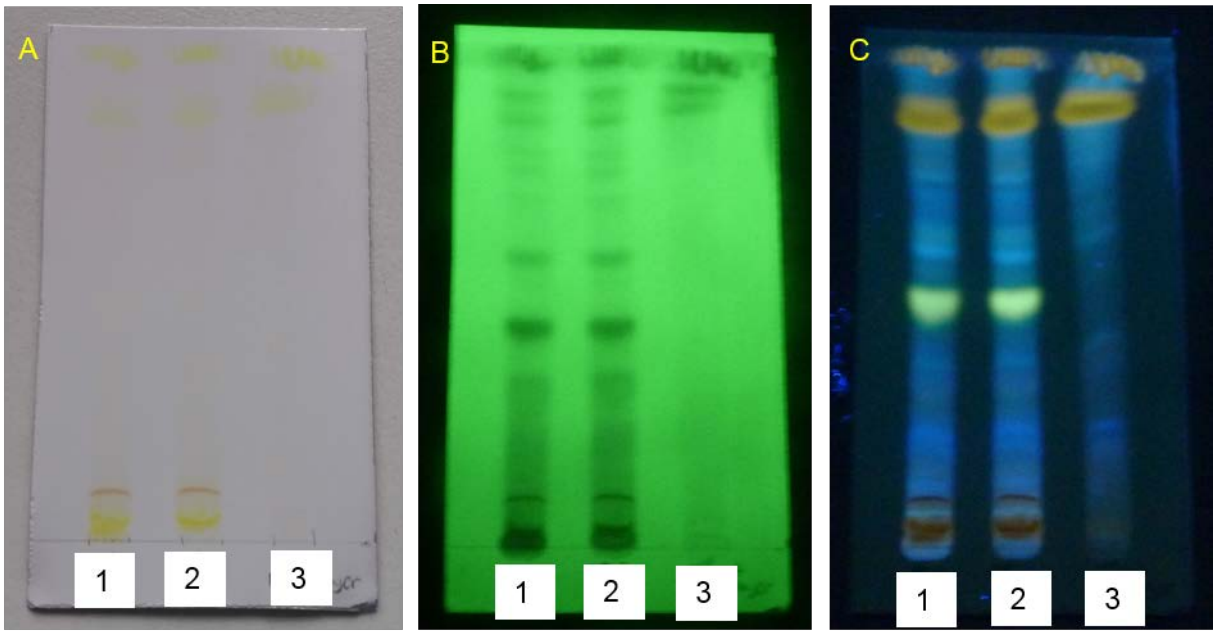


Figure 4.16 The TLC of lower phase after first extraction (lane 1 and 2 were duplicate), and third extraction (lane 3) developed with the lower phase of chloroform:methanol:water (7:13:8) under visual (A), UV 254 nm (B), and UV 365 nm (C) detections

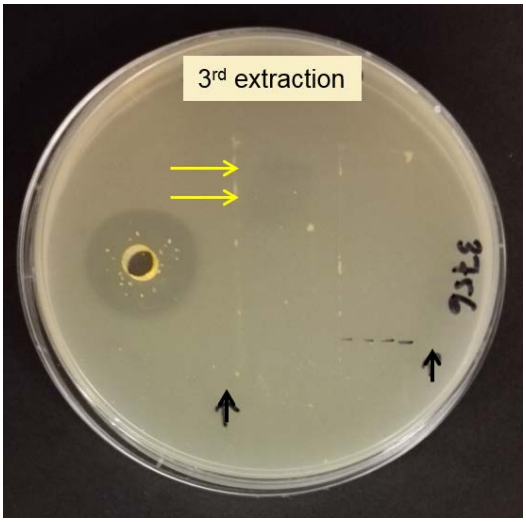


Figure 4.17 The bioautography of the lower phase after third extraction. Bands indicated having weak activity were indicated by the yellow arrows. The black arrows indicated the direction of TLC

TLC of upper phase and lower phases after the third extraction showed that most of the more polar impurities went to the upper (water) phase. The lower layer after the third extraction was relatively clean, even though some impurity bands seemed to be present (under UV 365 nm detection) in this phase (see figure 4.16 B and C). This was confirmed by TLC of the lower phase after first extraction, upper layer and lower layers after third extraction as demonstrated in figure 4.18.

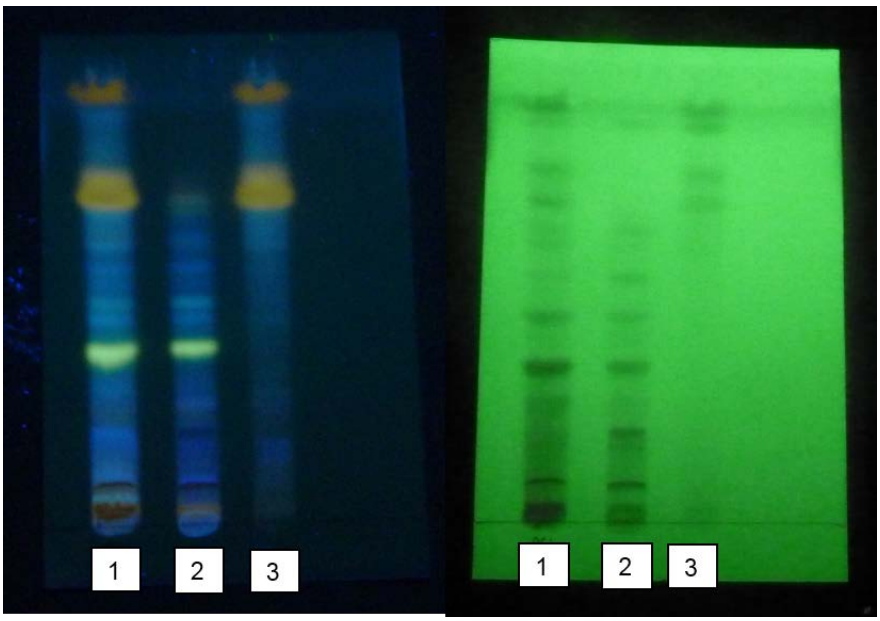


Figure 4.18 TLC of the lower layer after first extraction (1) upper (2) and lower layer (3) after third extraction showing the separation of impurities from the active compounds after repeated liquid-liquid partitioning

To confirm the result above, the upper layer after first, second, third extractions and the lower layer after first and third extractions were subjected to TLC developed with the lower phase of chloroform:methanol:water (7:13:8) as presented in figure 4.19 followed by bioautography. The results showed that all upper layers had antibiotic activity, with diameters of ZOI of 10, 12 and 8.5 mm after the first, second, and third extractions, respectively as presented in table 4.10.

The lower layer after the third extraction showed good activity having the same diameter of clear zone as the lower layer after the first extraction. Nevertheless, no active band was detected in this phase, including the more non-polar yellow active bands that were confirmed to be present in the previous bioautographies. Therefore, it was still in question where the activity indicated by the formation of a clear zone of 16.5 mm in diameter in the lower phase originated from (after third extraction).

It was noticed that the correct composition of the lower layer of chloroform:methanol:water seemed to be a critical factor for the presence or formation of yellow active bands in the organic phase after liquid-liquid extraction. It was affected by at least two factors, the equilibration time and the freshness of the mobile phase. The mixture of chloroform:methanol:water (7:13:8) needed to be equilibrated for at least 2 hours before separating the lower phase and it had to be used in less than 3 days.

Table 4.10 Activity against resistant *S. aureus* (SAK 3756) as measured by ZOI of all upper layers after repeated liquid extractions (three times) and the lower layer after the first and third extractions

Samples	Activity against SAK 3756	Rf or diameter of clear zone
Upper layer after first extraction	Active bands	Band at Rf < 0.1
	Sample in the well	10 mm
Upper layer after second extraction	Active bands	Band at Rf < 0.1
	Sample in the well	12 mm
Upper layer after third extraction	Active bands	No active band
	Sample in the well	8.5 mm
Lower layer after third extraction	Active bands	No active band
	Sample in the well	16.5 mm
Lower layer after first extraction	Active bands	Band at Rf < 0.1
	Sample in the well	16.5 mm

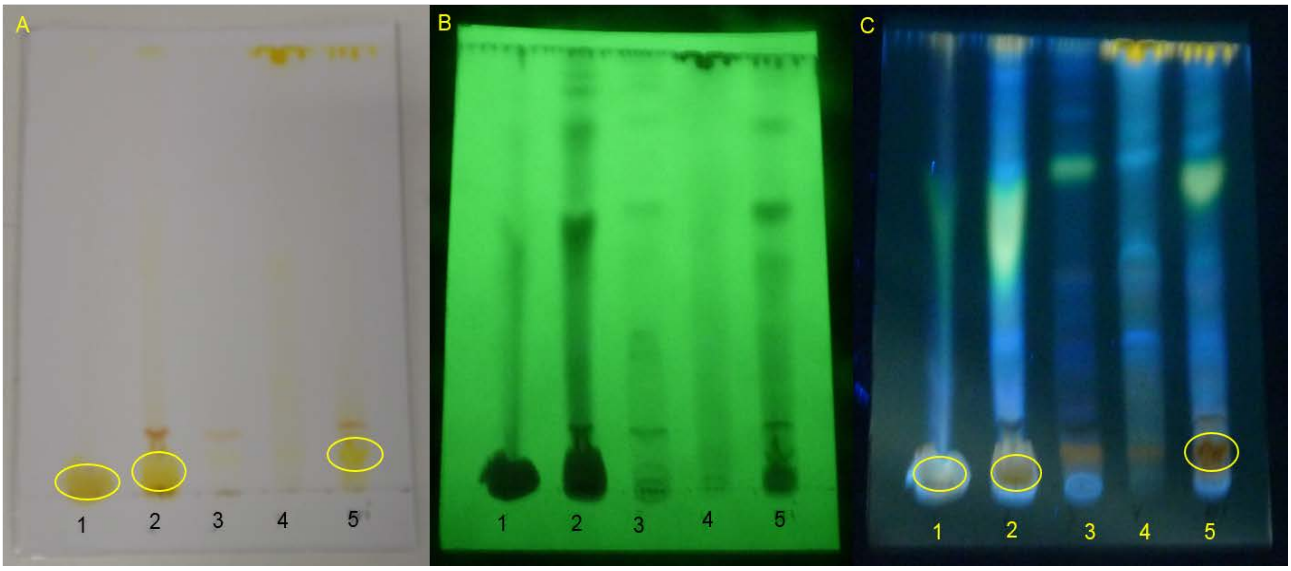


Figure 4.19 TLC of the upper layer after first (1), second (2) , and third (3) extractions and the lower layer after third (4) and first (5) extraction developed with chloroform:methanol:water (7:13:8) under visual (A), UV 254 nm (B) and UV 365 nm (C) detections. Circled bands were the active ones.

