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Identification and Characterization of Endophytic Actinobacteria Isolated from Plants and Sponges

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DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that 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 is made in the text.

Alhnouf Alqurashi

ABSTRACT

The majority of biologically active compounds that have been characterised are present in plants, although they are also abundant in microorganisms and other areas where life exists. Therefore, the effect of these metabolites on the existence and functioning of other organisms is important. Most microbial organic molecules that have an interactive function are called secondary metabolites, which consist of diverse chemical structures, their biological activities. Actinobacteria form a large phylum of Eubacteria and are known as prolific producers of these metabolites. The increase in pathogens resistant to antibiotics and the decline in introducing new antibiotics into the market has been the impetus for pharmaceutical companies to search for compounds produced by a range of actinobacterial cultures.

This study was carried out to identify and characterize endophytic Actinobacteria isolated from plants and sponges and determine whether they produce any novel compounds, especially antimicrobial activity against test organisms. Thirty-three endophytic Actinobacteria had been already isolated from Australian plants and sponges by previous students in the department of Medical Biotechnology at Flinders University. The isolates were cultured on ISP2 for up to 21 days in order to allow for production of metabolites. The plates containing the culture and agar were extracted with methanol (MeOH) and tested against three bacteria: *Staphylococcus aureus, Escherichia coli* and *Micrococcus luteus*. Only one strain ET11 showed a strong activity against the three test organisms compare to the other strains that showed weak activity against one or two of the test microorganisms.

ET11 was identified using 16S rRNA gene sequencing (ET11) and found to belong to the *Streptomyces* genus. BLAST searches based on the almost full length of the 16S rRNA gene sequence, showed that strain ET11 had the highest similarities

with *Streptomyces halophytocola* (KLBMP 1284T) (99.78 %) that been isolated from the surface-sterilized stems of a coastal halophyte *Tamarix chinensis* Lour. Phylogenetic trees reconstructed on the basis of 16S rRNA gene sequences revealed that strain ET11 formed a distinct lineage with *S. xiaopingdaonensis* DUT 180t and *S. halophytocola* KLBMP 1284T.

Fermentation techniques were used for inducing expression of secondary metabolites from the uncharacterised actinobacteria isolates. By using antimicrobial guided screening, it was determined that few of the isolates produced antimicrobial metabolites. Dominant antagonistic activity was detected from strain ET11 against Gram-positive bacteria. Nine fermentation media were used to cultivate the isolates for the production of antimicrobial metabolites. F33 medium was the most optimal medium for the production of antimicrobial metabolites. Various compounds were detected by using Thin Layer Chromatography (TLC) not identified indicating that endophytic Actinobacteria isolated from plants and sponges are prolific producers of secondary metabolites. Some of the compounds may have antimicrobial activity as some of the isolates cultured in liquid fermentation media and subjected to bioassay showed activity against one of the test microorganisms used in this study.

CHAPTER ONE

INTRODUCTION

1. General Overview

The majority of biologically active compounds exist in plants, although they are also abundant in microorganisms and other areas where life exists. Therefore, the effect of these metabolites on the existence and functioning of other organisms is significant, even if it is only secondary (Demain & Sanchez 2009)

This coexistence is important to the further understanding of how biology impacts life and how the balance between organisms remains important. (Grisham 2009) The idea that microorganisms can rely on and produce specific metabolites based on plant and sponge sources remains of interest because seeking the connection can also solve some of the life's mysteries. The impact of biological connections can be endless, because there are over 20,000 biologically active compounds derived from microorganisms. Many of which originate largely from one specific actinobacterial genus, *Streptomyces* (Fajardo & Martínez 2008). The great heterogeneity of secondary metabolites is important in the production of signals and toxins. Unfortunately, the number of effective antibiotics is declining, due to the high rate of antibiotic resistance in bacteria. But the human species still has a lot of truth to uncover about them, with many metabolites yet to be discovered (Solecka et al. 2012).

The biological world in which life takes place has many mysteries to reveal. This chapter, however, is focused on discussing whether new strains of actinobacteria found in native Australian plants and sponges will produce new compounds. One would believe the close relationship between actinobacteria and the source whether its

plant or sponge might produce specific outcomes. To hypothesize that new plants and sponges sources may be unique is innovative and seeks further exploration.

1.1 General Aspects of Actinobacteria

1.1.1 Morphological and genetics features of Actinobacteria

Actinobacteria are gram-positive, filamentous, spore-form soil bacteria (Goodfellow, Minnikin & bacteriology 1985). They have high guanine and cytosine in their DNA ranging from70 to 74%. They are responsible for the production of about half of the discovered bioactive secondary metabolites, antibiotics, antitumor, and enzymes from microbial sources (Hemashenpagam 2011). Of the Thousands of biologically active compounds obtained from microbes, Actinobacteria produce 45%, fungi 38% and only 17% from other bacteria (Gebreyohannes et al. 2013). Actinobacteria exhibit the greatest morphological differentiation among prokaryotic bacteria due to formation of various structures like hyphae, mycelia and a large range of spore types.

1.1.2 Classification of Actinobacteria

The name actinomycetes came from Greek means aktis (a ray) and mykes (fungus) and were wrongly classified as fungus from the initial observation of their morphology (Hemashenpagam 2011). They have a very close morphological resemblance to the higher fungi because they form arthrospores, aerial and vegetative mycelia, conidiospores and sporangiospores just like higher fungi. Although, actinobacteria, they belong to a different kingdom, actinobacteria are prokaryotes while fungi are eukaryotes.

1.1.3 Activities of Actinobacteria in the natural habitat

In nature, these bacteria can be found in cool, damp places as well as in water where certain groups of them grow on aquatic organisms. Actinobacteria are widely distributed in natural environments and played an important role in the degradation of organic matters. They are also well known as a rich source of bioactive compounds (Berdy 2005).

Some group of actinobacteria produce a chemical compound called geosmin which has an earthy aroma when they produce spores and that the reason behind the smell of soil after rain (Dwivedi et al. 2011). Their presence can influence crop production and maturity positively and negatively. In addition, they have important roles in ecosystem sustainability by degrading lignocellulosic plant residues and also recycling nutrients back to the environment. They can act as biological control agents to control fungal diseases and to enhance plant growth (Lechevalier 1988).

1.1.4 Endophytic Actinobacteria

Endophytic microorganisms can inhibit the interior tissues of plants such as leaves, branches and stems at one period of their life cycle. Endophytic microorganisms (bacteria or fungi), colonize the tissue of living plants and infect it without any symptoms of disease (Cao et al. 2004). Actinobacteria play an important role in improving crop performance as well as phytohormone production, removal of contaminants, direct suppression of pathogens via antibiotics or competition, and induction of plants defense responses (Franco et al. 2007).

1.2 Applications of Actinobacteria

1.2.1 Industrial application of Actinobacteria

Actinobacteria are known as prolific producers of secondary metabolites among microorganisms, of their bioactive compounds used as antibiotics (Berdy 2005). Actinobacteria have very versatile biotechnological applications such as the production of the important enzymes (Manivasagan et al. 2013) such as protease, lipase and amylase. They are used in the production of recombinant proteins (Nakashima, Mitani & Tamura 2005).

1.2.2 Agricultural application of Actinobacteria

Actinobacteria are the most abundant group of microorganisms in the soil. They play major roles in soils, for example, in the biogeochemical cycling of carbon and the mineralization of nitrogen compounds. Soil actinobacteria produce several compounds that have important activities for soil. These can be antifungal agents, which inhibit the synthesis of some enzymes and degrade cell walls of fungi. In addition, they are used commercially to produce some plant growth compounds like herbicides (Sharma 2014).

Actinobacteria are used as biological control agents to control crop diseases as an alternative to using fungicides and chemicals because they are more expensive and cause environmental problems. They are used to inhibit some plants diseases caused by other pathogenic microbes. Geldanamycin is an antibiotic produced by *S. hygroscopius* to control Rhizoctonia root rot in Pea plants (Dhanasekaran, Panneerselvam & Thajuddin 2012).

There are some disadvantages of Actinobacteria in agriculture too. Plants diseases such as scab diseases of potato tubers is caused by *Streptomyces scabies*. (Liu, Anderson & Kinkel 1995).

1.3 General aspects of Secondary Metabolites

1.3.1 Description of Secondary Metabolites

Secondary metabolites are microbial compounds with complex chemical structures; they are produced during the end of the growth phase of microbes under specific conditions. Endophytic bacteria can produce metabolites within plant parts such as leaves, stems and reproductive organs of the host plant (Miao & Davies 2010). Due to the metabolic need to adapt to particular plant environments, the ability of endophytes to produce metabolites is relatively well defined. In contrast, their soil-dwelling counterparts can grow in many types of soil (Miller et al. 2012).

The metabolic potential of endophytic Actinobacteria derived from botanical sources needs more research and documentation as it has been relatively neglected in favour of those from soil sources. The direct relationship between Actinobacteria and the plant source needs examination such as production of secondary metabolites *in planta.* The direct relationship needs further analysis and reflection because soil data has a limited correlation.

The rich variety of metabolites present in endophytic Actinobacteria shows that there are many more yet to be found but it remains a challenge to discover the connection between bacteria and plant sources. The discovery of hundreds of actinobacterial isolates as endophytes in diverse actinobacterial strains discovered in *Maytenus austroyunnanensis* further lends credence to this assertion (Qin et al. 2011). However what this also does is offer a lead for biological research in terms of how such plant sources can be used to better the human condition.

Analysis of endophytic Actinobacteria of therapeutic herbal plants using PKS and NRPS gene fragments analysis produced evidence of yet to be identified metabolites (Sturz, Christie & Nowak 2000). By actively asserting the relationship also means further discovery can take place, leading to further innovation such as plants for medicinal use and disease treatment to pursuing the exchange between bacteria and plant source. Other applications remain fruitful as well with concern for the physical design of new plants and that can remain resistant to ecological conditions and changes in the environment just by turning on certain gene combinations. It truly illustrates the beauty found in biology, how balances remains and further by products exist because of it. In this regard, the problem has many possible solutions and applications that make this research feasible and noteworthy.

1.3.2 Functions of Secondary Metabolites

The function of metabolites in the interaction between bacteria and plants is an area of interest for many scholars and researchers. The close interaction between bacteria and plants leads to the production of secondary metabolites. These metabolites are utilised as agents for nutrient absorption. Siderophores, for example, act as agents in the acquisition of iron (Barry & Challis 2009). These secondary metabolites can be beneficial to the plant source as they act as signals and receptors for nutrients and this can lessen the stress felt by plants as a result of the environment. Stress plays a significant role in determining the outcomes of the influence upon the plant life.

Researchers have observed the action of metabolites as toxins and in biofilm formation. (Glick 2012) witnessed extremely harmful factors or metabolites interfering with the ability of plants to signal hormones. This impacts the ability of the plant to thrive and multiply. These activities could affect plant pathogens as well as impact the environment on a level that changes production and patterns. Chemical imbalances

could lead to harm for the plants depending upon the desired outcomes. Similarly, difficulties may arise when trying to find a distinction between toxins and phytohormones because this can lead to missed signals.

Phytopathogens and endophytic actinobacteria are all known to produce hormones within the context of signalling, for instance (López, Bannenberg & Castresana 2008). These conditions exist in order to continue processes that promote homeostasis. In the process of development and stress responses by plants, they produce different types of phytohormones, such as auxins, abscisic acid, cytokinins, brassinosteroids and ethylene (Durbak, Yao & McSteen 2012). Plants manage to develop a defence and respond to stimuli by refining different phytohormone pathways all in response to changes in the environment but this may also lead to differing outcomes (Hardoim, van Overbeek & van Elsas 2008). This ability of a plant to carry out signalling can be compromised by associated bacteria. The addition of the bacteria changes the outcomes possibly by chance or intentionally by humans as to allow for certain outcomes to the crops and production goals. One sees this in farming where a bacterium is produced with the intent of suppressing certain signals by the plants so that their growth responds to different conditions such as drought. To manipulate the outcomes may be on purpose in response to known conditional changes or the plant simply adapts to its surroundings. What remains interesting is how bacteria can become a tool to control the hormonal signals, which also suggests a myriad of applications. While some may see this manipulation of nature a beneficial consequence of science, others may be appalled at the lack of ethical forethought. Respectfully there are arguments on either side that suggest manipulation allows for further plant sources to thrive in conditions that may have killed them or environments where such plants are not indigenous. Plant sources reflect the nutritional value and

this means such human intervention also creates food and wealth. Still the very action of manipulating the signal may have both positives and negatives.

