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Biological and enzymatic activity of actinobacteria associated with *Aloe ferox*

Nasreen Isaacs

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Supervisor: Dr Bronwyn Kirby-McCullough

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University of the Western Cape

Private Bag X17, Bellville 7535, South Africa

Telephone: ++27-21- 959 2255/959 2762 Fax: ++27-21- 959 1268/2266

Email: bfielding@uwc.ac.za

FACULTY OF NATURAL SCIENCE

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List of abbreviations

BLAST	Basis Local Alignment Search Tool
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide tri-phosphate
EDTA	Ethylendiaminetetraacetic acid
x g	Gravitational force
ISP	International *Streptomyces* Project
Kbp	Kilobase pair
LB	Luria Broth
OD	Optical Density
PCR	Polymerase Chain Reaction
PGP	Plant growth promotion
rRNA	Ribosomal ribonucleic acid
RPM	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
TAE	Tris-acetate Buffer
TE	Tris-EDTA buffer
V	Volts

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Abstract

Antimicrobial resistance is a global crisis that has been on the rise for decades, threatening the health and safety of many nations. This highlights the need to discover novel antimicrobial compounds to combat already resistant pathogens, as well as newly emerging pathogens. Historically soil bacteria, particularly actinobacteria, have been a source of novel bioactive compound, however, the constant re-isolation of known actinobacterial strains led to the need to explore unique environments. Higher plants, especially medicinal plants, represent a unique niche for the isolation of rare, bioactive actinobacterial species.

In this study, bioactive actinobacteria were isolated from *Aloe ferox*, a medicinal plant indigenous to South Africa. A large number (822) bacteria were obtained from soil samples, while 57 were obtained from leaf samples. Isolates were selected for antimicrobial screening, based on phenotypical characteristics, and ten isolates were selected for further testing based on the results of antimicrobial screening. The isolates were shown to have significant activity against at least one of the tested pathogens which included *Acinetobacter baumannii*, *Candida albicans*, *Enterococcus faecalis* Van A, *Escherichia coli*, *Mycobacterium aurum*, and *Staphylococcus aureus*. They were also revealed to produce significant siderophore activity, as well as amylases. A novel *Amycolatopsis* strain was identified and the name *Amycolatopsis aloensis*^{T} was proposed. This study highlights that plant actinobacteria are a source of antimicrobial compounds and other secondary metabolites, and systematic screening of medicinal indigenous species is warranted.

Keywords: Antibiotic resistance, actinobacteria, *Aloe ferox,* bioactivity.

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Chapter 1: Literature Review

1.1 Introduction

Antimicrobial resistance is a rapidly emerging global crisis that threatens the health and safety of many nations (Fong *et al*., 2018). While the misuse of antibiotics significantly contributes to resistance in bacteria, resistance has been observed for decades in bacteria isolated from remote locations unlikely to have been impacted by humans. As such, while antibiotic misuse is a major driver of antibiotic resistance development, it can also be a result of spontaneous mutations and horizontal transfer of antibiotic resistant genes from antibiotic producing strains (Alvan *et al*., 2011; Sengupta *et al*., 2013).

The source of the antimicrobial resistance determinants threatening us today is due to sporadic and reactionary infection control practices, irresponsible use and administration of antibiotics, and the blatant disregard of the warnings against the overuse of antibiotics. This leads to pathogens becoming increasingly resistant to the available antibiotics, leaving individuals vulnerable to untreatable infections. This highlights the dire need to discover novel antimicrobial compounds to combat already resistant pathogens, as well as newly emerging pathogens, and to aid in the reduction of the mortality rate associated with antimicrobial resistance (Gould and Bal, 2013; Wright, 2014).