The lower layer after third extraction (EC4) was subjected to HPLC/DAD analysis. Some major peaks were detected and the UV spectra were scanned as showed in figure 4.20, and 4.21 respectively. The peaks at RT 6.57 min, 6.81 min, 7.0 min, and 7.77 min indicated the presence of the yellow active compounds as the UV spectra of these peaks had UV absorption values at about 420-430 nm.

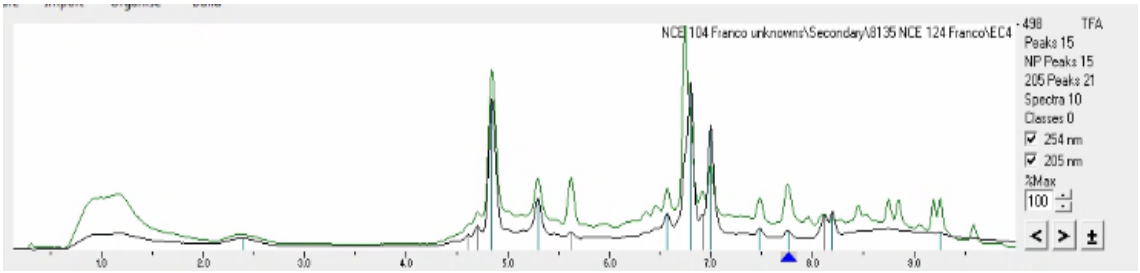


Figure 4.20 The HPLC chromatogram of the lower layer after liquid extraction using chloroform:methanol:water (65:35:10) of extract from submerged fermentation

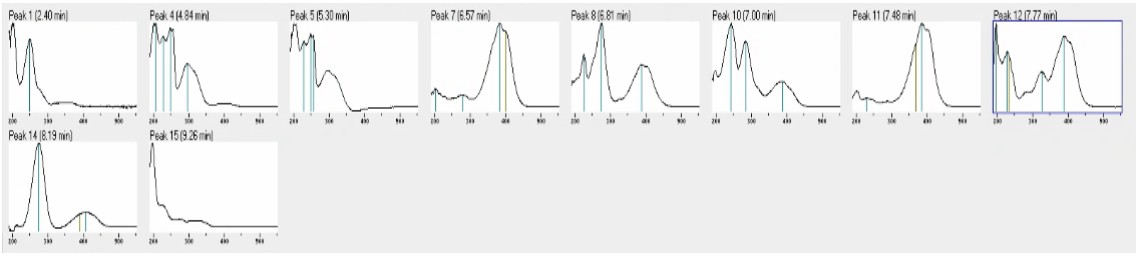


Figure 4.21 The UV spectra of the major peaks

However, the yellow active band at Rf about 0.82 (figure 4.16) did not always appear. This active band was not detected when TLC of the lower layer after extraction was repeated using the ssme solvent system (figure 4.19). This could be due to the poor reproducibility of the TLC results that were possibly affected by the solvent system used in the liquid-liquid extraction (chloroform-methanol-water = 65:35:10) in relation with the polarity of the active compounds. As some portions of methanol will go to the water phase, there was a chance that some active compounds were brought to water phase. This also happened when water was added to remove more impurities from the organic phase and more active compounds were brought to the water phase.

Ethyl acetate liquid-liquid extraction was then conducted to obtain better reproducibility. The ethyl acetate phase and the water phase had antibiotic activity against resistant *S. aureus*. TLC of the ethyl acetate phase developed with lower phase of chloroform:methanol:water (7:13:8) produced the active yellow band similar to that generated on the TLC of the lower phase after liquid extraction using chloroform:methanol:water (65:35:10). Figure 4.22 shows the TLC of the ethyl acetate phase and water phase after liquid-liquid extraction. The water phase which was not well separated showed

activity indicating the presence polar active compounds. Thus, strain DG1 in submerged fermentation produced polar and nonpolar active compounds.

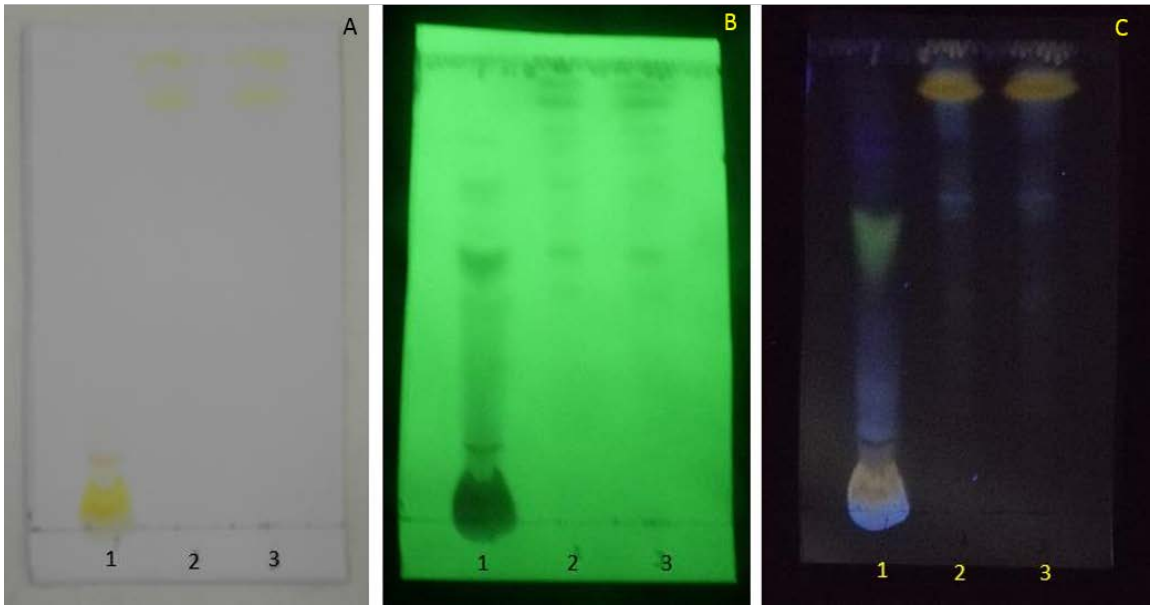


Figure 4.22 TLC of the ethyl acetate phase of dried fermentation extract after liquid-liquid extraction using ethyl acetate-water detected visually (), under UV 254 nm (B) and UV 365 nm

Based on the results of the bioassay guided purification of DG1 extract, generated from submerged fermentation, it was concluded that the active compounds were unstable with activity lost on contact with silica gel, and so the work to purify the antibiotic was stopped. Due to time limitations, it was decided that purification of the more stable antibiotic from the same culture grown on agar would yield results in a shorter time frame.

CHAPTER 5

PURIFICATION OF THE ANTIBIOTIC COMPOUNDS PRODUCED BY AGAR-CULTURE EXTRACTION

5.1 Introduction

It was noted that the activity of the original agar culture extract was more stable than the compounds produced by submerged fermentation. The differences in the production of compounds was noted in the chromatograms. Therefore, it was decided to scale up the production in an attempt to get sufficient pure compound for structure elucidation.

5.2 Methods

Solid state fermentation on rice as the substrate and scaled up solid state fermentation on ISP2 agar was trialled for the production of active compounds by strain DG1.

5.2.1 Solid state fermentation on rice

Twenty gram of rice was placed in each of five 250 ml Erlenmeyer flasks. A 5 ml volume of 0.1 % trace salt solution or HO-LE in LF 42 medium was then added into each flask followed by autoclaving at 121°C for 15 minutes. Seed culture was prepared as before by growing strain DG1 in IM22 liquid medium for 3 days incubated at 27°C in a shaker incubator. A 2 ml seed culture was inoculated into each flask followed by incubation at 27°C for 7 days. Sterile water was added if needed so the depth of the water was about 2 mm to prevent the media from getting dry. Samples were taken each day except day 5, where the approximately 0.8 g of whole rice culture per flask was taken and extracted with ethyl acetate and methanol. The samples were taken using a sterile spatula and put in Eppendorf tubes. A millilitre of methanol was subsequently added into each eppendorf tube, and well shaken for 15 minutes, followed by centrifugation to separate the mycelia from the methanol extract. The methanol extract was subjected to bioassay against the resistant *S. aureus* strain (SA3756) and susceptible *S. aureus* strain (SAK2703) using an agar well diffusion assay.

5.2.2 Solid state fermentation on ISP2 agar

In order to scale up the production of active compounds by strain DG1 on solid state fermentation, 46 plates of ISP2 agar were inoculated with 1 ml of spore suspension of the DG1 culture by spreading the spore suspension on the agar surface. This spores suspension was prepared by adding 100 ml sterile water into a 10 days old agar plate culture of DG1 that had been cut into small pieces. The 46 plates were extracted after 15 days incubation by chopping up the agar and adding the agar from each plate to 40 ml methanol. The methanol extracts from all the plates were pooled and concentrated to provide 3.6 g of crude extract.

5.2.3 HPLC method

The HPLC method used at Microbial Screening Technology (MST) is a proprietary protocol so the details cannot be divulged. It is a C18 column run with a Water-Acetonitrile gradient.

The diagnostic HPLC runs use a 10 cm column with a flow rate of 1 $ml.min^{-1}$, whereas the Preparative HPLC uses a larger column diameter at a flow rate of 2 $ml.min^{-1}$. Fractions were collected every 12 seconds from the Preparative HPLC-DAD-MS injected with a 1 mg sample, giving 400 µl eluate. This was further diluted with 1 ml methanol before testing against *S. aureus* SAK3756

5.3 Results and Discussion

The DG1 culture grew well on rice and produced a strong yellow pigment but no antibiotic activity was detected. As the methanol extract derived from agar culture of strain DG1 had good antibiotic activity, a scale up production of bioactive compounds was carried out on agar culture. The antibiotic activity of the extract was more stable compared to that of the extract from submerged fermentation. An active band was formed after the TLC plates developed with different ratios of chloroform:methanol and were subjected to bioautography. This band was at nearly the end point of the solvent development with Rf 0.93-0.98 depending on the mobile phase. The clear zone was also formed at the spotting point, indicating the very polar active compounds. The presence of polar and non-polar active compounds was confirmed by preparative TLC developed with chloroform:methanol (7:3) showing the active band at Rf= 0.9 and clear zone at the base line. The compounds eluted

from band at Rf = 0.9 also had antibiotic activity against resistant *S. aureus* (SAK 3756) whereas those eluted from the spotting line had weak antibiotic activity.