However, (Gebreyohannes et al. 2013) discovered that plant-based endophytic actinobacteria adapt to the plant and mimic the hormones found naturally in the plant as structural analogues. Coronatine, for example, mimics the active natural hormone (+)-7-iso-jasmonoyl-l-isoleucine by exhibiting phytotoxicity, which is characteristic of active jasmonate (Goodfellow, Minnikin & bacteriology 1985). Through mimicry, coronatine manages to suppress closure and defence responses of the stomata (Hemashenpagam 2011).

While mimicry may be natural at times to intentionally seek ways to manipulate such activity, they may have negative impacts on the plants and their sustainability. The issue here is how will the plant be used and in what capacity is the plant important. Researchers tried to elicit such mimicry in plants encoded by 16S rRNA and NRPS gene clusters with various degrees of success. However, it should be ethically understood how plant mimicry could be dangerous. To see the connection between a plant-bacteria relationship and apply it to another may have differing outcomes. To suggest that a copy is better than the original almost seems unnatural and goes against biology but the tools become innovative for a reason. To not see the opportunity for solving problems through plant mimicry and redesign falls short of seeing the influences signalling has. These are powerful connections in the plant environment that should be explored more fully yet carefully.

1.3.3 Screening for Secondary Metabolites

Identifying the influence of the host plant on metabolites was possible after isolating bacteria and the chemical screening of secondary metabolites. The next procedure was to grow the isolated bacteria *in vitro*. Analysis of the metabolites was done to provide a means of detecting and describing the metabolites produced from living plants. The objective was to detect metabolites in plant association. (Raaijmakers & Mazzola 2012) reveal that the concentration of compounds that resulted from the action of plant-associated bacteria in rhizophore and roots is typically less than 10 µg/g (Cotter, Ross & Hill 2013). This amount in their opinion is very low, and it negatively affects the direct structure elucidation. One of the most detected is the metabolite for Pseudomonas fluorescence CHA0 lipopeptides (Lahrmann et al. 2013). This has strong implication in terms of medical uses especially in the realm of immunology and oncology. However, (Moran 2002) concurs that there is still the challenge of detecting unknown compounds and further manipulation must be streamlined, parsed down to a single thread to see how the bacteria makes the product so that the purest form can be produced With sufficient amounts of a pure compound the chemical structure is obtained via analysis by Nuclear Magnetic Resonance (NMR) spectra and Liquid Chromatography-Mass Spectrum (LC–MS).

1.4 Native Australian Plants

1.4.1 Application and Innovation

Scholars are increasingly finding evidence about the potential of endophytic bacteria to produce yet to be described metabolites (Brader et al. 2014). To some extent, the circumstances under which these metabolites are produced and influenced are still unclear. However, the genomic revolution and growth of new, superior analytic techniques have the potential to make the discovery of metabolites and other

cryptic compounds easier than it is now. Once the coding of chemical structures and compounds are clearly understood, scientists may be able to explain in great detail the derivation of metabolites, endophytic or bacteria, and the influence of the host plant.

Controversy rages about whether new strains of Actinobacteria from native Australian plants will produce new compounds. The robust metabolic collection of endophytic bacteria allows investigations to be carried out on over 100 actinobacterial isolates existing in the form of endophytes in Australian trees. These isolates were also found in strains in *Maytenus austroyunnanensis* - a medicinal plant *(Qin et al. 2012)*. So far, however, these discoveries represent only a fraction of plant-associated Actinobacteria. Nonetheless, it is considered to be representative of potential new secondary metabolites sources. The connection between the bacteria and its influence on plant sources for production of metabolites remains unclear and several intensive investigations need to be done.

Such focus upon how Australian plants specifically could result in new compounds also brings up the implication of genetic modification for plants and designing new plants from this relationship between Actinobacteria and plant sources in the form of secondary metabolites. The impact is enormous upon the medical community as well as the large-scale food industry. Conversely, some bacterial endophytes produce metabolites that facilitate the manufacture and modification of ethylene and auxins, which help in the growth of the plant and stress response (Glick 2012); (Brader et al. 2014). Such outcomes can provide guidance on how to design plant growth around seasonal changes and other conditions that one can plan for.

To design a plant based upon how hormones function and exist in the current environment also promotes positive areas of research with concern for hunger and continuous value chains for producers. Such research emphasizes how signals are of

great importance in the plant and animal world. Endophyte-derived hormones also enable the plant to increase stress tolerance. The metabolite abscisic acid - a product of the endophytic bacterium, *Azospirillum lipoferum* - for example, helps in managing the drought stress in maize (Brader et al. 2014). Creating a corn plant that is resistant to drought is not only important to understand in times of changing weather patterns but also to grow corn in an arid climate like Africa. This has significant potential for solving growing hunger issues worldwide.

1.5 Marine Actinobacteria

1.5.1 Applications and Innovation

Seventy percent of the Earth's surface and harbouring most of the planet's biodiversity is covered by water. Even though marine plants and animals have received considerable attention as a resource for natural-product discovery, marine microorganisms remain relatively unexplored.

The marine environment is largely an untapped source for actinobacteria, having the potential to produce novel and useful bioactive natural products. Marine actinobacteria have unique enhancing and quite different biological properties including antimicrobial, anticancer, antiviral, and enzyme inhibitory activities. They have attracted global attention in the last ten years for their ability to produce pharmaceutically active compounds (Manivasagan et al. 2014). They are considered a major and rich source of novel natural bioactive compounds that belong to a variety of distinct structural classes (Fenical & Jensen 2006).

Most of the actinobacterial representatives can be isolated from seashores, sponges, bottom sediments, seaweeds, coastal waters molluscs, fishes, and mangroves (Manivasagan et al. 2014). New bioactive metabolites that have been discovered in

recent years have encouraged numerous studies and growing interest in marine actinobacteria in parallel with the need for new antibiotics. In recent years, several interesting and novel bioactive compounds produced by marine Actinobacteria with a variety of activities have been claimed. For instance, Griseorhodin A, Daryamides, Actinofuranones, Chartreusin, Cyclomarin A, and Komodoquinone A, bioactive compounds derived from marine Actinobacteria specifically genus *Streptomyces;* shows a variety of activities such as antitumor, antifungal, cytotoxic, Anti-inflammatory and Neuritogenic (Li, A & Piel 2002); (Asolkar et al. 2006); (Cho et al. 2006); (Xu et al. 2005); (Renner et al. 1999); (Itoh et al. 2003).

Marine actinomycetes have different characteristics from those of terrestrial counterparts due to the difference between marine environmental conditions and terrestrial ones; therefore, different types of bioactive compounds might be produced (Manivasagan et al. 2013). Marine actinomycetes had to adapt to the extreme living conditions within marine environments, which range from extremely high pressure (with a maximum of 1100 atmospheres) and anaerobic conditions at temperatures just below 0°C on the deep sea floor, to high acidic conditions (pH as low as 2.8) at temperatures of over 100°C near hydrothermal vents at the mid-ocean ridges (Lam 2006).

Recent studies claimed that the majority of actinomycetes from marine environments, such as sediments and sponges, couldn't be recovered by current culture-based and traditional methods. Due to the extreme conditions of marine life of Actinobacteria, they cannot be cultured with the laboratory conditions. Cultivation of these novel actinomycetes will facilitate the investigation of their ecological roles and provide an important source for discovery of novel metabolites (Lam 2006).

Novel actinomycete groups have been found in the Great Barrier Reef sponges *Rhopaloeides odorabile*, *Pseudoceratina clavata* and *Candidaspongia flabellate*, and the Mediterranean sponges *Aplysina aerophoba* and *Theonella swinhoei* (Kim, Garson & Fuerst 2005); (Burja & Hill 2001); (Hentschel et al. 2002); (Webster et al. 2001). Unique actinomycetes, belonging to *Micrococceae*, *Dermatophilaceae* and *Gordoniaceae*, have been isolated from marine invertebrates especially sponges (Lam 2006).

Marine sponge is known as an important source for marine drug development, and accumulated evidence suggests that symbiotic microorganisms could be the true source of at least some of the biologically active metabolites isolated from sponges. Sponges are considered as an ideal habitat for microorganisms because of the special two-layer structure of outer and inner endosome and special strategy for sequestering food by filtering seawater (Li, Z 2009).

There has been considerable groundwork set for the exploitation of marine actinomycetes as the next new source of novel secondary metabolites discovery. Marine actinomycetes represent an underexploited source for the discovery of novel secondary metabolites and their potential should not be overlooked. Culturing the 'unculturable', especially the unculturable marine actinomycetes, would represent a unique and promising source for the discovery of novel secondary metabolites.

1.6 General aspects of Antibiotic

1.6.1 History of Antibiotics

Figure 1. Has been removed due to copyright restrictions

Since the discovery of the first antibiotic (Penicillin) by Alexander Fleming in 1920s, for which he got a Nobel Prize (Drews 2000)**,** several new antibiotics had been discovered. One of the most interesting discovering of antibiotic was when Selman Waksman discovered streptomycin in his lab back in 1942 and he was the first to describe them as antibiotics (Clardy, Fischbach & Currie 2009). Streptomycin is an antibacterial drug for tuberculosis and its derived from the bacterium *Streptomyces griseus*.

Antibiotic is a Greek name meaning "against life" was suggested by Dr. Waksman in 1942. Antibiotics are drugs produced or derived from microorganisms (bacteria) to kill or stop another microorganism from growing.

1.6.2 Antibiotic mechanisms of action

Figure 2. Has been removed due to copyright restrictions

There are five basic mechanisms of antibiotic action against bacterial cells (Kohanski, Dwyer & Collins 2010):

1. Inhibition of cell wall synthesis: The cell wall is considered very critical for the life and survival of bacterial species. Therefore, A drug that targets cell walls can selectively kill or inhibit bacterial organisms. Examples: penicillins and vancomycin.

2. Inhibition of cell membrane function: Cell membranes are important barriers that segregate and regulate the intra- and extracellular flow of substances and a disruption or damage to this structure could result in leakage of important solutes essential for bacterial survival. Examples: polymixin B and colistin.

3. Inhibition of protein synthesis: Protein synthesis is an essential process necessary for the multiplication and survival of all bacterial cells. Several types of antibacterial agents target bacterial protein synthesis by binding to either the 30S or 50S subunits of the intracellular ribosomes. This activity then results in the disruption of the normal cellular metabolism of the bacteria, and consequently leads to the death of the organism or the inhibition of its growth and multiplication. Examples: Aminoglycosides, streptogramins, tetracyclines.

4. Inhibition of nucleic acid synthesis: DNA and RNA are keys to the replication of all living forms, including bacteria. Some antibiotics work by binding to components involved in the process of DNA or RNA synthesis, which causes interference with the normal cellular processes which will ultimately compromise bacterial multiplication and survival. Examples: quinolones, metronidazole, and rifampin.

5. Inhibition of other metabolic processes: Other antibiotics act on selected cellular processes essential for the survival of the bacterial pathogens. For example, both sulfonamides and trimethoprim disrupt the folic acid pathway, which is a necessary step for bacteria to produce precursors important for DNA synthesis.

1.6.3 Antibiotic Resistance in bacteria

Fig. 3. Has been removed due to copyright restrictions

As shown in Fig 3 the resistance of bacterial strains keeps increasing dramatically since 1980 until now because these bacteria adapted to these antibiotic. The resistance in bacteria is considered a serious problem worldwide especially at healthcare institutions. The increasing problem of bacterial resistance against antibiotics in hospitals complicates treatment of these pathogenic microorganisms and increases the financial costs of treatments as well (Tenover 2006).

The widespread use of antibiotics and using them inappropriately, and the increase in the number of people travelling are some factors of multidrug resistance in bacteria globally. One solution for bacterial resistance is by developing a much

stronger and new drug, but it may be temporary because of the speed with which bacteria will adapt to it at the end (Laxminarayan et al. 2013).