Historically, the most successful source of novel bioactive compounds are natural products, such as secondary metabolites, produced by microorganisms (Gould and Bal, 2013; Spellberg and Gilbert, 2014). Actinobacteria are of particular interest when it comes to the isolation of bioactive compounds as they are known to be prolific producers of secondary metabolites. They produce a number of beneficial enzymes, as well as potent antibiotics, the majority of which have been isolated from *Streptomyces* species (Arifuzzaman *et al.*, 2010; Duraipandiyan *et al.*, 2010). Streptomycetes are the most abundant actinobacterial species isolated from soil, which results in the frequent re-isolation of the same *Streptomyces* strains. This unfortunately limits the probability of finding novel antimicrobial compounds. Despite this, bioprospecting studies done by Trabelsi *et al.* (2016), Nithya *et al.* (2015), Ceylan *et al.* (2008), Taddei *et al.* (2006), and Oskay *et al.* (2004) highlight the importance of continuing to look at soil for the isolation of potentially novel and bioactive actinobacteria.

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While soil isolates are still promising, researchers need to explore new environmental sources in the hopes of isolating rare species of actinobacteria which have been shown to produce antibiotics including *Actinomadura, Actinoplanes, Amycolatopsis, Dactylosporangium, Kibdelosporangium, Microbispora, Micromonospora, Planomonospora, Planobispora,* and *Streptosporangiu* (Lazzarini *et al.*, 2000). Alternative sources of isolation that have shown to be promising include aquatic environments, as well as plant associated environments.

Higher plants, especially medicinal plants, represent a unique niche for the isolation of rare, bioactive actinobacterial species. This potential has been explored in research performed by Verma *et al.* (2009), Qin *et al.* (2009), and Zhao *et al.* (2011). South Africa has a rich diversity of plants with ethnobotanical history (which can be defined as the knowledge and use of indigenous plants by the local population). These plants provide several unique and potentially significant sources for isolating important epi- and endophytic actinobacteria (Abdalla and Mcgaw, 2018). One such source includes the various *Aloe* species which have been used by countless people in traditional medicine. *Aloe ferox* is of particular interest in this study as it is the main *Aloe* species used in traditional medicine in the Western Cape.

Although there are a number of promising unexplored environments which could be screened for actinobacteria, this review will focus on terrestrial habitats, specifically those associated with medicinal plants. The literature discussed in this review serves to indicate that the potential of actinobacteria, particularly those colonising indigenous South African medicinal plants, has not been fully explored. The review will touch on the antibiotic crises and the diversity of indigenous plants within South Africa, before directing attention to *Aloe ferox.* Some unique habitats of actinobacteria will be referenced but emphasis will be on the terrestrial and plant associated habitats. The potential of actinobacteria will be explored focusing on their ability to produce important antimicrobial and enzymatic compounds.

1.2 Antibiotic crises

One of the major advances in modern medicine has been the discovery of antibiotics. Antibiotics have contributed to increasing the average life span by limiting the effects of bacterial infections, as well as aiding in the success of advanced medical practices (World Health Organization, 2014). The discovery of penicillin by Alexander Fleming in 1928, along with several reports of microorganism producing antimicrobial compounds,

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inspired Selman Waksman to study microbes as producers of antimicrobial compounds. His research was instrumental in the discovery of soil-dwelling actinomycetes which were prolific producers of antimicrobial compounds (Hutchings *et al*., 2019). This essentially instigated the 'Golden Age' of antibiotic discovery that occurred between the 1940s and 1960s (Katz and Baltz, 2016). Since this period, very few novel antimicrobial compounds have been discovered. This, coupled with the gross misuse of antibiotics in both humans and animals, has led to the development of antimicrobial resistance in bacteria (Bbosa *et al.*, 2014).

There is a steady increase in the amount bacteria becoming resistant to traditional antibiotics in clinical, as well as non-clinical settings. Initially, the most concerning cases were the Gram-positive bacteria, specifically vancomycin resistant *Enterococcus* and methicillin resistant *Staphylococcus* species. The focus has since shifted to include Gram-negative bacteria as the increase in resistance is faster in these bacteria while the development of antibiotics against them is much slower (Kumarasamy *et al.*, 2010). Antimicrobial resistance is seen as a key factor in the recovery of patients in the intensive care unit (ICU), where extremely ill and potentially highly contagious patients are confined and administered antibiotics. This results in antibiotics becoming less effective for specific pathogens and eventually resulting in antimicrobial resistance (Kollef and Fraser, 2001). This reiterates the need for novel antimicrobial compounds to effectively treat multi-drug resistant pathogens and combat rapidly emerging infections (Gohain *et al.*, 2019). A source of interest is medicinal plants as research based on ethnobotanical knowledge has revealed the pharmacological properties of these plants. Additionally, it has been shown that medicinal plants are a unique source for the isolation of rare, bioactive actinobacteria (Abdalla and Mcgaw, 2018).