The active compounds produced in submerged fermentation were also recovered after liquid-liquid extraction using ethyl acetate-water as shown in figure 4.17 and figure 4.21.

Therefore, the dried extract derived from agar culture was subjected to liquid-liquid extraction using the same system considering that polar and nonpolar active compounds were present in this extract. After acetate extraction, 33.3 mg of dried extract was recovered. Some bands were formed after the ethyl acetate phase derived from liquid-liquid extraction was subjected to TLC developed with the lower phase of chloroform:methanol:water (7:13:8). The dried extract of DG1 obtained from submerged fermentation and agar culture showed different banding patterns indicating the production of different compounds in both cultures. A ZOI was formed at the top bands (figure 5. 1, circled) after bioautography against resistant *S. aureus*. Thus, it was likely that the active compounds produced in agar culture were different from those produced in submerged fermentation. A yellow band detected visually and under UV365 nm at Rf 0.39 was formed (figure 5.1), indicated by the arrows), but it was not active. This result revealed that the yellow band did not always represent the active compounds.

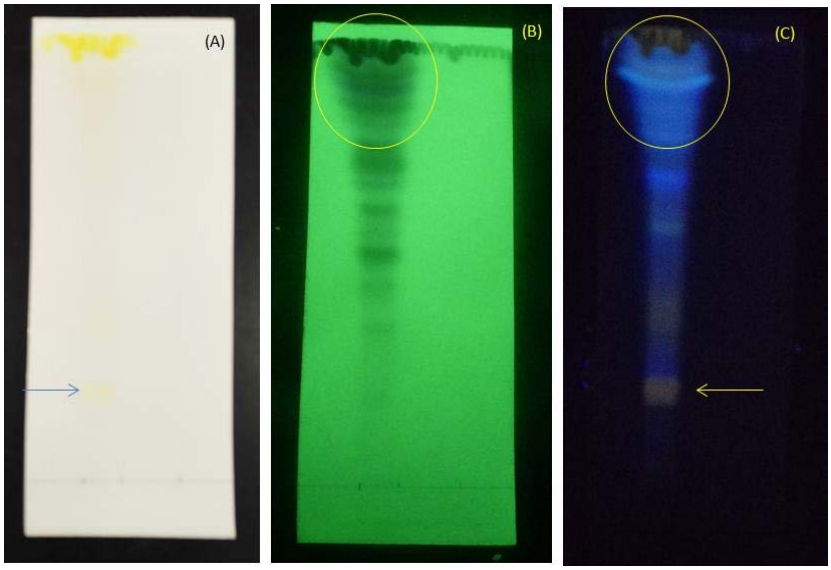


Figure 5.1 TLC of ethyl acetate phase of agar culture extract after ethyl acetate-water liquid-liquid extraction detected visually (A), under UV 254 nm (B), and UV 365 nm (C)

The production of different active compounds by strain DG1 grown in submerged fermentation and solid fermentation on agar culture was supported by different slopes of the concentrations versus antibiotic activities of partially purified extracts from the submerged fermentation (figure 5.2) and agar culture (figure 5.3).

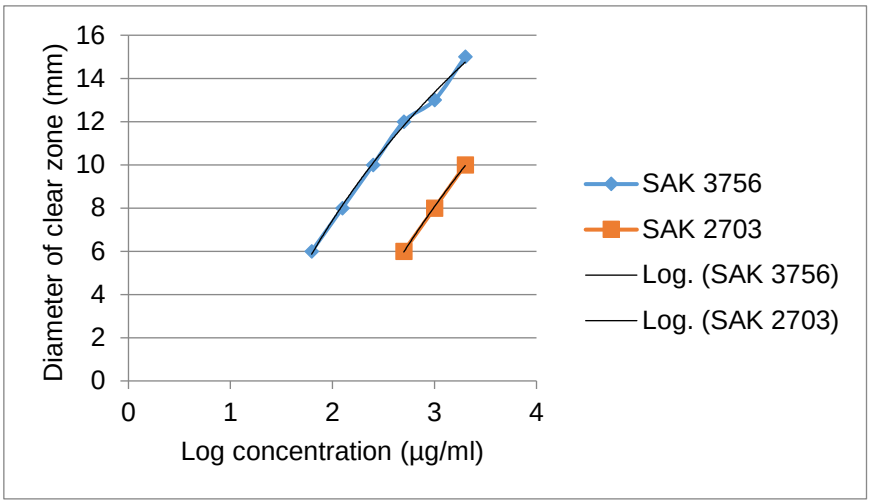


Figure 5.2 Double dilution assay of partially purified submerged fermentation extract

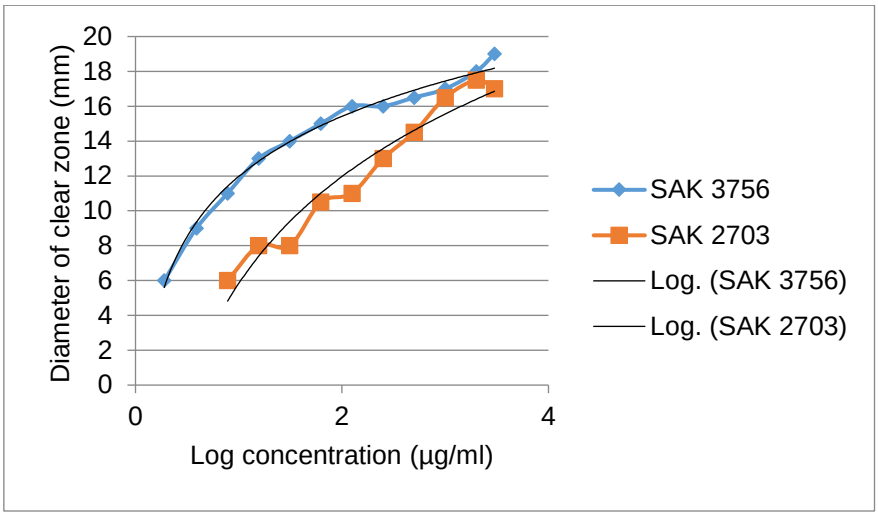


Figure 5.3 Double dilution assay of partially purified agar extract

The production of different secondary metabolites by a single strain of bacteria or fungi through the alteration of cultivation media composition has been investigated before which is known as One Strain-Many Compounds (OSMAC) approach. The capability of most fungi or bacteria to produce a number of chemically diverse secondary metabolites was confirmed by the whole genome

sequencing showing a number of gene clusters responsible for several secondary metabolite biosynthetic pathways in one strain. It has been reported that up to 20 different secondary metabolites were isolated from a single organism (Bode *et al.*, 2002).

A number of studies to explore the production of secondary metabolites using OSMAC approach have been reported. VanderMolen *et al.* (2013) examined the effect of media composition on the diversity of secondary metabolites produced by fungi involving 6 liquid media and 5 solid media. The UPLC-ELSD profile showed that the secondary metabolites produced by a fungal strain varied widely between media, especially in liquid media. *Streptomyces antibioticus* Tü6040 synthesized a diverse series of four different simocyclinones depending on the carbon and nitrogen composition in the fermentation media (Schimana *et al.*, 2001). It was interesting that *Isaria cateinnanulata* produced a significantly different proportion of volatile alkenes and alkanes when it was cultivated in solid and liquid media. It produced 57.6% alkenes and 9.19 % alkanes in solid medium. In contrast, 7.85% alkenes and 22.4% alkanes were produced in liquid medium. Moreover, certain types of polycyclic aromatic hydrocarbons were only synthesized in liquid or solid medium. The cultivation of *Isaria cateinnanulata* in solid and liquid media resulted in different compositions of a number of volatile polycyclic aromatic hydrocarbons (Zhang et al, 2011).

The small quantities of active compounds produced by strain DG1, especially the nonpolar ones, lead to difficulties in their isolation and identification. After solvent partitioning of 1 gram of dried extract from submerged fermentation with ethyl acetate for example, only 9.2 mg of active extract was obtained and about 20 metabolites were detected in this extract based on HPLC analysis. A similar amount of dried extract was gained from combined active fractions after HPCPC. If it could have been purified, only micro grams of the active compounds would have been obtained and this amount was not enough for structure analysis, for example NMR.

One of the limitations of the natural products based discovery is that they are often synthesized in small amounts. Therefore, a scale up fermentation is commonly run to increase the yield. A 100 litres submerged fermentation has been done to purify and isolate two novel homologous series of

macrolactam antibiotics produced by *Actinomadura* sp. SCC1778 (Cooper *et al.*, 1992). Four novel antitumor antibiotics which are close to rebeccamycin structurally, were purified and isolated from 110 litres of submerged fermentation of *Actinomadura melliaura* (Matson *et al.*, 1989)

In the case of DG1, a larger solid state fermentation scale up would be needed to produce sufficient compounds from strain DG1 for identification purposes. Depending on the molecule size, about 30-50 mg of material is required for proton NMR whereas carbon NMR requires 10-100 mg material, approximately. About 10 mg of partially purified active extract, which may give up to 1 mg of pure compound, was obtained from 1 g of dried crude extract derived from agar. Forty plates yielded 9 g crude extract which would be equivalent to 6 - 9 mg of pure compound. Therefore the equivalent of about 350 plates should yield approximately 50 mg of pure active compounds. It is a limitation in this study that the facilities for such a scale of upstream as well as downstream processing were not available.

Despite the limitation of this study to produce enough pure compounds for their identification, the LC/MS analysis of the extract from agar culture of DG1 showed the production of a number of secondary metabolites with unique UV spectra indicating unknown compounds. Figure 5.4 depicts the chromatogram of a partially purified agar culture extract after HPLC analysis. The UV spectra of the peaks and the molecular weight of the compounds are presented in table 5.2-5.4. The chromatogram and the UV spectra of the peaks detected in the ethyl acetate extract of the agar culture demonstrated that strain DG1 produced unique major active compounds in the agar culture and submerged fermentation. The chromatogram and the UV spectra of compounds produced in submerged fermentation was presented in figure 4.20 and 4.21.

To assess if the compounds that have activity against resistant *S. aureus* overexpressing *NorA* are the ones that have unique UV spectra, the extract was subjected to preparative HPLC. Sixty fractions were collected (each fraction is equivalent to 12 seconds) and some of them showed antibiotic activity as presented in table 5.1.