Scientists and researchers face a problem of the rediscovery of the same bacterial strains as well as same biologically active compounds. So, while the bacterial resistance is increasing, the new active antibiotics are declining. To solve this problem, looking for new strains in new places and new organisms need to be done. Also, using some biotechnological technics in research to avoid the rediscovery of the same strains over and over again. So the aims in this study are, Actinobacteria strains that had been isolated from native Australian plants and sponges will be purified for new antimicrobial compounds. Also, characterize their features by using morphological, physiological and biochemical methods. While the hypothesis is new strains of Actinobacteria from native Australian plants and sponges will produce new compounds.

1.7 Detection of Secondary Metabolites

The separation of plant extracts still remains a big challenge for the process of identification and characterization of bioactive compounds due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities (Sasidharan et al. 2011).The separation and purification of bioactive compounds present in plant or sponge extracts are mainly carried out using chromatographic techniques based on their size, shape, or charge. Isolation of these bioactive compounds commonly done by using a number of different separation techniques such as TLC, column chromatography, flash chromatography, and/or Sephadex chromatography. Also, there are a number of non-chromatographic techniques used to obtain and facilitate the identification of the bioactive compounds

such as immunoassay, which use monoclonal antibodies (MAbs), and phytochemical screening assay (Sasidharan et al. 2011).

The most common chromatographic techniques are High Pressure Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) to analyse the bioactive compounds present in microbial extracts.

1.7.1 Thin Layer Chromatography (TLC)

TLC is one of the oldest chromatography techniques but is still utilized as a practical technique for many large-scale multiple drug screening programs. TLC is a sensitive technique in analytical chemistry used to separate, identify and determine the purity of the compounds, and follows the progress of the reaction**.** TLC is a simple, quick, and inexpensive procedure that gives the researcher a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the Rf of a compound is compared with the Rf of a known compound. It is commonly used for the rapid and positive analysis of drugs and drug preparations (Sasidharan et al. 2011).

Thin layer chromatography has several advantages in terms of identification and analyzing of components (Touchstone 1992):

1. Samples are readily recoverable.
2. Not limited by the lack of volatility or thermal stability of the sample.
3. Minimal requirements needed for sample preparation and clean up.
4. Very sensitive and very fast to get the results comparing to other techniques.
5. The amount of sorbent used is small
6. The cost of TLC plates is low.
7. The polarity and type of solvents can be changed in minutes

1.7.2 High Pressure Liquid Chromatography (HPLC)

Analytical HPLC is a widely used technique in analytical chemistry to separate, identify and quantify each component from crude extracts or microbial fermentation broth (Inamdar et al. 1996).

High-pressure liquid chromatography offers several interesting advantages in terms of identification and separation of components:

1. Requires small quantity of samples
2. It takes few minutes up to 30 mins maximum.
3. Very accurate.

In conclusion, the aims for this study were to detect and purify antibacterial, antifungal and anticancer compounds from novel strains of actinobacteria. Also, to characterise the new isolates by using morphological, physiological and molecular methods. The hypothesis was "New strains of actinobacteria from native Australian plants and sponges will produce new compounds."

CHAPTER TWO: MATERIALS AND METHODS

2.1 Microorganisms used

2.1.1 Actinobacteria used

Thirty-three strains of actinobacteria that were previously isolated from Australian plants and sponges were screened for antibacterial compounds activity.

Actinobacteria strains were maintained on Mannitol Soy Agar (MS) and Yeast Extract Malt Extract Agar (ISP2). They have been regrown from the stock culture every 3 months and the plates were stored at 4℃. Some strains were obtained from glycerol stocks that were kept at -80℃

The identity and purity of strains were confirmed by visual observation of colony morphology (form and colour of colony and spores) and the incubation period to obtain full growth in culture plates.

2.1.2 Test organisms

The microorganisms used to detect the spectrum of antimicrobial compounds produced by the Actinobacteria were three species of bacteria because they are commonly used and available in laboratories. All the test microorganisms were obtained from stock cultures maintained in our laboratory in the Department of Medical Biotechnology.

Bacterial species:

1. *Staphylococcus aureus* (Gram positive)
2. *Micrococcus luteus* (Gram positive)
3. *Escherichia coli* (Gram negative).

Bacterial species were maintained in glycerol stocks at -20℃, regrown on Tryptone Soy Broth (TSB) medium every month and stored at 4℃.

2.2 Morphological Characterization of Actinobacteria

Actinobacteria strains were preliminary identified according to the morphological criteria, including characterization of colonies on the agar plate, morphology of substrate and aerial mycelium, colour of spores and pigment produced. The Actinobacteria strains that been isolated from native Australian plants were cultured on ISP2 medium (International Streptomyces Project) which consisted of (g/L) (Yeast Extract 4.0; Malt Extract 10.0; Glucose 4.0; Agar 18.0); while Actinobacteria strains that been isolated from marine sponges were cultured on a modified ISP2 medium which contained 200ml sea water per liter medium. The isolates were streaked onto the media and incubated at 27°C for 21 days. The colony morphology was observed and recorded at this stage (Shirling & Gottlieb 1966).

2.3 Extractions and Processing of Actinobacteria Culture Extracts

A general extraction procedure adopted in this study involved extracting metabolites from the whole broth with methanol (MeOH). The broth supernatant following centrifugal separation was retained for analysis. Following solvents extraction the fractions were tested in well-type and plug type assays to determine if they contained any biological activity. Fractions containing bioactivity were further analysed using bioautography to determine the active component. Inactive culture were analysed using thin layer chromatography for secondary metabolites production.

2.3.1 Actinobacteria agar metabolites extraction

Extraction of metabolites from the actinobacteria cultures was carried out using methanol HPLC grade (BDH, Cat. No.15250) after growth on ISP2 or its modification for 7-10 days. The agar was removed from the petri dish, chopped into small pieces, and placed into 50 ml centrifuge tubes and immersed in 15 ml of MeOH. Tubes were capped and placed onto an orbital mixer over night at room temperature. The extracts were filtered through Whatman® No.1 filter paper 70 mm diameter to remove agar pieces. Lastly, the extracts were centrifuged to remove any solids at 3000 rpm for 5 min. The extracts were collected and kept at 4℃ to analyse for antimicrobial assays.

2.3.2 Small-scale Actinobacteria fermentation metabolites extraction

Actinobacteria cultures were fermented in various production media for 10-14 days and 1 ml was taken out by siphoning it off with a micropipette in 1.5 ml centrifuge tube. Tubes were centrifuged at 4000 rpm for 10 min to pellet the mycelium. After centrifugation a micropipette was used to separate the broth supernatant that was kept for testing. $50\mu ls$ of MeOH was added to the pelleted mycelium to extract metabolites. Following the addition of MeOH to the mycelium a vortex mixer was used to mix it thoroughly. The tubes were left to stand for 10 min before being placed onto an orbital mixer to shake at 5000 rpm over night at room temperature. After incubation, the mycelium extracts were centrifuged at 4000 rpm for 10 min, and a micropipette was used to separate off the MeOH mycelia extracts. Both the broth supernatant and MeOH mycelia extracts were tested for bioactivity.

2.3.3 Large scale production and recovery of antimicrobial metabolites

According to the directed screening, a number of the environmental actinobacterial cultures were identified as producers of antimicrobial metabolites. Four of these cultures were selected for large-scale production and purification studies. These cultures were ET11, ET77, Lup77, and PL12 that derived from sponges and plants respectively. Using optimal fermentation conditions for each of the cultures, the fermentation was scaled up to 1 liter.

In order to partially identify and characterize the active compounds from the selected Actinobacteria strains. The purification step involved using 1 liter of actinobacterial fermented cultures, dispensed into 50 ml centrifuge tubes and centrifuged at 3000 rpm for 20 min to pellet the mycelium. The broth supernatant was placed into 250 ml flask and twice the volume of the broth was added of Ethyl acetate (EtOAc) to obtain the non-polar extractable metabolites and was placed onto an orbital mixer for 4 hours at room temperature. Following the mixing with EtOAc, flasks were allowed to stand for 1 hour until two layers were visible, the top organic layer and an extracted broth spent bottom layer. The mixtures were separated using a separation flask and broth supernatant and EtOAc extract were collected in different flasks. Both the broth supernatant, the spent and the organic layer were tested for antimicrobial activity.

2.4 Concentration of the extract

MeOH and EtOAc extracts were subjected to a rotary evaporator. The speed setting of the rotary evaporator was 4 m/s and no heat was required. The organic solvents were evaporated off and a powder was collected. Filtrate of the MeOH and

EtOAc extracts were placed in 1.5 ml tubes and concentrated in a Savant Centrivap for 2hr until the MeOH and the EtOAc were evaporated off. Following concentration the extracts were freeze-dried using (VirTis freeze drier benchtop K) with the settings at 13mT and -85.2℃ over night.

2.5 Bioassay of secondary metabolites

2.5.1 Plug type bioassay

Screening the actinobacterial cultures for the production of antimicrobial compounds were carried out by extruding 6 mm plugs from 7-10 days Actinobacteria cultures using a stainless steel cylinder cork borer (6 mm inner diameter, 8 mm outer diameter, and 10 mm length). The plugs were transferred to a bioassay agar Antibiotic Medium No.1 (AAM) that consisted of (g/l) Peptone 6.0; Tryptone 4.0; Yeast Extract 3.0; 'Lab-lemco' powder 1.5; Glucose 1.0; Agar 11.5; at pH 6.5. The bioassay medium (AAM) was seeded with 1% (v/v) inoculum of test cultures (see table 1), grown in Tryptone Soy broth (TSB) medium consisting of (g/l) Pancreatic digest of casein 17.0; Enzymatic (contains Papain) digest of soya bean 3.0; Sodium Chloride 5.0; Di-potassium hydrogen phosphate 2.5; Glucose 2.5 at pH 7.2 to an optical density of 0.2 at 600mm. Optical measurements were performed on a UV-visible Spectrophotometer Shimadzu model UV-160A. Bioassay agar plates were incubated at 37℃ and zone of inhibition were recorded after 1-3 days.

Table 1: Test organisms for bioassay

Test organisms	Test activity
Micrococcus luteus	Anti-bacterial (gram positive)
Staphylococcus aureus	Anti-bacterial (gram positive)
Escherichia coli	Anti-bacterial (gram negative)

2.5.2 Well type bioassay

Screening was also performed using the conventional well type bioassay; 6 mm wells were extruded from AAM plates by using a stainless steel cylinder cork borer (6 mm inner diameter, 8 mm outer diameter, and 10 mm length). Seeded with test organisms (listed in table 1). 50μl aliquot of resuspended extract, fermentation broth supernatant or mycelia extracts were pipetted into the wells using micropipette. The plates were incubated at 37℃ and zones of inhibition were recorded after 1 day.

2.6 Fermentation of Actinobacteria

The aims of fermentation of Actinobacteria were to find suitable media and length of cultivation to optimize antimicrobial metabolite production from each actinobacterial strain. Also, to determine optimal fermentation conditions for selected Actinobacteria isolates. Two types of fermentations were done using various media that are submerged, shake-flask fermentation and solid-state fermentation.

2.6.1 Small Scale Submerged Shake-Flask Fermentation

Actinobacterial cultures were grown on ISP2 agar medium for 14-21 days. Plugs of well-grown Actinobacterial cultures were extruded and used to inoculate 50ml of IM22 inoculum medium in a 250ml-baffled Erlenmeyer flask. The flasks were incubated on an orbital shaker (Ratek, Australia) at 120 rpm at 27℃. Following 3 days of fermentation, 5ml of inoculum medium IM22 was transferred by 5000μl Pipette into the flasks containing 50 ml of selected production media as shown in Table 2.

Fermentations were carried out over a 10-day period, with samples were taken every day for biological activity assessment. One ml was taken out from the fermentation flasks by siphoning it off with a micropipette in 1.5 ml centrifuge tube.

Tubes were centrifuged at 4000 rpm for 10 min to pellet the mycelium. After centrifugation a micropipette was used to separate the broth supernatant out and it was kept for testing for any microbial activity. 50μls of MeOH was added to the pelleted mycelium to extract metabolites.