1.3 Indigenous plants in South Africa

Internationally, South Africa is known as a botanical diversity hotspot, being home to roughly 31 400 species of plants (Reinten and van Wyk, 2018). It boasts a rich diversity of medicinal plants with 24 000 taxa being reported in the region (Germishuizen and Meyer, 2003; Abdalla and Mcgaw, 2018), and 3 000 species being used medicinally with an estimated 350 commonly used in traditional herbal medicine (van Wyk *et al*., 2009). One of the most unique floral regions in South Africa, with the greatest concentration of higher plant species in the

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world, is the Cape Floral Region. This region alone is home to 8 600 plant species of which 68% are endemic to the region (Kuete, 2013). Despite the rich floral diversity, as well as the well- documented ethnobotanical literature, very limited scientific information is available of the medicinal uses of these plants. In the past few decades however, there has been an increase in research concerning the biological activity, as well as the chemistry of plants used in traditional healing (van Wyk and van Staden, 2002; van Vuuren, 2008).

A study by van Vuuren and colleagues (2006) investigated the antimalarial, antimicrobial, and the toxicity profiles of the essential oil, crude extract, and the helihumulone isolated from the extracts of *Helichrysum cymosum* (licorice plant). They found that crude extract had a higher antimicrobial value compared to the essential oil. In contrast, the essential oil had the highest antimalarial activity compared to the extract, highlighting the importance of testing both sources for bioactivity. Mathabe and associates (2008) isolated four known terpenoid compounds from *Spirostachys Africana* (tamboti) and investigated the antibacterial activity of these compounds against diarrhoeal causative microorganisms. The results suggested that the compounds may be responsible for the medicinal properties of *S. Africana.* The above-mentioned plants have both been used in traditional medicine with the licorice plant being used to treat various topical infections as well as respiratory infections, while tomboti is used to treat diarrhoea and dysentery (van Vuuren *et al.*, 2006; Mathabe *et al.*, 2008).

The use of herbal medicines has become increasingly popular, both in conjunction with western medicine, as well as being used as the primary treatment for many ailments. The World Health Organization (WHO) estimates that 60% of the world's population makes use of herbal medicines, while 80% of the population in developing countries depends entirely on herbal medicine. They have also determined the annual growth rate of the trade in medicinal plants, herbal raw material, and herbal drugs to be around 15% (Ahmad Khan and Ahmad, 2018).

In 2000, the United States spent $17 billion on traditional herbal medicines. The WHO estimated that the annual global market for herbal medicine was worth $60 billion in 2003, while the global industry in traditional Chinese medicine alone was reportedly worth $83 billion in 2012 (Allkin, 2017). As of 2020, the value of botanical and plant-derived drugs is estimated to grow from $29.4 billion in 2017 to $39.6 billion by 2022. The WHO anticipates the global market value of herbal products will be worth $5 trillion by the year 2050

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(Carvalho, 2020). The estimated number of indigenous medicine consumers within South Africa, along with the supporting industry, is roughly 27 million individuals with an increased number of herbal gatherers and traders noted (Chen *et al.*, 2012). The statistics stated serves as proof that the use of indigenous medicinal plants is no longer limited to traditional healers but has been introduced into the formal entrepreneurial sectors of not just South Africa, but the international economy (Dold and Cocks, 2002).

In addition to being used in traditional herbal medicine, medicinal plants are also used in commercial goods and cosmetics. The addition of plant extracts such as mint, aniseed, clove, olive leaf, and neem leaf extract in toothpaste enhances its activity by providing additional antimicrobial activity. The addition of aloe ferox extract in toothpaste, in combination with other herbal extracts, could be used to treat inflammation and ulcers (Bodiba *et al.*, 2018). Medicinal plants are also often consumed in herbal teas such as rooibos and honeybush (McKay and Blumberg, 2007).