The active compounds were predicted to be in fractions 24 to 32 (Rt 4.6 to 6.4 minutes) as these fractions showed activity with ZOI of between 10-19 mm. The DAD and MS of the active fractions showed 2 compounds with Rt 4.79 and 6.21 minutes with masses of 281 and 295 Daltons, and unique UV spectra of 218, 252 (major), 290 and 320 nm, and 214, 254 (major), 286 and 322 nm, respectively. This indicates the 2 compounds are related with a possible difference as a methine (-CH2-) group.

Table 5.1 The fractions after preparative HPLC of purified extract from agar culture that showed activity against resistant *S. aureus* (only the active fractions are shown)

Fractions	Diameter of ZOI (mm)	Fractions	Diameter of ZOI (mm)
1	10	27	15
2	22	28	17
3	13	29	18
4	9	30	19
17	9	31	19
18	10	32	16
23	9	33	13
24	10	34	10
25	13	35	10
26	14	36	10

Searches of databases revealed no known compounds with the same UV spectra. The nearest, based on UV spectra with multiple peaks in a similar region are quinolones: burkholone produced by *Burkholderia sp*. (Mori *et al.*, 2007), intervenoline produced by *Nocardia* sp. (Kawada *et al.*, 2013), and a series of 8 new quinolones produced by a *Pseudonocardia* sp. (Dekker *et al.*, 1997). The UV spectra of active compounds produced by strain DG1 in agar culture is also similar to an indolzidine alkaloid designated as A58365 produced by *Streptomyces chromofuscus* (Nakatsukasa *et al.*, 1985). It was therefore concluded that at least 2 potentially novel compounds active against multi-drug resistant *Staphylococcus aureus* have been discovered.

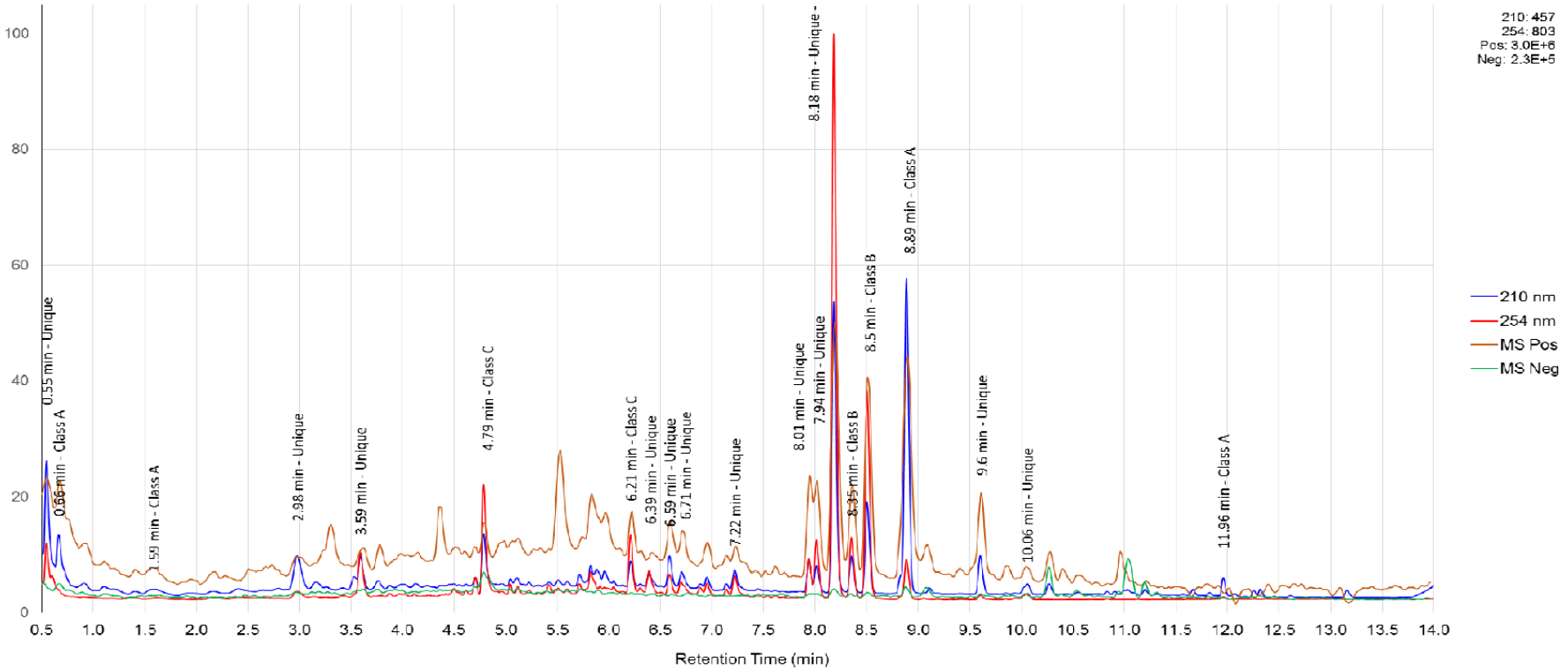


Figure 5.4 The chromatogram of HPLC-DAD analysis of partially purified agar culture extract

Table 5.2 The UV spectra and MS data of the peaks detected in the extract from agar culture

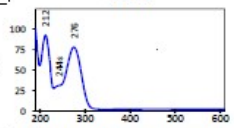
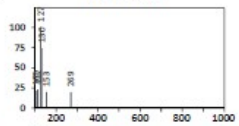
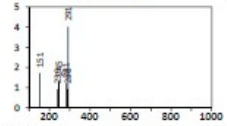
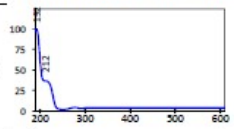
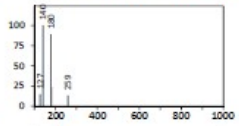
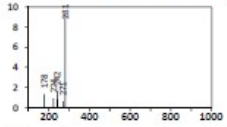
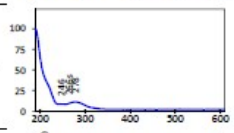
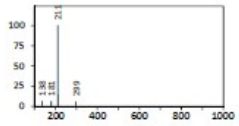
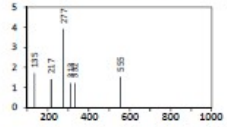
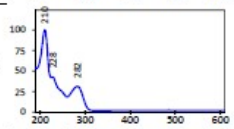
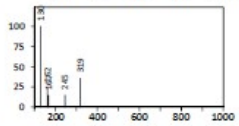
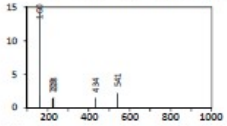
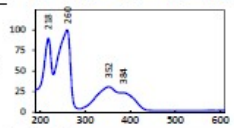
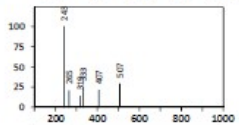
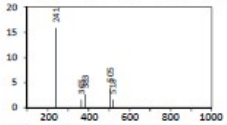
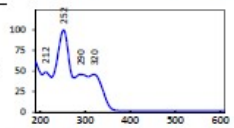
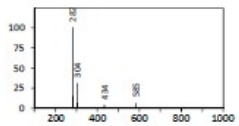
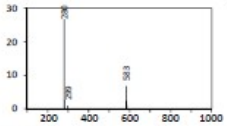
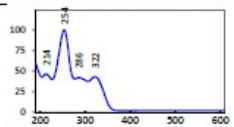
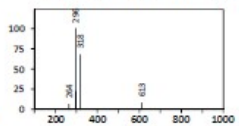
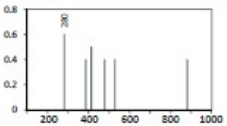
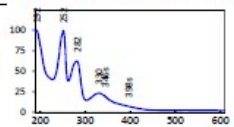
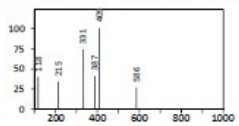
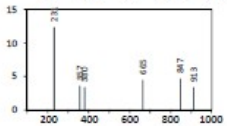
Peak	RetTime	Area	%Area	MS(+)	MS(-)	MW	Pos/Neg	UV Peaks	ID	UV-Vis	MS Positive	MS Negative
1	0.55	326.6	6.32	127 (100) 130 (73.9) 112 (22.6) 102 (21.1) 153 (19.6) 269 (19.4) TIC: 1.7E+05	291 (4) 151 (1.7) 245 (1.3) 281 (1.2) 288 (0.9) 239 (0.9) TIC: 6.9E+03	246* 152	24.7	212 (92) 244s (31) 276 (77)				
2	0.66	152.5	2.95	140 (100) 180 (88.6) 181 (23.1) 141 (21) 127 (14.6) 259 (12.8) TIC: 2.2E+05	281 (9.3) 242 (1.6) 178 (1.3) 224 (0.9) 243 (0.8) 271 (0.6) TIC: 2.1E+04	179**	10.7	192 (100) 212 (36)	Class A			
7	1.59	67.6	1.31	211 (100) 212 (14.5) 181 (6) 138 (5.9) 299 (5.2) 180 (4.6) TIC: 8.7E+04	277 (3.9) 135 (1.7) 555 (1.5) 217 (1.4) 313 (1.2) 332 (1.2) TIC: 3.4E+03	278	25.5	246 (8) 266s (9) 278 (11)	Class A			
13	2.98	239.2	4.63	130 (100) 319 (35.5) 162 (23.8) 245 (14.9) 167 (14.4) 131 (8.5) TIC: 9.2E+04	160 (14.1) 541 (2.1) 161 (1.8) 228 (1.5) 434 (1.4) 223 (1.3) TIC: 1.3E+04	161	7.1	210 (100) 228 (42) 282 (31)	Unique			
17	3.59	101.6	1.97	243 (100) 507 (29) 333 (24.1) 407 (21.1) 265 (20.5) 319 (13.4) TIC: 6.1E+04	241 (15.8) 505 (3.9) 242 (2.6) 383 (2.6) 518 (1.5) 365 (1.5) TIC: 9.6E+03	242***	6.3	218 (89) 260 (100) 352 (31) 384 (23)	Unique			
23	4.79	219.1	4.24	282 (100) 304 (31.1) 283 (15.3) 305 (6.5) 585 (6.1) 434 (3.7) TIC: 2.3E+05	280 (26.6) 583 (6.6) 281 (2.6) 584 (2.3) 585 (1.1) 299 (0.8) TIC: 6.1E+04	281****	3.8	212 (48) 252 (100) 290 (45) 320 (46)	Class C			
36	6.21	94.1	1.82	296 (100) 318 (67.5) 297 (22.3) 319 (9.7) 613 (7.8) 264 (5.8) TIC: 2.2E+05	280 (0.6) 414 (0.5) 478 (0.4) 529 (0.4) 387 (0.4) 881 (0.4) TIC: 2.2E+03	295*	99.3	214 (46) 254 (100) 286 (42) 322 (43)	Class C			
38	6.39	61.1	1.18	409 (100) 331 (73.7) 387 (41) 118 (39.9) 215 (34) 586 (26.6) TIC: 1.8E+04	231 (12.3) 847 (4.6) 665 (4.4) 357 (3.5) 380 (3.3) 913 (3.3) TIC: 2.2E+03	386	8.1	192 (100) 252 (99) 282 (62) 330 (23) 346s (18) 398s (7)	Unique			