Table 2: Production media used for the Secondary Metabolites

Medium	Main ingredients (per Liter)
Isp2	Malt Extract 10g, Yeast Extract 4g, Glucose 4g, Potassium nitrate 2g, Sodium chloride 3.5g
F26	Glucose 20g, Soy Bean Flour 10g, CaCo3 4g, CoCl2.6H2O 1g, Sodium Chloride 2g
F28	Glucose 10 g, Soluble starch 10 g, Malt Extract 7.5 g, Peptone 7.5 g, MgSo4.7H2O 1 g, Sodium Chloride 3 g, Trace elements: CuSo4.5H2O 7mg, FeSo4.7H2O 1mg, MnCl2.4h2O 8mg, ZnSo4.7H2O 2mg
F31	Glycerol 15 g, Glucose 5 g, Pharmamedia 20 g, Yeast Extract 5 g, Kh2PO4 1 g, MgSo4.7H2O 0.5 g, CaCo3 2 g, Sodium Chloride 2g Trace elements: CuSO4.5H2O 7mg, FeSo4.7H2O 1mg, MnCl2.4H2O 8mg, ZnSo4.7H2O 2mg
F33	Glucose 5 g, Soluble starch 15 g, Proflo 20 g, MgSo4.7H2O 2 g, Sodium Chloride 2 g, CaCo3 3 g
Tryptone Glucose Yeast Extract (TGY)	Glucose 1g, Yeast Extract 5g, Tryptone 5g, CaCo3 5g, K2HPo4 1g
Tryptone Fructose Yeast Extract (TFY)	Tryptone 5g, Yeast Extract 5g, Fructose 1g, K2Hpo4 1g, CaCo3 4g
Sucrose Production Medium	Sucrose 20g, CaCo3 2.5g, K2Hpo4 0.5g, MgSo4.7H2O 0.5g, Sodium Chloride 0.5g, FeSo4.7H2O 1g

Table 2: Production media used for the Secondary Metabolites (continued)

Medium	Main ingredients (per Liter)
M-2	Starch 10g, cornmeal 4g, K2hpo4 0.3g, MgSo4.7H2O 1g, CaCo3 4g
IM22	Glucose 15g, Soy Bean Flour 15g, Corn Steep 10g, Sodium Chloride 10g, CaCo3 4g

Following the addition of MeOH to the mycelium a vortex mixer was used to mix it thoroughly. The tubes were left to stand for 10 min before being capped and placed onto an orbital mixer to shake at 5000 rpm over night at room temperature. After incubation, the mycelium extracts were centrifuged at 4000 rpm for 10 min, and a micropipette was used to separate off the MeOH mycelia extracts. Both the broth supernatant and MeOH mycelia extracts were tested every day of sampling for antibacterial activity.

The flasks were maintained at 27℃ $\pm$ 2℃ on a shaking platform. Flasks were then harvested on day 10.

2.6.2 Large Scale Submerged Shake-Flask Fermentation

Two liquid fermentation media were selected to scale up the fermentation media to 1 litre; F31 and F33 had shown good results in terms of the production of antimicrobial compounds.

Actinobacterial cultures were grown on ISP2 agar medium for 10-14 days. Plugs of well-grown Actinobacterial cultures were extruded and used to inoculate 50ml of IM22 inoculum medium in (250 ml) baffled Erlenmeyer flasks. The flasks were incubated on an orbital shaker at 120 rpm at 27℃. Following 3 days of fermentation, 5ml of inoculum medium IM22 was transferred by 5000μl Pipette into baffled 500 ml

flasks containing 250 ml of F31 and F33. Fermentations were run over 10 days period, with samples taken at days 6, 7, 8, 9 and 10 for biological activity assessment.

2.6.3 Solid state Fermentation

Solid substrate medium (rice) was dispensed into 250 ml Erlenmeyer flasks that were then autoclaved for 1 hour at 121℃. Following autoclaving, sterile liquid supplements (Trace Salts Solution: 0.1g Sodium Tartrate: 0.01g ZnSO4.5H2O, 0.01g FeSO4.7H2O, 0.1g KH2HPO4, 0.1g MgSO4.7H2O, 0.2g Yeast Extract made up to1000 ml with distilled water and sterilised) were added. LF42 Solution: 5g Yeast Extract, 5g Peptone, 5g Soya flour, 40 ml glycerol solution, 2g Soluble Starch, 2g CaCO3, 5g NaCl made up to 1000 ml with distilled water and sterilised), were added to serve as a nutrient source and to maintain the moisture content. 2 ml of seeded IM22 inoculum was transferred to baffled 250 mL Erlenmeyer flasks containing rice as a solid fermentative medium. The flasks were maintained at 27℃ on a static platform. Flasks were then harvested on day 10. The solid substrate was extracted with 50 ml Methanol.

2.7 Detection of the compound

2.7.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was done to separate and identify compounds either from the cell extract, the freeze-dried cell extract or the elution product of purification. Samples were spotted onto TLC sheet using a 20μl pipette for several applications and allowed to dry. The samples were then run using a solvent mixture, chloroform: methanol (9:1) and Butanol: Acetic acid: Water (4:1:1).

Next, the separate compound bands were visualized under UV light at wavelengths of 254 nm and 365 nm. Each band was numbered and RF values were recorded.

2.7.2 Bioautography

Bioautography was used to determine the band of active compounds after separation by TLC. It was carried out, by placing the TLC sheet that had single bands and had been recorded and numbered, on top of the (AAM) medium seeded with the test organism. It was left overlaid for 2-3 hours that allowed the compound to diffuse into the agar. Bacteria seeded in AAM were prepared the same as described in section 2.5. The TLC pieces were then removed from the agar plates before the plates were incubated at 37°C overnight to check for the presence of an inhibition zone, which would determine which band contained the active compound.

2.7.3 High Performance Liquid Chromatography (HPLC)

The isolate extracts were sent to Microbial Screening Technologies in Sydney (http://www.microbialscreening.com/) for evaluation in their proprietary HPLC-DAD system.

2.8 Identification of endophytic actinomycetes based on 16S rRNA gene sequencing

2.8.1 Extraction of Bacterial DNA

Two loops full of well-sporulated actinomycetes culture were scraped from the surface of a ten day-old colony on yeast extract - malt extract agar. They were

resuspended in 500μl tris-EDTA (10M tris-10mM EDTA pH 8.7) in a 1.5 ml sterile eppendorf tube. Ten microliters of 10mg/ml lysosome (Sigma) was added, followed by incubation at 37 ℃ for 60 minutes. In the same time, 10$\mu l\ of$ 1% (w/v) proteinase K (sigma) and 32.5 μl of 10% SDS were added and the mixture was incubated at 55℃ for 60 minutes. Following this step was the addition of 100 μl of 5M NaCl and 65 μl of 10 g Cetyl trimethylammonium bromide (CTAB) in 4.1 g/L Sodium Chloride (NaCl) solution. The mixture was then subjected to a further incubation at 55 ℃ for 10 minutes. After incubation, 500 μl chloroform was added and the mixture was left at room temperature for 30 minutes with intermittent shaking. The mixture was then centrifuged at 12,000 rpm for 15 minutes. Then, the upper layer, which is the aqueous layer, was transferred to a new sterile eppendorf tube. Followed by adding 0.1x volume of 3M Sodium Acetate and 3x volume of 100% ethanol. The mixture was left at 4 ℃ overnight to precipitate the DNA, followed by centrifuging at 12,000 rpm for 5 minutes. The supernatant was then removed without disturbing the pellet. The pellet was washed twice with 70% ethanol followed by drying the pellet by placing the tube in the heating block at 55 ℃ with the lid open for approximately 10 minutes or until the pellet was dry. Then, the pellet was resuspended in 50 μl sterile water or water for injection. Storage of the DNA extracts was kept at -20˚C (Coombs & Franco 2003).

2.8.2 Polymerase Chain Reaction (PCR)

PCR was conducted prior to sequencing the DNA. The primer used in this PCR was universal 16S rRNA gene primers and designated as 27f (AGAGTTTGATCMTGGTCAG, M= A+C) and 1492r (CGG TTA CCT TGT TAC GAC TT). PCR was done by adding 36 μl of PCR mixture to 14 μl of DNA in a PCR

tube. PCR mixture consists of 25.75μl water for injection, 2μl forward primer (27f) 2μl reverse primer (1492r), 1 μl dNTP (a mixture f 10 μl dATP, dGTP, dCTP, and dTTP), 0.25 *Taq* polymerase, and 5 μl Standard *Taq* Reaction Buffer.

The reaction was run using the following cycles: 94 ℃ to 98 ℃ for 1 minute, 94 ℃ 70 ℃ to 80 ℃ for 10 to 60 seconds Amplicon and DNA polymerase dependent, 70 ℃ 80 ℃ for 5 minutes for the final cycle. This was done using Thermal Cycler (TECHNE) (Coombs & Franco 2003).

2.8.3 Polymerase Chain Reaction (PCR)

The DNA has to be purified from primers and others PCR reagents before sequencing. Purification of PCR product was done using UltraClean ™ PCR Clean up kit according to the manufacture's protocols. A 5x volume of buffered salt solution was added to the PCR product. This mixture was transferred to a spin filter unit and centrifuged at 13,000 rpm for 30 seconds. The spin filter basket was removed, the liquid was discarded by decanting and the spin filter basket was returned to the same tube. An ethanol based wash solution was then added to remove any traces of unwanted contaminates while allowing the desired PCR product DNA to stay bound to the silica spin filter membrane followed by centrifuging at 13,000 rpm for 30 seconds. The spin filter basket was removed and the liquid was discarded by decanting. Then, the spin filter basket was put back into the same tube for further centrifugation at 13,000 rpm for 60 seconds. The spin filter basket was transferred to a new tube followed by adding 5 μl of elution buffer (10mM Tris) or sterile water and centrifuged at 13,000 rpm for 60 seconds to release the purified DNA.

2.8.4 Agarose Gel Electrophoresis

DNA sample was analysed using agarose gel electrophoresis. The concentration of agarose was 0.8% and prepared by dissolving 0.32 g agarose in 40 ml 0.5x TBE buffer. The mixture was heated in a microwave until well dissolved. The solution was cooled down and 3 μl of ethidium bromide was added followed by pouring the gel in a gel mould. Ten microliters of the samples mixed with 2 μl of loading dye were then loaded to each well of the gel. Next, the gel was submerged in the gel tank containing 0.5x TBE buffer and the electrophoresis was run at 60v for one hour. The gel was visualized using a UV Transluminator.

2.8.5 16S rRNA gene sequencing

The 16S rRNA gene obtained was submitted to the DNA sequencing facility (Macrogene) in Korea (http://www.macrogen.com/). A big dye® terminator V 3.1 cycle sequencing kit was used to sequence the DNA. The DNA was then run in automated DNA sequencer using capillary electrophoresis system, ABI3730XL DNA analyser. The sequence was compared to a database available at EzTaxon.

2.8.6 Phylogenetic Analysis

The nucleotide and protein sequences incorporated in the construction of the phylogenetic trees were retrieved from EZtaxon (http://www.ezbiocloud.net/eztaxon). The sequences were uploaded in FASTA format. Sequences were aligned across specific regions, which included only the secondary metabolite biosynthetic gene under investigation; these alignments were performed using the ClustalW alignment program EZTAXON using blastn program (altschul et al., 1990).

CHAPTER THREE: RESULTS

3.1 Morphological Characteristic of Actinobacteria cultures

Morphological characterization was carried out based on ISP method (Shirling & Gottlieb 1966) involving the characteristics of substrate and aerial mycelia, spore colour, and soluble pigments. Thirty-three Actinobacteria cultures used in this colony characterization were grown on ISP2 medium and modified ISP2 medium. The colony characterizations of the cultures are shown in table 3 and 4.