Table 5.3 The UV spectra and MS data of the peaks detected in the extract from agar culture (continued)

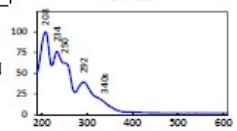
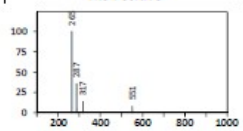
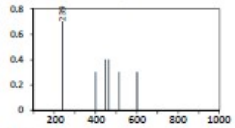
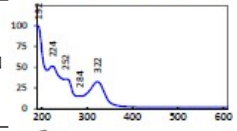
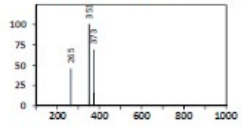
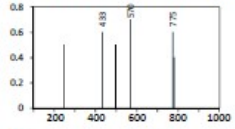
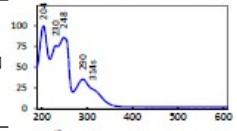
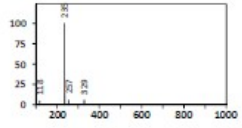
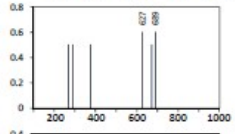
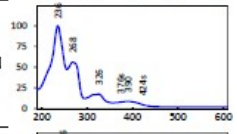
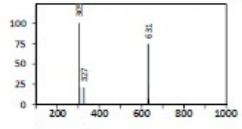
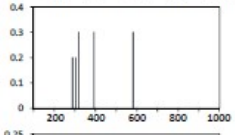
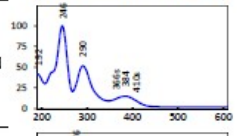
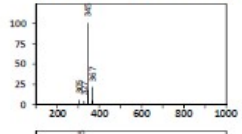
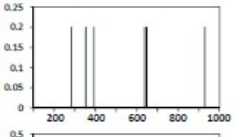
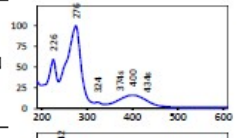
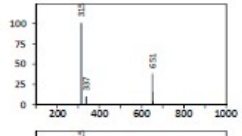
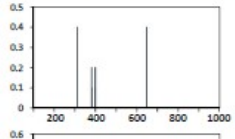
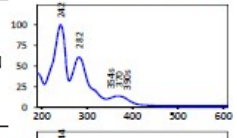
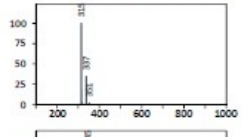
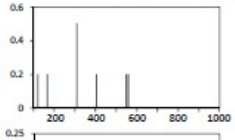
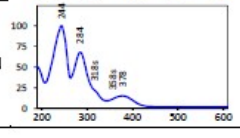
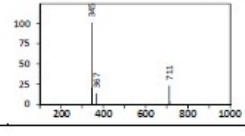
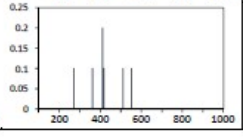
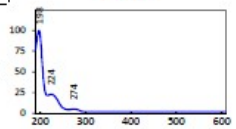
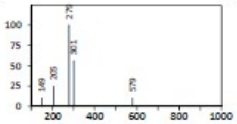
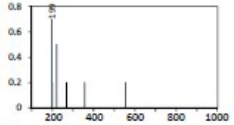
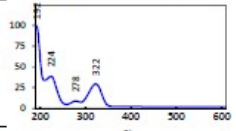
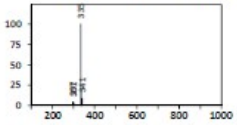
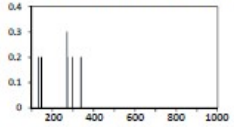
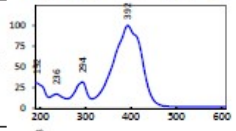
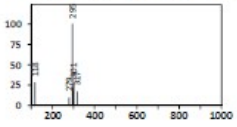
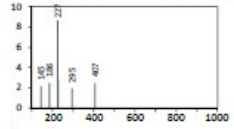
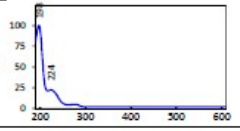
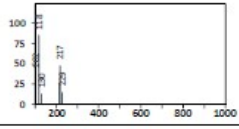
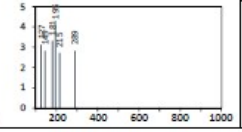
Peak	RetTime	Area	%Area	MS(+)	MS(-)	MW	Pos/Neg	UV Peaks	ID	UV-Vis	MS Positive	MS Negative
39	6.59	120.8	2.34	265 (100) 287 (36) 266 (15.2) 317 (13.7) 551 (8.1) 288 (6) TIC: 2.5E+05	239 (0.7) 464 (0.4) 449 (0.4) 515 (0.3) 601 (0.3) 401 (0.3) TIC: 1.6E+03	264*	151.6	208 (100) 234 (76) 250 (62) 292 (39) 340s (14)	Unique			
40	6.71	70.9	1.37	351 (100) 373 (68.5) 265 (45.3) 352 (23) 374 (15.5) 353 (9) TIC: 1.5E+05	570 (0.7) 433 (0.6) 775 (0.6) 248 (0.5) 498 (0.5) 781 (0.4) TIC: 1.0E+03	350	143.2	192 (100) 224 (51) 252 (35) 284 (15) 322 (32)	Unique			
44	7.22	91.6	1.77	235 (100) 236 (13.3) 257 (5.7) 329 (5.5) 118 (4.7) 237 (3.4) TIC: 1.8E+05	627 (0.6) 689 (0.6) 375 (0.5) 267 (0.5) 289 (0.5) 672 (0.5) TIC: 1.4E+03	234	124.4	204 (100) 230 (76) 248 (86) 290 (35) 314s (23)	Unique			
46	7.94	89.7	1.74	305 (100) 631 (74.4) 632 (24.7) 327 (20.8) 306 (20.8) 633 (7.3) TIC: 4.5E+05	582 (0.3) 318 (0.3) 393 (0.3) 289 (0.2) 288 (0.2) 304 (0.2) TIC: 1.9E+03	304*	231.6	236 (99) 268 (56) 326 (17) 376s (8) 390 (8) 424s (4)	Unique			
47	8.01	79.1	1.53	345 (100) 367 (21.3) 346 (17.9) 305 (5.4) 368 (4.9) 327 (4.1) TIC: 6.1E+05	648 (0.2) 637 (0.2) 930 (0.2) 393 (0.2) 353 (0.2) 281 (0.2) TIC: 1.5E+03	344	407.2	192 (41) 246 (100) 290 (51) 366s (12) 384 (14) 410s (8)	Unique			
48	8.18	1227.4	23.74	315 (100) 651 (37.7) 316 (19.2) 652 (15.6) 337 (9.7) 653 (3.9) TIC: 1.4E+06	313 (0.4) 649 (0.4) 381 (0.2) 399 (0.2) 398 (0.1) 382 (0.1) TIC: 6.5E+03	314*****	223.4	226 (59) 276 (100) 324 (7) 374s (11) 400 (16) 434s (8)	Unique			
49	8.35	151.3	2.93	315 (100) 337 (34.6) 316 (17.8) 338 (6.3) 317 (3) 351 (1.9) TIC: 5.7E+05	309 (0.5) 547 (0.2) 558 (0.2) 167 (0.2) 403 (0.2) 121 (0.2) TIC: 3.0E+03	314	189.6	242 (100) 282 (60) 354s (12) 370 (13) 390s (9)	Class B			
50	8.50	390.8	7.56	345 (100) 711 (22.8) 346 (20.7) 367 (13.1) 712 (9) 713 (3.2) TIC: 1.2E+06	411 (0.2) 362 (0.1) 271 (0.1) 418 (0.1) 553 (0.1) 511 (0.1) TIC: 3.4E+03	344**	355.5	244 (100) 284 (67) 318s (21) 358s (12) 378 (15)	Class B			

Table 5.4 The UV spectra and MS data of the peaks detected in the extract from agar culture (continued)

Peak	RetTime	Area	%Area	MS(+)	MS(-)	MW	Pos/Neg	UV Peaks	ID	UV-Vis	MS Positive	MS Negative
51	8.89	1395.2	26.99	279 (100) 301 (56.1) 205 (24.4) 280 (19.7) 149 (10.1) 579 (9.9) TIC: 9.1E+05	199 (0.7) 221 (0.5) 269 (0.2) 200 (0.2) 555 (0.2) 357 (0.2) TIC: 6.7E+03	278***	136.5	198 (100) 224 (23) 274 (5)	Class A			
54	9.60	171.7	3.32	335 (100) 336 (20.2) 341 (8.7) 337 (6) 297 (4.2) 301 (3.2) TIC: 5.5E+05	271 (0.3) 339 (0.2) 147 (0.2) 131 (0.2) 299 (0.2) 272 (0.2) TIC: 1.9E+03	340 300	289.8	192 (100) 224 (38) 278 (8) 322 (29)	Unique			
55	10.06	60.4	1.17	295 (100) 118 (28.1) 301 (26.8) 296 (21.5) 317 (16.9) 279 (9.3) TIC: 6.3E+04	227 (8.6) 229 (2.8) 186 (2.4) 407 (2.4) 145 (2.1) 295 (1.9) TIC: 5.4E+03	294*	11.6	192 (30) 236 (17) 294 (31) 392 (100)	Unique			
64	11.96	59.2	1.15	118 (84.8) 217 (47.6) 102 (37.8) 215 (26.5) 229 (14.5) 130 (12.9) TIC: 2.2E+04	195 (4.1) 181 (3.3) 127 (3.1) 289 (2.8) 145 (2.8) 215 (2.7) TIC: 5.2E+03	216	4.2	198 (100) 224 (22)	Class A			

CHAPTER 6

MAJOR FINDINGS AND FUTURE DIRECTIONS

6.1 Major findings

6.1.1 Isolation of endophytic actinobacteria

A total of 59 endophytic actinobacteria were isolated from the trunks of three Australian eucalyptus trees, Sydney blue gum (*Eucalyptus saligna*), Lemon scented gum (*Corymbia citriodora*), and an unidentified eucalyptus tree, tentatively classified as a stringy bark (*Eucalyptus obliqua).* Most actinobacteria were isolated from one eucalyptus tree, tentavely identified as a stringy bark (*Eucalyptus obliqua)*. At least 3 factors affected the number of actinobacteria isolated from the wood: the form of the sample plated onto the isolation media, the low abundance of actinobacteria residing the wood requiring more wood samples to be plated out onto a larger number of plates, and the isolation media used.