Table 3: Colony Characterization of Actinobacteria cultures isolated from Australian plants grown on ISP2 medium

Strain	Characteristics	Medium / ISP2
Lup 7	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Cream Brown Grey Brown White
Lup 77	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Cream Cream White Light brown White
Lup 38	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Brown White Grey Light purple Light brown
Lup 75	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Colourless Cream White Cream -
Lup 26	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Brown Grey White/grey Light brown Cream

Table 3: Colony Characterization of Actinobacteria cultures isolated from Australian plants grown on ISP2 medium (continued)

Strains	Characteristics	Medium / ISP2
Lup 19b	Substrate mycelium	Light brown
	Aerial mycelium	Grey
	Spore colour	Light grey
	Reverse pigments	Cream
	Soluble pigments	Cream
Lup 19 w	Substrate mycelium	Cream
	Aerial mycelium	Light grey
	Spore colour	Grey / White
	Reverse pigments	Cream
	Soluble pigments	Cream
Lup 76 b	Substrate mycelium	Cream
	Aerial mycelium	Cream
	Spore colour	White
	Reverse pigments	Yellow
	Soluble pigments	-
Lup 42 b	Substrate mycelium	Dark brown
	Aerial mycelium	Grey
	Spore colour	Grey
	Reverse pigments	Light orange
	Soluble pigments	Grey
MF 12 a	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	Grey / White
	Reverse pigments	Light red
	Soluble pigments	-
MF12 b	Substrate mycelium	Dark brown
	Aerial mycelium	White
	Spore colour	Grey
	Reverse pigments	Brown
	Soluble pigments	-
Pl 12	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	White
	Reverse pigments	Light yellow
	Soluble pigments	-
Pl 21	Substrate mycelium	Brown
	Aerial mycelium	Grey
	Spore colour	Grey / White
	Reverse pigments	Yellow
	Soluble pigments	-

Table 3: Colony Characterization of Actinobacteria cultures isolated from Australian plants grown on ISP2 medium (continued)

Strains	Characteristics	Medium / ISP2
CM 6	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	White White Grey Yellow -
PP 13	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Colourless White White Cream -

Table 4: Colony characterizations of Actinobacteria cultures isolated from Sponge marine grown on modified ISP2 medium

Actinobacteria strain	Characteristics of	Medium / ISP2
ACT001	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Brown Cream Grey Brown White
ACT004	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Cream Brown Grey Brown Brown
ACT005	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Colourless Cream Cream Cream -
ACT010	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Colourless Cream White Yellow -
ACT014a	Substrate mycelium Aerial mycelium Spore colour Reverse pigments Soluble pigments	Cream Brown Grey Light brown -

Table 4: Colony characterizations of Actinobacteria cultures isolated from Sponge marine grown on modified ISP2 medium (continued)

Actinobacteria strain	Characteristics of	Medium / ISP2
ACT022a	Substrate mycelium	Black
	Aerial mycelium	Brown
	Spore colour	Grey
	Reverse pigments	Brown
	Soluble pigments	Light brown
ACT025	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	White
	Reverse pigments	Light brown
	Soluble pigments	Cream
ACT039b	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	Cream
	Reverse pigments	Yellow
	Soluble pigments	-
ACT052b	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	Cream
	Reverse pigments	Cream
	Soluble pigments	-
ACT053	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	Cream
	Reverse pigments	-
	Soluble pigments	-
ACT069	Substrate mycelium	Red
	Aerial mycelium	Cream
	Spore colour	Red
	Reverse pigments	Orange
	Soluble pigments	-
ACT069g	Substrate mycelium	Brown
	Aerial mycelium	Grey
	Spore colour	Grey
	Reverse pigments	Blue
	Soluble pigments	Grey
ACT040	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	Cream
	Reverse pigments	-
	Soluble pigments	-

Table 4: Colony characterizations of Actinobacteria cultures isolated from Sponge marine grown on modified ISP2 medium (continued)

Actinobacteria strain	Characteristics of	Medium / ISP2
ACT014c	Substrate mycelium	Colourless
	Aerial mycelium	Brown
	Spore colour	Grey
	Reverse pigments	Light red
	Soluble pigments	-
ACT017	Substrate mycelium	
	Aerial mycelium	Colourless
	Spore colour	Cream
	Reverse pigments	Cream
	Soluble pigments	Yellow
		-
ACT079	Substrate mycelium	Brown
	Aerial mycelium	Cream
	Spore colour	White
	Reverse pigments	Cream
	Soluble pigments	-
ACT077	Substrate mycelium	Colourless
	Aerial mycelium	Cream
	Spore colour	Cream
	Reverse pigments	-
	Soluble pigments	-
ET11	Substrate mycelium	Green
	Aerial mycelium	Yellow
	Spore colour	Yellow
	Reverse pigments	Green
	Soluble pigments	Yellow

3.2 Primary antibacterial assay

First study to find actinobacterial strains that have high production of antibacterial compounds was conducted by well type antibacterial assay of metabolites extracted from agar. It involved testing metabolites extracted with methanol (MeOH) from 10 days old cultures grown on ISP2 medium.

Thirty-three of Actinobacteria strains were extracted with MeOH and tested against *E. coli* , *S. aureus* and *M. luteus* . Table 5 shows that only 5 of the cultures were active against *S. aureus* while 8 of them showed activity against *M. luteus* which both represent gram-positive bacteria. No antagonist effect was detected against the gram-negative *E. coli* bacteria except one strain that is ET11. The metabolite producing isolates were subjected to further studies using submerged liquid fermentation media (Section 3.3).

Table 5. The activity of Actinobacteria strains against *S. aureus, M. lutues* and *E. coli*

Strains	Zone of inhibition		
	S. aureus	***M. luteus***	***E. coli***
Lup 7	+	++	_
Lup 26	-	-	-
Lup 38	-	-	-
Lup 19b	-	-	-
Lup19 w	-	-	-
Lup 42 b	-	-	-
Lup 75	-	-	-
Lup 76 b	-	-	-
Pl 12	+	+	-
Pl 21	-	-	-
PP 13	-	-	-
Mf 12 a	-	-	-
Mf 12 b	-	-	-
CM 6	+	+	-
Lup 77	-	+	-

Table 5. The activity of Actinobacteria strains against *S.aureus, M.lutues* and *E.coli* (Continued)

Strains	Zone of inhibition		
	S.aureus	***M.luteus***	***E.coli***
ACT 001	-	-	_
ACT 004	+	++	-
ACT 005	-	-	-
Act 010	-	-	-
ACT 14 a	-	-	-
ACT 014 c	+	+	_
ACT 017	-	-	-
ACT 022 a	-	-	-
ACT 025	-	-	-
ACT 039 b	-	-	-
ACT 040	-	-	-
ACT 052 b	-	-	-
ACT 053	-	-	-
ACT 069	+	+	-
ACT 069 g	-	-	-
ACT 077	+	++	-
ACT 079	-	-	-
ET 11	++	+++	+

Key: +++ = Strong activity (zone inhibition ≥ 20 mm); ++ = moderate activity (zone inhibition ≥ 10 mm); + = week activity (zone inhibition < 10 mm); - = no activity

3.3 Fermentation

3.3.1 Submerged Shake-Flask Fermentation

Further screening of Actinobacterial strains was conducted by cultivating the four active strains in nine different fermentation media because the other strains showed no activity against the test microorganisms. Two types of fermentation were done (Submerged liquid fermentation and solid state fermentation).

Four isolates were chosen to be cultivated on submerged state fermentation, which were ET11, ACT77, Lup7 and Pl12. However, they did not grow well in the first batch of liquid fermentation media. The first batch was conducted on five different liquid media that were TGY, TFY, M-2, F26 and Sucrose production medium. All of

the four isolates cultivated in the first batch of fermentation had weak activity against *S. aureus, M. luteus* and *E. coli.*

Second batch of liquid fermentation, was done by using another production media, F28, F31, F33, and ISP2 liquid medium. Three of the isolates did not grow well on the second batch of fermentation as well which are ACT77, Lup7 and Pl12. They have weak activity against the test organisms so there were not analysed further.

Only isolate ET11 had grown very well on three fermentation media of the second batch, F31, F33 and ISP2 liquid medium. The F28 fermentation medium was found to be a poor medium compared to others. No activity or weak activity against test microorganisms was observed with the methanol extracts of inoculated F28 whereas the methanol extracts of ET11 grown on other liquid media at the same sampling time showed greater activity against the same test microorganisms.

3.3.2 Solid State Fermentation

Further screening of Actinobacterial strains was conducted by cultivating the one active strain in rice as a solid fermentative medium. ET11 was the only strain that showed good activity against the test organisms grown on some of the submerged liquid fermentation media used in this study.

Isolate ET11 had not grown well on the solid fermentation medium. Solid-state fermentation medium (rice) was found to be a poor medium compared to the submerged liquid media. No activity against test microorganisms was observed with the methanol extracts of inoculated (rice medium) whereas the methanol extracts of ET11 grown on liquid media at the same sampling time showed greater activity against the same test microorganisms.

3.4 Antibacterial activity by submerged fermentation

3.4.1 Antibacterial Activity Against *S. aureus*

The activity of Actinobacteria against *S. aureus* was summarized in table 6. Antibacterial activity was detected from most of the actinobacterial strains tested. However, consistent activity from only two cultivation media tested was found from strain lup77 and ET11. The best activity was displayed by ET11 with the clearest and largest zone of inhibition compared to the other strains.

Table 6. Activity of cell extracts and supernatants of actinobacterial strains against Staphylococcus aureus. Actinobacterial cultures were grown in 4 media by submerged fermentation for 14 days.

Medium	Strain	Zone of inhibition (mm) on day													
		Cells							Supernatant						
		2	4	6	8	10	12	14	2	4	6	8	10	12	14
Isp2	ET11	-	11	12	12	13	11	11	10	10	9	9	11	11	11
	ACT77	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lup77	7	8	8	7.5	9	9	8	8	9	9	7	10.5	8	8
	Pl12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F28	**ET11**	**-**	**-**	**8**	**9**	**9**	**9**	**7**	**-**	**6**	**8**	**8**	**7**	**7**	**6**
	ACT77	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lup77	-	-	-	4	5	4	6	7	6	7	-	-	-	-
	Pl12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F31	**ET11**	**9**	**10.5**	**10**	**11**	**11**	**11**	**10**	**11**	**12**	**14**	**14**	**12**	**11**	**13**
	ACT77	-	-	-	-	-	-	-	-	7	-	-	-	-	-
	Lup77	-	-	6.5	6.5	7	7	-	-	8	9	7	7	-	-
	Pl12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F33	**ET11**	**10**	**11**	**11**	**13**	**13**	**15**	**12**	**12**	**11**	**12**	**12**	**12**	**12**	**10**
	ACT77	-	-	-	-	-	-	-	-	-	-	7.5	-	-	-
	Lup77	-	-	7	6	6	10	6	6	7	10	10	8	6	7
	Pl12	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.4.2 Antibacterial Activity against *M. luteus*

The activity of Actinobacteria against *M. luteus* was summarized in table (7). Antibacterial activity was detected from most of the actinobacterial strains tested. However, consistent activity from only two cultivation media tested was found from

strain Lup77 and ET11. The best activity was displayed by ET11 with the clearest and largest zone of inhibition compared to the other strains.

Table 7. Activity of cell extracts and supernatants of actinobacterial strains against Micrococcus luteus. Actinobacterial cultures were grown in 4 media by submerged fermentation for 14 days.

Medium	Strain	Zone of inhibition (mm) on day													
		Cells							Supernatant						
		2	4	6	8	10	12	14	2	4	6	8	10	12	14
Isp2	ET11	-	13	13	14	14	11	11	11	10	11	9	11	10	10
	ACT77	-	7	7	-	-	-	-	-	8	10	6.5	-	-	-
	Lup77	9	8	8	7.5	11	8	8	8	11	11	8	10	7	8
	Pl12	-	5	6	6	-	-	-	-	7	8	7	-	-	-
F28	**ET11**	**-**	**10**	**9**	**13**	**12**	**12**	**10**	**11**	**10**	**13**	**13**	**14**	**12**	**12**
	ACT77	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lup77	-	7	7	9	8	8	7.5	9	9	8	10	10	9	9
	Pl12	-	-	-	-	-	-	-	-	-	7.5	7	-	-	-
F31	**ET11**	**12**	**12**	**14**	**13**	**13**	**12**	**10**	**11**	**12**	**14**	**14**	**12**	**11**	**13**
	ACT77	-	-	-	-	-	-	-	-	7	-	-	-	-	-
	Lup77	-	-	6	6	7	7	-	-	8	10	7	7	-	-
	Pl12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F33	**ET11**	**12**	**12**	**14**	**15**	**15**	**15**	**17**	**14**	**16**	**12**	**17**	**20**	**18**	**15**
	ACT77	-	-	-	-	-	-	-	-	-	-	7.5	-	-	-
	Lup77	-	7	10	0	13	13	12	8	9	12	12	12	10	10
	Pl12	-	-	-	-	-	-	-	-	-	8	7	-	-	-

3.5 Secondary Metabolites detection

3.5.1 Thin Layer chromatography (TLC)

Based on the results from primary antibacterial assay activity and fermentation bioassays, *Streptomyces* ET11 grown in F33 fermentation medium was selected in order to extract and identify antibacterial compound produced by this strain. The methanol extract and ethyl acetate extract at the 14th day of the fermentation were run

in two TLC systems; Chloroform: Methanol (9:1) and Butanol: Acetic acid: Water (4:1:1).

The first TLC results demonstrated that isolates PL12, Lup77, ET11, and ACT77 produced relatively more non-polar compounds. The reason was that most of the spots appeared on TLC plates eluted with Chloroform: Methanol system and only a few compounds resolved on TLC eluted with Butanol: Acetic acid: Water system. Figure 4 demonstrates TLC plates of isolates Lup77, Pl12, ET11, and ACT77 that had been extracted with MeOH and concentrated, after elution in C: M and B: A: W systems under two wavelength 254 and 365 nm UV light detection. Table 8 summarized the retention factors (Rfs) and colour of the spots under UV light detection representing the compounds produced by each isolate.

The results also indicated that some of the same compounds seem to be produced by most isolates, as spots with same Rf and colours under 254 nm or 365 nm UV detection were observed on TLC plates. For instant, after developing with BAW solvent system, two compounds detected from MeOH extracts of two isolates (ET11 and ACT77) had the same Rf under 254 nm UV detection. Three spots appeared on TLC plates eluted in C: M solvent system, two were detected under 254 nm and one was detected under 365 nm also had the same Rf and colour.

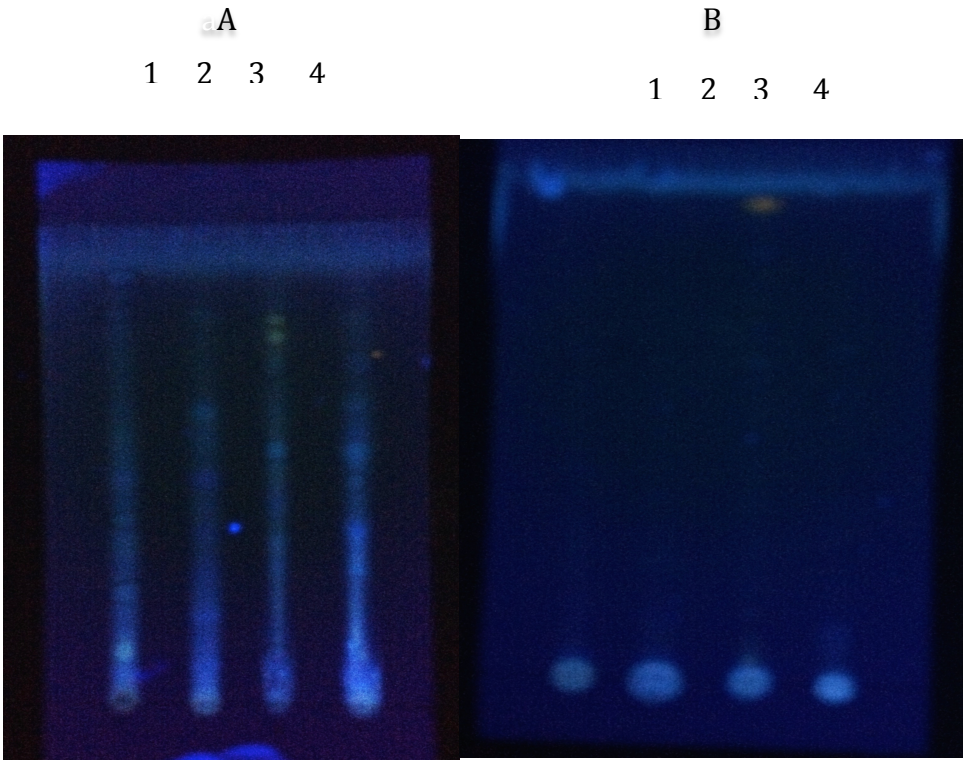

Figure 4: TLC plates of isolate Pl12 MeOH extract (1); isolate Lup77 MeOH extract (2); isolate ET11 MeOH extract (3); isolate ACT77 MeOH extract (4) eluted in two solvent systems; (A) butanol: acetic acid: water (4:1:1) and (B) Chloroform: Methanol (9:1) detected with 365 nm UV light.

As shown in figure 4, in TLC plate (A) where the isolates were eluted in BAW solvent system, a compound with Rf 0.97 was observed from two isolates (Pl12 and ET11) under 65 nm UV detection. Under 254 nm UV light, two compounds were detected with Rf 0.1 and 0.85 respectively from isolates ET11 and ACT77. As shown clearly in the TLC plates (figure 4), al the compounds produced by the four isolates Pl12, Lup77, ET11 and ACT77 in the two solvent systems were light blue spots. However, only two spots were yellow at Rf 0.93 and 0.97 produced from isolate ET11 that eluted in B: A: W solvent system while only one spot was clearly yellow with Rf 1.1 under 365 nm UV light. All the isolates were more dissolved in butanol: acetic acid: water system and more bands were produced than the chloroform: Methanol system.

Table 8. The Rf and colours of spots shown on TLC plates eluted with two different solvent systems under 254 nm and 365 nm UV light detection

Isolate	Butanol: Acetic acid: water= 4:1:1				Chloroform: Methanol 9:1			
	UV detection 254 nm		UV detection 365 nm		UV detection 254 nm		UV detection 365 nm	
	Rf	Colour	Rf	Colour	Rf	Colour	Rf	Colour
Pl12	0.0	Dark green	0.13 0.24 0.37 0.78 0.85	Light blue	0.0	Dark green	0.67	Light blue
Lup77	-	-	-	-	0.0	Dark green	0.85 0.67	Light blue
ET11*	0.10 0.85	Dark green	0.49 0.93 0.97	Light blue Yellow* Yellow*	0.0 0.10 0.91	Dark green	0.06 0.16 0.67 0.76 1.1	Light blue Yellow *
ACT77	0.10 0.49 0.59 0.68	Dark green	0.42	Light blue	0.0 0.10 0.31 0.44 0.91	Dark green	0.58 0.67	Light blue

* = Isolate ET11 generated visible yellow spot on TLC plates at Rf 0.93, 0.97 and 1.1 respectively after eluted in BAW and CM systems.

As shown in table 8, most of the compounds produced by the four isolates were more non-polar and moved through out the TLC plates in both solvents systems. Only few spots were generated near the original pipetted spot, which indicated that they were non-polar compounds.

The second TLC plates in Figure 5 shows the results of TLC plates of extracts of isolate ET11 cultivated in F33 production medium and were extracted with Methanol and Ethyl acetate. The two extracts were eluted with two solvent systems as well that were Chloroform: Methanol (9:1) and Butanol: Acetic acid: Water (4:1:1), then detected under two wavelength 254 nm and 365 nm UV light detection.

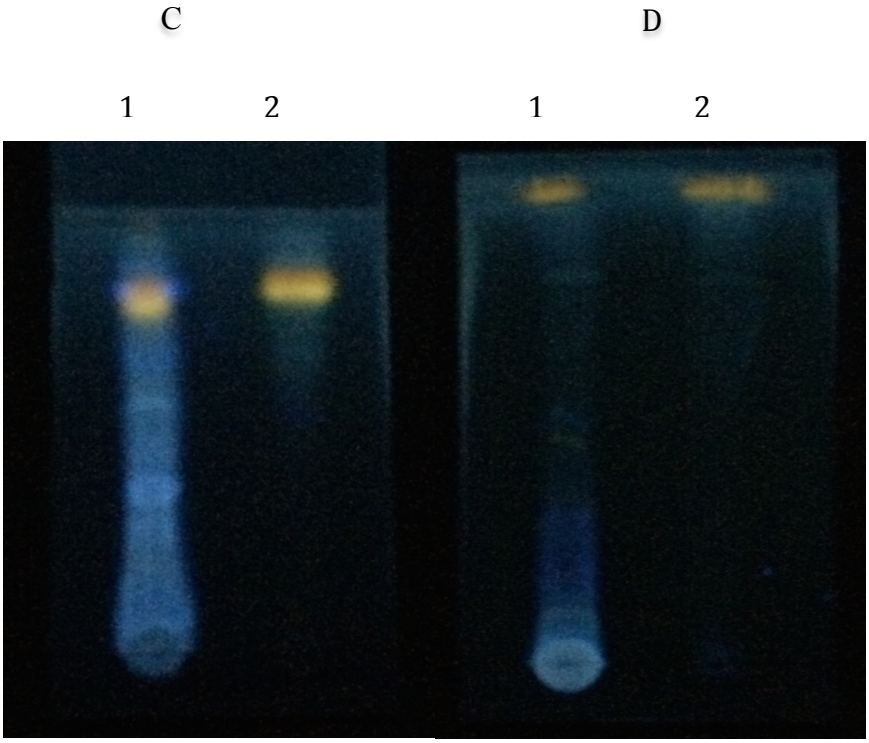

Figure 5: TLC plates of isolate ET11 MeOH extract (1); isolate ET11 EtOAc extract (2) eluted in two solvent systems; (C) Chloroform: Methanol (9:1) and (D) butanol: acetic acid: water (4:1:1) detected with 365 nm UV light.

The second TLC results demonstrated that isolate ET11 after been cultivated in F33 production medium for 14 days and harvested and then extracted with MeOH and EtOAc solvent produced relatively more non-polar compounds.

The results also indicated that some of the same compounds seem to be produced by both extracts, as spots with same Rf and colours under 254 nm or 365 nm UV detection were observed on TLC plates. For example, after developing with BAW solvent system, two compounds detected from MeOH extract and EtOAc extract had the same Rf under 365 nm UV detection and one compound observed had the same Rf as 0.81 and same colour under 254 nm UV light detection. Only one spot appeared on TLC plates eluted in C: M solvent system was detected under 365 nm UV light detection.

As shown in Figure 5, in TLC plate (c) where the extracts were eluted in CM solvent system, a compound with Rf 0.56 was observed from the two extracts (MeOH and EtOAc) under 365 nm UV detection and generated a yellow spot. Under 254 nm UV light, one compound was detected with Rf 0.56 from both extracts. As shown clearly in the TLC plates (figure 5), all the compounds produced by both extracts in the two solvent systems were light blue spots. However, only 3 spots were yellow at Rf 0.32, 0.37 and 0.81 produced from MeOH and EtOAc extract that eluted in B: A: W solvent system while only one spot was clearly yellow with Rf 0.56 under 365 nm UV light.

Table 9. The Rf and colours of spots shown on TLC plates eluted with two different solvent systems under 254 nm and 365 nm UV light detection

Extract	Butanol: Acetic acid: water= 4:1:1				Chloroform: Methanol 9:1			
	UV detection 254 nm		UV detection 365 nm		UV detection 254 nm		UV detection 365 nm	
	Rf	Colour	Rf	Colour	Rf	Colour	Rf	Colour
MeOH	0.0	Dark green	0.08 0.32* 0.37* 0.59 0.81*	Light blue Yellow Yellow Blue Yellow		Dark green	0.27 0.37 0.56*	Light blue Yellow
EtAc	-	-	0.59 0.81*	Blue Yellow	0.0	Dark green	0.56*	Yellow

* = Isolate ET11 extracted with MeOH and EtOAc generated visible yellow spot on TLC plates after eluted in BAW and CM systems.

As shown in table (9), most of the compounds produced by both extracts were more polar and moved through out the TLC plates in both solvents systems. Only few spots were generated near the original spot, which indicated that they were non-polar compounds.

3.5.2 Bioautography

Bioautography of the single band either from the cell extract or freeze dried of cells shows the inhibition zone of one test organism *M. luteus* is displayed by an Rf value 0.67 and did not have any antibacterial activity against *S. aureus* nor *E. coli.*

3.5.3 High Performance Liquid Chromatography (HPLC)

As shown in table 10, eleven peaks were observed for isolate ET11 at different retention times with different wavelengths.