Wood powder was the most successful source for isolation of actinobacteria with more than 85% of isolates in this study was obtained from the wood powder. Powdering the wood enlarged the surface area of the sample that was in contact with the isolation media compared to wood shaving samples. It also aided the release of endophytic actinobacteria that commonly reside in the intercellular spaces (Jiao *et al.*, 2006). Therefore, there was a bigger chance for endophytic actinobacteria to access and grow on the isolation media.

The varation in numbers of isolated actinobacteria among the wood samples used in this study was proposed to be associated with different abundances of actinobacteria residing within the tree. However, no strong conclusion could be drawn from the wood shaving samples where only 3 and 5 isolates were obtained. These came from the first and second isolation where the isolation media used for the isolation was different from the third isolation (chapter 2 table 2.1). Therefore, while the wood samples were obtained from different Eucalyptus species which grew at different locations, no

conclusion could be drawn on the diversity of the populations of actinobacteria, or whether the type of soils and other environmental factors affected the abundance of the endophytic actinobacteria.

The isolation media influenced the number of actinobacteria isolated from the wood. The highest number of actinobacteria was obtained on HVA. These ares in accordance with a number of previous studies showing that HVA is a good medium to isolate actinobacteria. Isolation medium supplemented with amino acids was also found to support the growth of wood endophytic actinobacteria. Xylem sap contains various amino acids such as arginine, aspartic acid, citruline, glutamic acid (Barnes, 1963), proline, arginine (Escher *et al.*, 2004). Therefore, the addition of amino acids in the isolation media provided the nutrients present in the host plants.

A higher number of endophytic actinobacteria in the present study were obtained from an area within the young xylem. Nonetheless, there was no pattern of the distribution of actinobacteria inhabiting the wood along the cut cross section. Thus, it was proposed that endophytic bacteria randomly colonized the wood compartment.

6.1.2 Wood serves as a unique niche for rare actinobacteria

A diverse genera of actinobacteria were identified among the actinobacteria isolated from the wood, which were *Streptomyces*, *Actinomadura*, *Asanoa*, *Actinoallomurus*, and *Actinoplanes*. Interestingly, except for *Streptomyces*, the other genera are categorized as rare. In addition, one of the *Streptomyces* isolate was slow growing and possibly novel. Woods originated from endemic native trees, i.e. Australian trees represent unique niches for actinobacteria. Therefore, they are a potential source for other rare actinobacteria and slow growing *Streptomyces*. These niches are relatively unexplored part of plants offering a greater probability for isolation of novel actinobacteria.

6.1.3 Bioprospecting of endophytic actinobacteria to reverse antibiotic resistance

One actinobacterium isolated from wood designated as DG1 produced compounds more active against multidrug resistant *S. aureus* overexpressing NorA efflux pump than its sensitive partner. This isolate belongs to the rare genus *Actinomadura*. Based on the LC/MS analysis, partially purified

DG1 extract from solid fermentation contained a number of compounds having UV spectra that did not match with those in the MST proprietary data base of secondary metabolites produced by actinobacteria. The compounds that had activity against MDR bacteria are possibly novel ones. The results indicated that rare endophytic actinobacteria are potential producers of novel secondary metabolites.

6.1.4 Strain DG1 produced different active compounds in solid and submerged fermentation.

Partially purified DG1 extract generated from submerged fermentation in F40 medium contained different active compounds from those obtained from solid fermentation using ISP2 agar medium. Dissimilar active bands were detected on TLC plates of both partially purified extracts and confirmed by HPLC analysis. The active compounds produced in the solid fermentation was more active and more stable compared to the active compounds produced in submerged fermentation. It These results agree with previous studies that fermentation media influence the production of secondary metabolites.

6.1.5 Whole cell assay targeting NorA efflux pump led to the discovery of actinobacteria producing active compounds against MDR bacteria.

The efflux mechanism is one of the major causes of resistance, and is the first barrier for antibiotics to enter the MDR bacterial cell. Thus, the bacterial efflux pump was proposed to be an excellent screening target to search for novel antimicrobial agents, especially to reverse antibiotic resistance. This study demonstrated that using a well-defined MDR bacteria overexpressing *NorA* efflux pump in a whole cell assay led to the detection of active compounds against MDR bacteria. The whole cell assay targeting the efflux pump would generate not only *in vitro*, but also *in vivo* antibiotic activity as it has a specific target while maintaining the bacterial intact cellular function (Donadio *et al.*, 2002). This assay could be used as an up-front examination of antimicrobial activity to discover novel antibiotics (Genilloud *et al.*, 2011)

To conclude, the wood of native trees represents a unique niche that provided a diverse genera of rare actinobacteria as well as slow growing *Streptomyces.* A number of factors increased the success of isolation of actinobacteria from wood in terms of number and variety: 1) powdering the wood samples, 2) use of HVA as the isolation medium, 3) supplementing the medium with amino acids, 4) use of large numbers of isolation plates, and 5) extended incubation time. A screening strategy involving the use of rare actinobacteria and whole cell assay targeting efflux pump overexpressing bacteria led to the detection of active compounds against MDR bacteria.

6.2 Future directions

1. Isolation of actinobacteria from wood of a wide range of plants.

Various genera of rare actinobacteria and slow growing *Streptomyces* have been isolated from wood of Australian trees. This suggests that further exploration of wood from various native plants or plants that grow on unique environmental settings, for example high biodiversity areas, are worthy sources to isolate these unusual groups of actinobacteria. Rare actinobacteria as well as slow growing *Streptomyces* would provide chemically diverse unknown secondary metabolites, which are very important for the screening of new drugs.

2. Increase the productivity of strain DG 1.

The active compounds produced by strain DG1 are promising to overcome antibiotic resistance and based on the LC/MS analysis there is a high probability that these compounds are new ones. However, low levels of active compounds were produced by strain DG1 both in submerged fermentation and in solid fermentation. Thus, increasing the productivity of these compounds is needed to be able to further isolate and identify the pure active compounds.

Based on the TLC analysis, different active compounds were produced by strain DG1 in solid and submerged fermentation. The compounds produced in solid fermentation were more active and stable. Therefore, it is suggested that scaled up solid fermentation be used to obtain enough active compounds for further isolation and identification.

Strain selection and improvement is also an effective approach to increase antibiotic production. The advances in whole genome sequencing facilitate evaluation of the genes and the biosynthetic pathways responsible for antibiotic production. Mutation of certain genes has been proven to increase the yield of antibiotic product.

3. Exploring other useful secondary metabolites produced by strain DG1.

Based on the LC/MS analysis, strain DG 1 that belongs to the genus *Actinomadura* produced several potentially new compounds. Thus, the exploration of strain DG using different activity assays may lead to the discovery of other new active compounds for different applications.

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APPENDICES

APPENDIX 1. MEDIA RECIPES

A. Isolation media

1. Humic Acid Vitamin B Agar

	g/L
Humic acid	1*
Na_2HPO4	0.5
KCl	1.71
$MgSO_4.7H_2O$	0.05
$FeSO_4.7H_2O$	0.01
$CaCO_3$	0.2
Agar	15

Vitamin B (100X) (add 1 ml/L media after autoclaving)

	Per 100 ml RO water
Thiamine hydrochloride	5 mg
Riboflavin	5 mg
Niacin	5 mg
Pyridoxine-hydrochloride	5 mg
Inositol	5 mg
Ca-pantothenate	5 mg
p-aminobenzoic acid	25 mg
Biotin	25 mg
Adjust pH to 4.5 and filter sterilized	

2. VL70

	Per litre
3-(N-morpholino) propanesulfonic acid	2.09 g
$MgSO_4$	0.2 mM
($MgSO_4$ 24.09 mg; $MgSO_4.7H_2O$ 49.3 mg)	2 mM
K_2HPO_4 (27.02 mg)	0.2 mM
Growth substrate[a]	
Selenite tungstate solution[b]	1 ml
Trace element solution SL-10[c]	1 ml
Vitamin solution 1[d]	1 ml
Vitamin solution 2[e]	3 ml

[a]Growth substrate:

AA[f]: Amino acid mixture with an addition of 0.08 g L tryptophan

per 100 ml of stock solution added at the ratio of 10 ml stock solution per litre of medium (filter sterilized using 0.22 µm Millipore filter)

GGXA: Mixture of D-glucose, D-galactose, D-xylose and L-arabinose 0.5 mM each (filter sterilized using 0.22 µm Millipore filter)

GGCMC: Carboxymethyl cellulose 0.05% (w/v) added before autoclaving

[b]Selenite tungstate solution:

NaOH	0.5 g
$Na_2SeO_3.5H_2O$	3 mg
$Na_2WO4.2H_2O$	4 mg
RO water	1 L

[c]Trace element solution-SL10:

HCl (25%,7.7M)	10 ml
$FeCl_2.4H2O$	1.5
(or $FeCl_3$. 6H2O 2.04 g)	
$ZnCl_2$	70 mg
$MnCl_2.4H_2O$	100 mg
H_3BO_3	6 mg
$CoCl_2.6H_2O$	190 mg
$CuCl_2.2H_2O$	2 mg
$NiCl_2.6H_2O$	24 mg
(or $NiCl_2$ 13 mg)	
$Na_2MoO_4.2H_2O$	36 mg
RO water	990 ml

The pH was adjusted to 2.5

First dissolved FeCl2 in 7.7M HCl, then dilute in the water, add and dissolve the other salts. Make the solution up to 1 L.