Table 10. Commet analysis of isolate ET11- HPLC trace under TFA conditions

Peaks	Number of peaks
Non-polar (254 nm)	15
Non-polar (205 nm)	21
Total	19
Resolved UV spectra	11

Table 11. Resolved UV spectra of isolate ET11 – HPLC trace under TFA conditions

	RT	Height	UV Description
5	2.14	73288	211 (p, 100%), 240 (s, 20%), 299 (p, 15%)
6	2.47	38904	206 (p, 100%), 248 (p, 17%), 283 (s, 3%), 315 (p, 10%)
10	3.27	30582	209 (p, 27%), 252 (p, 85%), 261 (s, 74%), 319 (p, 100%)
11	3.49	15645	201 (p, 100%), 253 (p, 45%)
12	3.91	55307	201 (p, 33%), 310 (p, 10%), 386 (s, 73%), 404 (p, 100%), 424 (p, 95%)
13	3.98	23363	250 (s, 0%), 298 (p, 13%), 384 (p, 75%), 402 (p, 100%), 426 (p, 78%)
14	4.03	71345	204 (p, 33%), 256 (p, 17%), 318 (p, 28%), 41 (p, 100%)
15	4.11	26648	204 (p, 33%), 220 (s, 25%), 255 (p, 17%), 311 (s, 34%), 319 (p, 38%), 415 (p, 100%)
16	4.17	7459	318 (p, 47%), 413 (p, 100%), 430 (s, 88%)
18	4.94	15928	235 (p, 100%)
19	5.13	19676	218 (p, 100%), 269 (s, 31%)

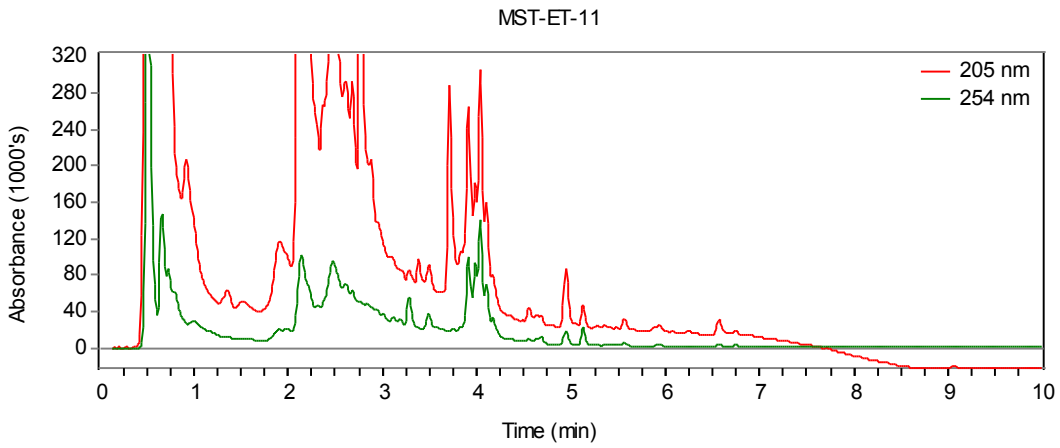

Figure 6. The HPLC chromatogram of isolate ET11 extract

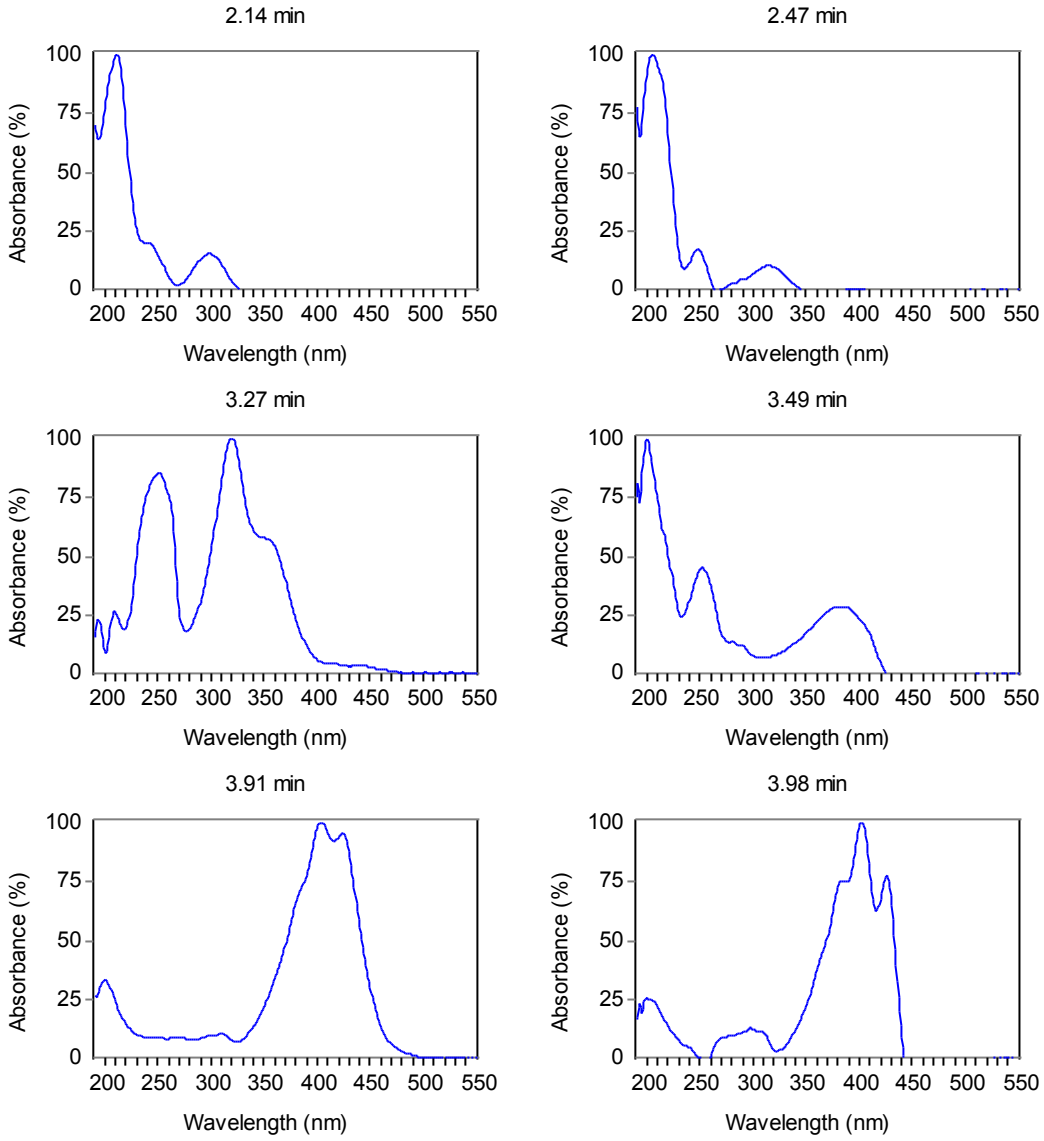

Figure 7. The UV absorption spectrum of compounds at different retention times

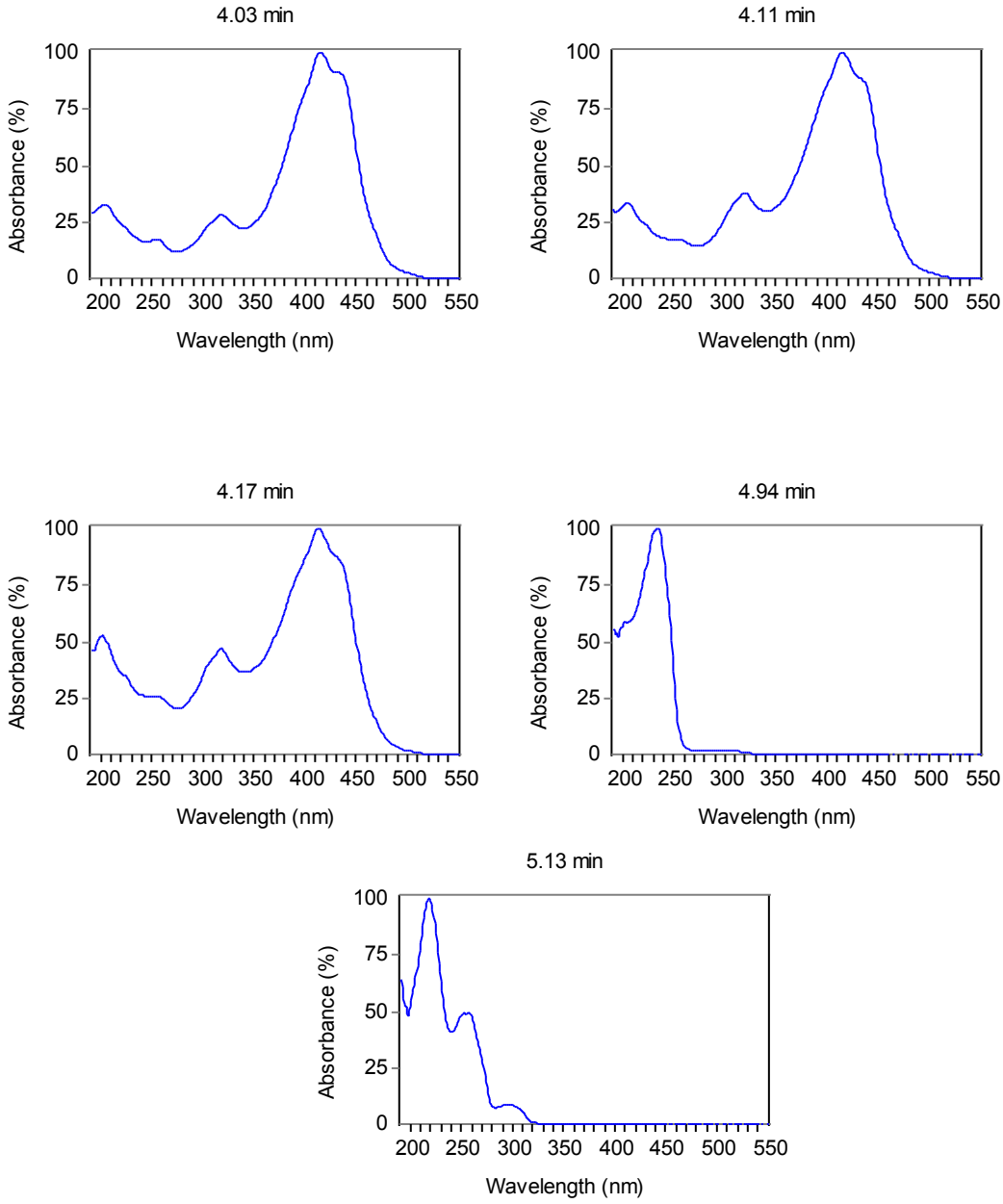

Figure 7. The UV absorption spectrum of compounds at different retention times (continued)

3.6 Identification of endophytic actinomycetes using 16S rRNA gene sequencing

3.6.1 16S rRNA Sequencing

Only one isolate (ET11) of the thirty-five strains was identified based on the 16S rRNA gene sequencing. Table 12 show the identification result of the isolate compared to the gene database available at EzTaxon website using Blastn search. The bit score and the identity indicate the similarity between hit and a query. One genus of actinomycetes was identified which is Streptomyces.

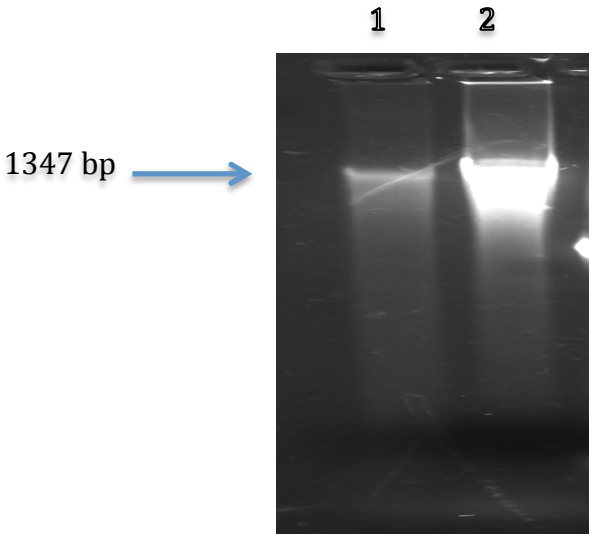

Figure 8. PCR amplification of 1347 bp from marine actinobacterial isolate ET11. Both lanes are replicate of isolate ET11.

As shown in table 12, isolate ET11 had the highest similarity with strain *Streptomyces halophytocola* KLBMP 1284(T) with a 99.78 % similarity. The second highest similarity was with strain *Streptomyces xiaopingdaonensis* DUT 180(T) with a 99.7 % similarity.

Table 12. Results of 16S rRNA gene sequencing shows the top 3 actinobacteria strains that are similar to strain ET11

Rank	Name	Strain	Accession	Pairwise Similarity(%)
1	*Streptomyces halophytocola*	KLBMP 1284 (T)	JQ819259	99.78
2	*Streptomyces xiaopingdaonensis*	DUT 180 (T)	EF577241	99.7
3	*Streptomyces sulphureus*	NRRL B-1627 (T)	DQ442546	99.7

3.6.2 Phylogenetic tree

Figure 9 represent the phylogenetic tree of isolate ET11 among species of the genus Streptomyces. The almost-complete 16S rRNA gene sequence (1347 bp) of strain ET11 was compared with sequences in the EzTaxon database. The results indicated that the isolate belonged to the genus *Streptomyces*. Strain ET11 showed the highest 16S rRNA gene sequence similarity with *Streptomyces sulphureus* NRRL B-1627 ^{T} (100%). Phylogenetic tree constructed revealed that strain ET11 formed a distinct lineage with species *S. xiaopingdaonesis* DUT 180 ^{T}. It also formed a distinct lineage with species *S. halophytocola* KLBMP 1284 ^{T} and loosely associated with the other closest phylogenetic neighbours.

The 16S rRNA gene sequence similarity to other species of the genus *Streptomyces* was less than 97%. It is evident from the phylogenetic tree (Figure 9) that strain ET11 1347 was clustered to *S. sulphureus* NRRL B-1627 ^{T}, and they formed a distinct subclade with a high bootstrap value of 100% by neighbour-joining analysis. This relationship was also found in trees constructed using the maximum-parsimony and maximum-likelihood algorithms.

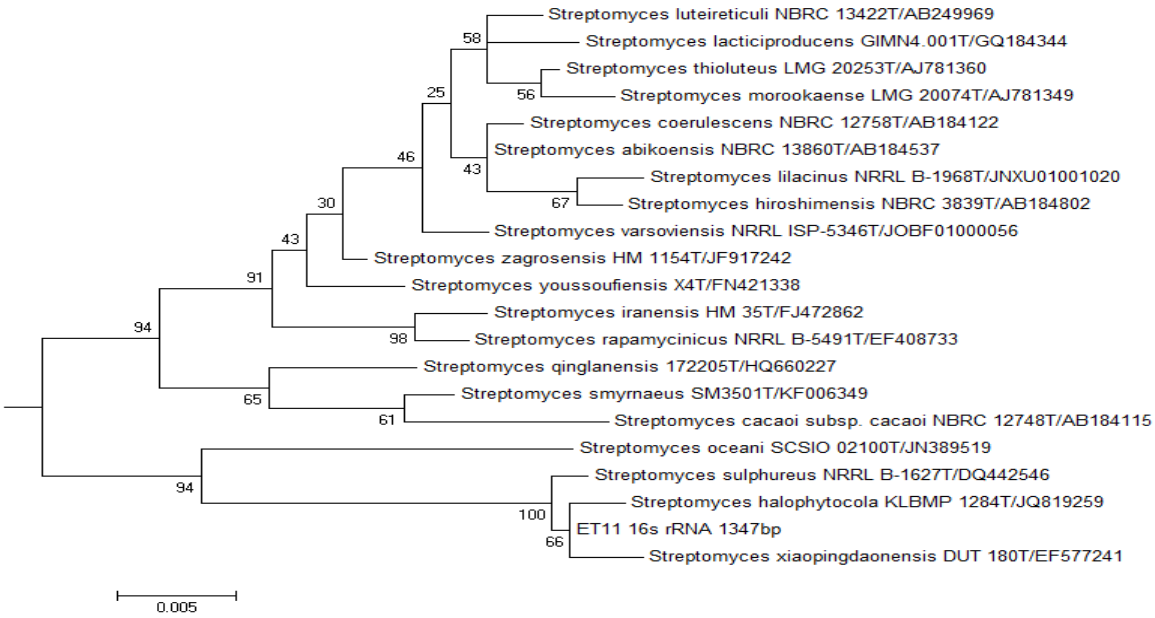

Figure 9: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence, showing the position of strain ET11 among species of the genus Streptomyces. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. Asterisks indicate branches of the trees that were also found using the maximum- parsimony and maximum-likelihood tree-making algorithms. Bar, 0.005 substitutions per nucleotide position.

Chapter Four: Discussion

In this thesis, characterization and identification of endophytic Actinobacteria associated with healthy plants and marine sponges were studied as well as their antimicrobial activity to look for new secondary metabolic compounds.

The active strain ET11 was identified according to the 16s rRNA sequencing that it belonged to the genus *Streptomyces*. The morphological observation of a 21-day culture of strain ET11 grown on ISP2 medium revealed that strain ET11 had the typical characteristics of members of the genus *Streptomyces*. It formed a highly branched substrate mycelium and aerial hyphae with smooth spores. The strain grew very well on ISP2 medium modified with fresh seawater instead of distilled water at 28 ˚C for 21 days. It formed strong yellow aerial mycelia and yellow to strong green substrate (Shirling & Gottlieb 1966).

The closest strain that show (99%) similarity with ET11 is *Streptomyces halophytocola* (KLBMP 1284$_T$) which been isolated from the surface-sterilized stems of a costal halophyte Tamarix chinensis Lour, when grown on ISP2 medium, it formed yellowish to orange-yellow aerial mycelium and yellowish-white to orange-yellow substrate mycelium (Qin et al. 2013). The two strains grow very well on ISP2 medium and they both formed yellow aerial mycelium.

4.1 16S rRNA sequencing

Based on the 16S rRNA sequencing, one genus of actinomycetes, *Streptomyces* was identified as the genus for the isolate ET11. This result was in accordance with different studies by (Taechowisan, Peberdy & Lumyong 2003) finding that *Streptomyces* being dominant among the endophytic actinomycetes isolated, followed

by *Microbispora* and other genera, such as *Nocardia spp*., *Micromonospora spp*., and *Streptosporangium spp*. Other reports also showed that the genus *Streptomyces* constituted a majority of the isolates found within plant tissue (Coombs & Franco 2003); (Sardi et al. 1992). These findings would suggest that *Streptomyces* is widely distributed in plants. Moreover, isolate ET11 displayed a very high level of 16S rRNA sequence similarity to known species. According to the sequencing results, it belongs to the genus *Streptomyces* with closest similarity to strain *Streptomyces halophytocola* (KLBMP 1284T).

4.2 Fermentation

Among the nine media used in fermentation only few media were suitable for the tested Actinobacteria that showed activity against the tested organisms. Those media were nutrient poor media as noticed that the isolates did not grow well and had week activity against test organisms. F33 medium was found as the most effective medium for the cultivation of secondary metabolites within Actinobacteria strains, compare to the other media used in this study. It has been found that Proflo improved the yield of productivity of some fermentation processes. Also, it has been used widely for the production of antibiotics like tetracycline and penicillin. Few compounds were detected in the fermentation extracts of the isolates grown on SmF media.

Antibiotic formation usually occurs during the late growth phase of the producing microorganism. The temporal nature of their formation is certainly genetic, but expression can be influenced greatly by environmental manipulations. Therefore, synthesis of antibiotics is often brought on by exhaustion of a nutrient, addition of an inducer and/or by a decrease in growth rate (Sanchez et al. 2010).

Formation of antibiotics is also regulated by nutrients (such as nitrogen, phosphorous and carbon source), metals, growth rate, and feedback control and enzyme inactivation. In bacteria and other microorganisms, glucose, usually an excellent carbon source for growth interferes with the formation of many antibiotics.

The solid fermentation did not offer appropriate conditions for the production of secondary metabolites by the one isolate ET11 that worked very well on the SmF media.

4.3 Bioassay

All four isolates subjected to bioassay showed weak to strong activity against at least one bacterium. Some of the isolates had broad-spectrum activity as they inhibited all the tests organisms, and some showed narrow spectrum activity as they only inhibited only one test organism. It was not surprising that these isolates exhibited antibacterial activity since actinomycetes are known as prolific source of secondary metabolites, especially antimicrobial agents.

Isolate ET11 showed a good activity against *S. aureus* and *M. luteus* while other isolates showed week or no activity against them. Strains that have been already isolated from marine sources were reported to have antimicrobial activity against pathogenic microorganisms, and it was not surprising that the majority were belonging to the genus *Streptomyces*. For instant, antagonistic activity against MRSA with a zone of 42 mm was reported from *Streptomyces rubrolavendulae* ICN3 (Kannan, Iniyan & Vincent 2014).

Another study by (Rajan & Kannabiran)of strain *Streptomyces sp*. VITBRK1 showed significant antibacterial activity against MRSA strains with the zone of inhibition of 24 mm against *Staphylococcus aureus* (ATCC 29213), and antibacterial

activity against VRE strains with a zone of inhibition of 25 mm against *Enterococcus faecalis* (ATCC 29212). In addition, marine *Streptomyces* reported to have activity against different cancer cell lines like strain *Streptomyces parvus*. It was tested against human liver cancer cell line, mouse lymphoma cell line, breast cancer cell line and human colon cancer cell showed interesting inhibition activities against all of them (Abd-Elnaby et al. 2015).

4.4 Detection of Secondary metabolites

Based on the results of TLC most of the compounds produced by the isolates obtained from plants and sponges used in this study were relatively more polar compounds. There were spots detected on TLC plates after eluted in the more polar solvent system Butanol: acetic acid: water that came out at the end of the separation process.

The spots produced were light blue in color except for the last one that was strong yellow in color. Also, in the second solvent system that been used in this study (Chloroform: Methanol), there were more polar spots observed on the TLC plate. Even though, the time limitation of this study did not allow identifying the compounds produced, it is possible that new compounds were produced by the strains used in this study.

Chapter Five

Conclusions and Future Direction

5.1 Conclusions

Thirty-three strains of endophytic Actinobacteria already isolated from Australian plants and sponges, were used in this study. All of them grew very well on ISP2 medium agar for 21 days and were extracted with MeOH. The extracts were tested for antibacterial activity against three bacterial cultures *E. coli, S. aureus and M. luteus*. Most of the extracts showed weak to moderate activity against at least one of the test microorganisms. Strain ET11 shows strong activity against *S. aureus* and *M. luteus* while having minor activity against *E. coli*.

F33 medium was the most effective fermentation medium for the cultivation of the endophytic Actinobacteria used in this study. As the strains show good activity against the microorganisms used, while the other media used were not a good medium for the Actinobacteria to produce any antibacterial activity against the test bacterial cultures.

Strain ET11 was identified to belong to *Streptomyces* spp. based on the 16S rRNA gene sequencing. This result corresponds to other studies on the isolations of endophytic Actinobacteria and suggests that *Streptomyces* is the most widely distributed in marine and terrestrial environments.

5.2 Future Directions

Various identification and characterization techniques need to be done to get better information of endophytic Actinobacteria inhabitation of plants and sponges as many genera can be identified and characterized with appropriate techniques.

Realisation of an isolates full potential for producing secondary metabolites, fermentative media design for SM screening needs to be well defined for each individual isolate. It was shown that certain constituents in the liquid media such as dextrin and glycerol favour antimicrobial activity, whereas liquid media supplemented with various refined oils enhanced antimicrobial activity. Various fermentation media need to be used (solid-state fermentation media and submerged fermentation media) to induce the secondary metabolites compounds from the isolate.

Not all the Actinobacteria has the same activity against bacteria or fungi or even cancer cells. In this study, we focused on the antibacterial property of the isolates against three bacterial cultures *S. aureus, M. luteus and E. coli* and we have not tested against fungi or even cancer cells. So, it needs to be tested against several bacterial cultures, fungi and cancer cells because it may have antifungal or anticancer activity against specific type of them.

(Suneetha & Khan 2010)

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