[d]Vitamin solution 1 (filter sterilized)

	Per litre RO water
4-aminobenzoate	40 mg
(+)-Biotin	10 mg
Nicotinic acid	100 mg
Calcium D(+) panthothenate	50 mg
Pyridoxine hydrochloride	150 mg
Thiamine hydrochloride	100 mg
Cyanocobalamin	50 mg

The pH was adjusted to 4.5

[e]Vitamin solution 2

	Per litre RO water
DL-6,8 thioctic acid	10 mg
Riboflavine	10 mg
Folic acid	4 mg

The pH was adjusted to 4.5

[f]Amino acid mixture

	Per 100 ml RO water
Aspartic acid	470 mg
Threonine	210 mg
Serine	320 mg
Glutamic acid	960 mg
Proline	690 mg
Glycine	150 mg
Alanine	200 mg
Valine	220 mg
Methionine	90 mg
Isoleucine	110 mg
Leucine	250 mg
Tyrosine	70 mg
Phenylalanine	140 mg
Lysine	350 mg
Histidine	100 mg
Arginine	150 mg
L-tryptophan	80 mg

All isolation media were supplemented with Nalidixic acid 20 µg/ml and Benomyl 50 µg/ml to control contamination by bacteria and fungi respectively.

B. Media for morphological characterization

1. Yeast extract-malt extract agar (ISP 2)

	g/L
Malt extract	10
Yeast extract	4
Glucose	4
Agar	20

2. Oatmeal Agar (ISP3)

	g/L
Oatmeal	20
Trace solution*	1 ml
Agar	18

*trace solution

	g/100 ml
$FeSO_4.7H_2O$	0.1
$MnCl_2.4H_2O$	0.1
$ZnSO_4.7H_2O$	0.1

3. Inorganic salts starch agar (ISP4)

	g/L
Soluble starch	10
$CaCO_3$	2
$(NH4)_2SO_4$	2
K_2HPO_4	1
$MgSO_4.7H_2O$	1
NaCl	1
$FeSO_4.7H_2O$	1 mg
$MnCl_2.7H_2O$	1 mg
$ZnSO4.7H_2O$	1 mg
Agar	20

4. Glycerol asparagine agar (ISP5)

	g/L
Glycerol	10
L-asparagine	1
K_2HPO4	1
Trace salt solution*	1 ml
Agar	20

The pH was adjusted to 7.4

*trace salts solution

	g/100 ml
$FeSO_4.7H_2O$	0.1
$MnCl_2.7H_2O$	0.1
$ZnSO_4.7H_2O$	0.1

5. Tyrosine agar (ISP 7)

	g/L
Glycerol	15
L-tyrosine	0.5
L-asparagine	1.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.5
NaCl	0.5
$FeSO_4.7H_2O$	0.01
Trace solution HO-LE*	1.0 ml
Agar	20

The pH was adjusted to 7.3

Trace element solution HO-LE

	g/L
H_3BO_3	2.85
$MnCl_2.4H_2O$	1.8
Sodium tartrate	1.77
$FeSO_4.7H_2O$	1.36
$CoCl_2.6H_2O$	0.04
$CuCl_2.2H_2O$	0.027
$Na_2MoO_4.2H_2O$	0.025
$ZnCl_2$	0.020

6. Bennet's medium

	g/L
Glucose	10
Pancreatic digest of casein	2
Yeast extract	1
Beef extract	1
Agar	15

The pH was adjusted to 7.0

7. Half strength potato dextrose agar (HPDA)

Media was prepared by adding 19.5 g potato dextrose agar (Oxoid CM139) and 7.5 g agar to 1 L RO water

8. Nutrient Agar (NA) (Oxoid CM01)

NA medium was prepared following the manufacturer's instruction for Nutrient Broth medium preparation and added with 1.5% agar.

C. Media for submerged fermentation

1. IM22 (seed culture medium)

	g/L
Glucose	15
$CaCO_3$	2
NaCl	5
Soytone	15
Pharmamedia	5

The pH was adjusted to 7.2

2. Medium F26

	g/L
Glucose	20
Soy bean flour	10
$CaCO_3$	4
$CoCl_2.6H_2O$	1

The pH was adjusted to 7.2

3. Medium F31

			g/L
Glycerol			15
Glucose			5
Pharmamedia			20
Yeast extract			5
KH_2PO_4			1
$MgSO_4.7H_2O$			0.5
$CaCO_3$			2
Trace element	**:**	$CuSO_4.5H_2O$	1 mg
		$FeSO_4.7H_2O$	7 mg
		$MnCl_2.4H_2O$	8 mg
		$ZnSO_4.7H_2O$	2 mg

4. Medium F33

	g/L
Glucose	5
Soluble starch	15
Proflo	20
$MgSO_4.7H_2O$	2
NaCl	2
$CaCO_3$	3

The pH was adjusted to 7.2

5. Medium F40

			g/L
Glucose			0.5
Soluble starch			15
Malt extract			5
Proflo			3
Corn steep liquor			2
$CaCO_3$			2
$MgSO4.7H_2O$			1
NaCl			2
Trace element	:	$CuSO_4.5H_2O$	1 mg
		$FeSO_4.7H_2O$	7 mg
		$MnCl_2.4H_2O$	8 mg
		$ZnSO_4.7H_2O$	2 mg

The pH was adjusted to 7.2

6. SI medium

	g/L
Sucrose	20
$CaCO_3$	2.5
KNO_3	1
KH_2PO_4	0.5
$MgSO_4.7H_2O$	0.5
NaCl	0.5

The pH was adjusted to 7.2

D. Medium for solid fermentation using rice

LF 42 medium

	g/L
Yeast extract	5
Peptone	5
Soy flour	5
Glycerol	4 ml
Soluble starch	2
CaCO3	2
NaCl	2
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.5
Trace salt solution*	1 ml

The pH was adjusted to 7.3

*Trace salt solution

	mg/L
$FeSO_4.7H_2O$	10
$CUSO_4.5H_2O$	5
$ZnSO_4.7H_2O$	5
$MnSO_4$	5
$NaMoO_4.2H_2O$	5
$CoCl_2.6H_2O$	5

E. Medium for bioassay

Antibiotic medium No.1 (agar seed, AAM) (Oxoid CM0327)

The medium was prepared based on the manufacturer's instructions.

F. Medium to grow *Staphylococcus aureus*

Mueller Hinton (II) broth (Oxoid CM405)

The media was prepared based on the manufacturer's instructions. Mueller Hinton agar was prepared by adding 15 g agar per litre Mueller Hinton broth.

APPENDIX 2. LIST OF ENDOPHYTIC ACTINOBACTERIAL ISOLATES FOR SCREENING

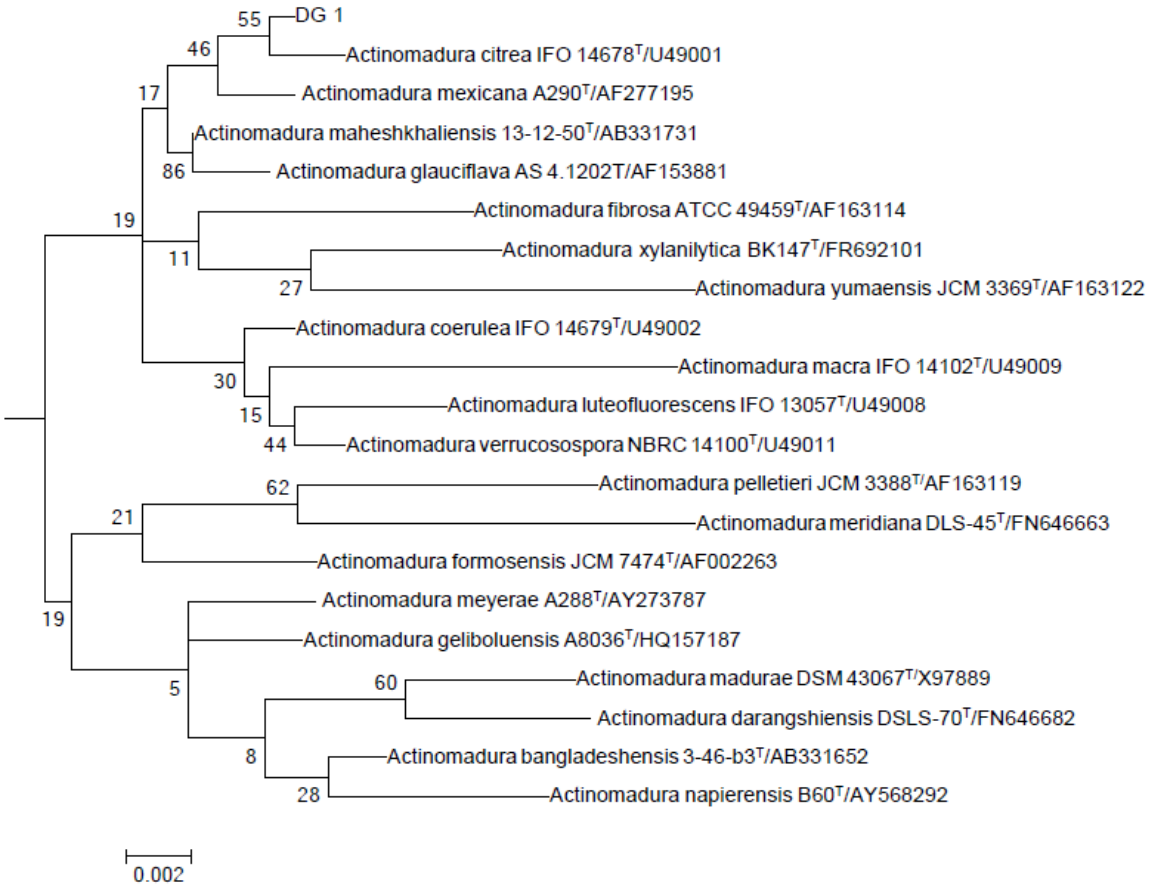
1. Endophytic actinobacteria from wood of Australian Eucalyptus tentatively classified as Eucalyptus oblique (stringy bark)

No	Isolate Code	Isolation week	Isolation spot
1	AA2(a)1	2	2
2	AA4(a)1.1		4
3	AA4(a)1.2		4
4	AA4(a)1.3		4
5	AA4(a)1.4		4
6	AA9(a)1		9
7	HV4(h)1		4
8	HV4(h)2		4
9	HV6(g)1		6
10	HV9(a)1		9
11	HV9(a)2		9
12	CMC4(a)1		4
13	AA9(b)2	3	9
14	AA9(d)5		9
15	CMC9(a)2		9
16	HV4(a)3		4
17	HV4(b)4		4
18	HV6(a)2		6
19	AA9(c)3	4	9
20	AA9(a)6		9
21	HV9(a)3		9
22	HV9(a)4		9
23	HV9(a)5		9
24	AA9(c)4.1	5	9
25	AA9(c)4.2		9
26	AA9(c)4.3		9
27	HV4(e)6		4
28	HV4(f)7		4
29	HV4(f)7.1		4
30	HV9(a)6		9
31	HV9(a)7		9
32	HV9(a)8		9
33	HV9(a)9		9
34	HV4(a)8	6	4
35	HVA9(b)10		9
36	HV9(a)12		9
37	Hv4(f)9	7	4
38	HV6(g)3		6
39	HV6(g)4		6
40	AA9(g)9	8	9
41	AA4(a)3	9	4
42	CMC9(d)5		9
43	AA9(b)11	11	9
44	AA9(a)10		9
45	CMC6(a)2		6
46	CMC2(a)4		2
47	HV9(c)18		9
48	HV 4(f) 20	14	4
49	HVA6(a)5	16	6
50	HVA6(d)6		6
51	HVA6(c)3	No record	6

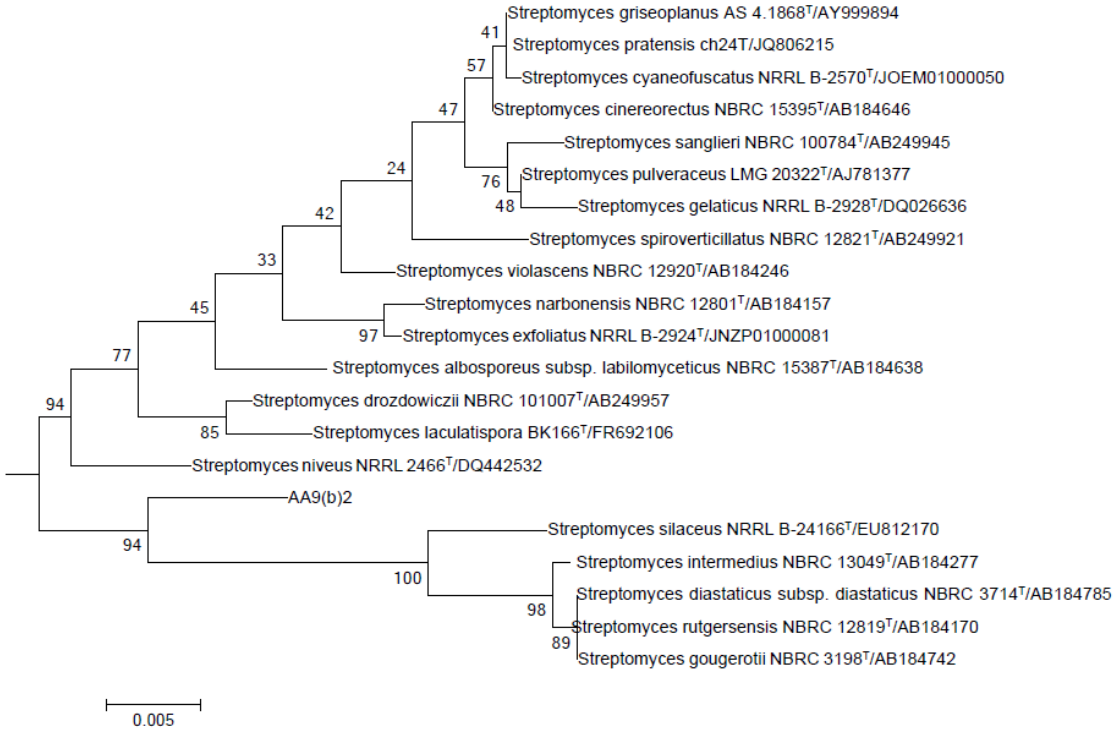
2. Endophytic actinobacteria from native Australian plants (Kaewkla and Franco, 2013)

No	Isolate Codes	Isolation week		No	Isolate Codes	Isolation week
1	CAP 28	6		26	EUC 66	5
2	CAP 53	5		27	EUC 75	12
3	CAP 69	3		28	EUM 63	1
4	CAP 76	7		29	EUM 130	1
5	CAP 103	4		30	EUM 165	3
6	CAP 149	4		31	EUM 168	1
7	CAP 168	10		32	EUM 199	1
8	CAP 208	6		33	EUM 209	7
9	CAP 214	7		34	EUM 220	8
10	CAP 215	7		35	EUM 238	8
11	CAP 230	10		36	EUM 242	4
12	CAP 238	4		37	EUM 244	5
13	CAP 250	8		38	EUM 253	5
14	CAP 261	1		39	EUM 296	6
15	CAP 266	1		40	EUM 356	6
16	CAP 288	7		41	EUM 359	4
17	EUC 4	9		42	PIP 48	1
18	EUC 6	4		43	PIP 111	4
19	EUC 7	4		44	PIP 146	5
20	EUC 8	5		45	PIP 154	11
21	EUC 19	7		46	PIP 156	11
22	EUC 20	4		47	PIP 196	11
23	EUC 33	4		48	PIP 201	11
24	EUC 45	7		49	PIP 245	4
25	EUC 49	1				

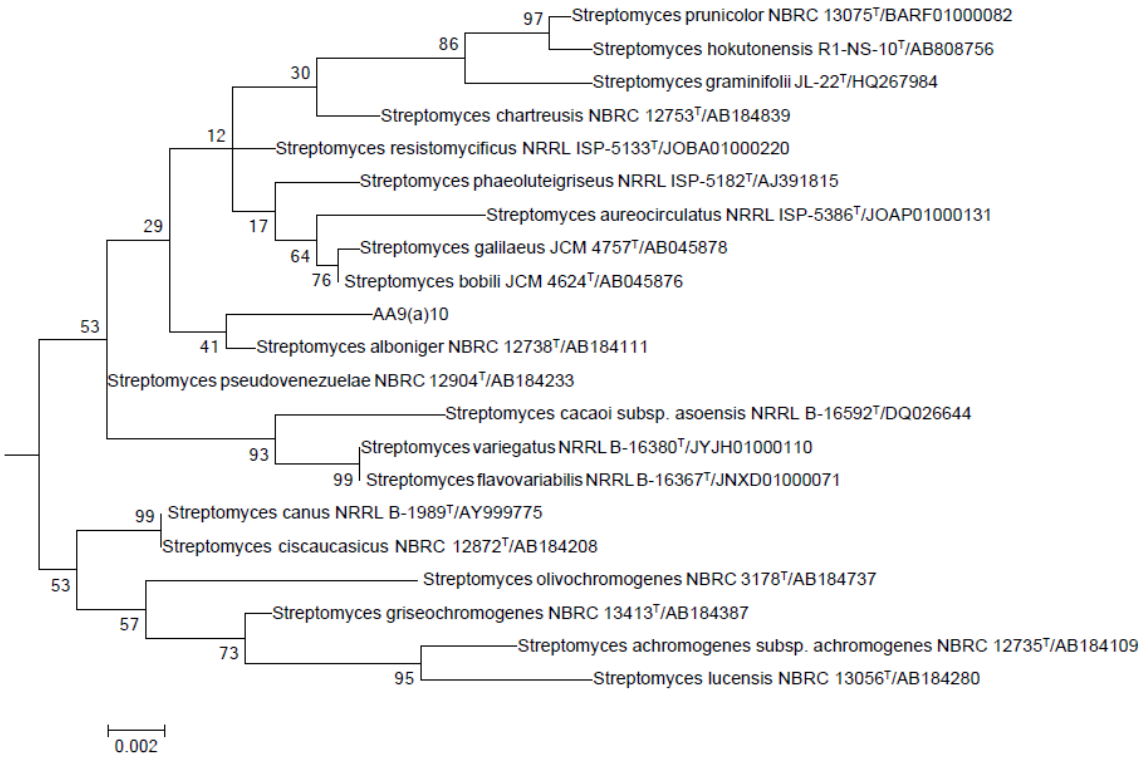
APPENDIX 3. MAXIMUM LIKELIHOOD TREES FOR THREE SELECTED POTENTIALLY NOVEL STRAINS



Maximum likelihood tree demonstrated the relationship between strain DG1 and representative related members of the genus *Actinomadura*



Maximum likelihood tree demonstrated the relationship between strain AA9(b)2 and representative related members of the genus *Streptomyces*



Maximum likelihood tree demonstrated the relationship between strain AA9(a)10 and representative related members of the genus *Streptomyces*

APPENDIX 4. ABBREVIATIONS

ABL	Antibiotic Literature Database
ADRA	Amplified Ribosomal DNA Restriction Analysis
AFLP	Amplified Fragment Length Polymorphism
bp	Base pair
C	Degree Celcius
CFU	Colony Forming Unit
cm	centimeter
CRE	Carbapenem Resistant Enterobacteriaceae
DAP	Diaminopimelic acid
DNA	Deoxyribonucleic Acid
DPG	Diphospatidylglycerol
EPIs	Efflux pump inhibitor(s)
ESBLs	Extended Spectrum β-lactamase
f	forward
FAMEs	Fatty Acid Methyl Esters
g	gram
GGAA	Gellan Gum Amino Acid
GGCMC	Gellan Gum Carboxymethylcellulosa
GSK	GlaxoSmithKline
HPCPC	High Performance Centrifugal Partition Chromatography
HPDA	Half Potato Dextrose Agar
HPLC	High Performance Liquid Chromatography
HTS	High Throughput Screening
HV	Humic acid Vitamin
HVA	Humic acid Vitamin Agar
IDSA	Infectious Diseases Society of America
ISP	International Streptomyces Project

IU	International Unit
Mb	Mega base pairs
MDR	Multidrug resistant
mg	miligram
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimal Inhibitory concentration
MIC	Minimum Inhibition Concentration
min	minute
ml	mililiter
mm	milimeter
MRSA	Methicilin Resistant *Staphylococcus aureus*
NA	Nutrient Agar
Nov.	In biological nomenclature, Latin for “new”
NRPs	Non-ribosomal Peptide synthetase
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIms	Phosphatidylinositol mannosides
PKS	Polyketide synthetase
r	reverse
Rf	Retention Factor
RNA	Ribonucleic Acid
RND	Resistance Nodulation Division
rpm	Rotation per minute
rRNA	Ribosomal Ribonucleic Acid
Rt	Retention Time

SAM	S-adenosyl methionine
SAMs	S-adenosyl methionine synthetase
sp	species
spp.	species plural
TLC	Thin Layer Chromatography
TSB	Tryptic Soy Broth
TWYE	Tap Water Yeast Extract
UV	Ultra Violet
VISA	Vancomysin Intermediate Resistant *Staphylococcus aureus*
VRE	Vancomycin Resistant Enterococcus
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WHO	World Health Organization
ZOI	Zone of Inhibition