The African continent is home to an abundance of indigenous, medicinally important plants although some species have been found in other continents, such as Europe, including species from the genus *Helichrysum* (within the sunflower family, *Asteraceae*). The genus consists of roughly 500 species that are used for the treatment of various medical conditions, with 245 being indigenous to South Africa (Lourens *et al.*, 2004). Other plant species indigenous to South Africa include *Hypoxis hemerocallidea* ("African potato"), *Harpagophytum procumbens* (devil's claw), *Cyclopia genistoides* (honeybush), *Aspalathus linearis* (rooibos), *Sutherlandia frutescens* or *Lessertia frutescens* (cancer bush), and, the focus of this study, *Aloe ferox* (bitter aloe) (Van Wyk, 2011).

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1.3.1 Aloe

The *Aloe* genus forms part of the *Asphodelaceae* family. The genus is broadly dispersed in Eastern European as well as the African continent, with species spread almost throughout the world. The genus consists of succulent plants adapted to severe climates and the ability to survive in diverse habitats including beaches, mountains, deserts, and grasslands (Ndhlala *et al.*, 2009; O'Brien, *et al.*, 2011; Chen *et al.*, 2012). While *Aloe vera* and *Aloe ferox* are the most researched species, there have been phytochemical studies that included other members of the genus. The various species are noteworthy sources of bioactive compounds in a number

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of classes including alkaloids, chromones, flavonoids, glycoproteins, and pyrones (Dagne *et al*., 2005; Loots *et al*., 2007). A few aloe species produce highly toxic compounds. For example, *Aloe ruspoliana Baker,* a species found in northern Kenya, has been used to poison stray dogs and hyenas. Some toxic species from Tanzania, such as *Aloe elata* and *Aloe ballyi,* are used in arrow poisons in Kenya (Amir *et al.*, 2019).

Evidence has been found throughout history of *Aloe* being used as a traditional remedy to treat a multitude of ailments by various cultures. This included Arabians, Moroccans, Tunisians, Greeks, Romans, Chinese, Hebrews, Indians, Algerians and indigenous communities in Africa (Barcroft and Myskja, 2003). Aloe has been used as an immune stimulant, an antiseptic, as a treatment for ailments such as insect bites, gastrointestinal disorders, skin burns and other skin wounds. It has also been reported to have laxative, antitumor, anti-ulcer, antidiabetic, and anti-inflammatory capabilities (ElSohly *et al*., 2004; Loots *et al*., 2007). Aloe extract have become increasingly prevalent in a multitude of commercially available products in the form of aloe gels, creams, powders, capsules, as well as aloe drinks. "Aloe 4 U", for example, is a South African product consisting of different aloe species combined to reportedly give a stronger effect to the healing properties of aloe (ElSohly *et al*., 2004).

Some of the *Aloe* species that have been reported to have therapeutic properties include *Aloe arborescens, Aloe perryi baker, Aloe vera,* and *Aloe ferox* (Jia *et al*., 2008). Most of the scientific research published on the medicinal properties of *Aloe* species focuses on *A. vera.* However, the *Aloe* species used in traditional medicines is highly dependent on the availability of the plant within that community. In South Africa the species most commonly used in the Eastern and Western Cape provinces is *A. ferox* (Loots *et al*., 2007).

1.3.2 Aloe ferox

Aloe ferox, also known as Cape Aloe, is widely distributed throughout the eastern and western parts of South Africa. It is a succulent plant consisting of single-stemmed erect orange to scarlet flowers surrounded by broad, thick fleshy leaves arranged in a compact rosette around the stout stem. The leaves have a thorn rim and appear reddish- green in winter, while they are a dull green in summer. *A. ferox* is known for its bitter taste and historical use in traditional medicine (Chen *et al.*, 2012).

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Figure 1.1: An *Aloe ferox* plant photographed on the side of the road. (Photo by Thabo Maphisa https://www.operationwildflower.org.za/index.php/albums/aloes/aloe-ferox-thabo-2-2198).

A. ferox is one of the few plants that have been illustrated in San rock paintings, reiterating the fact that it has been used or at least appreciated for centuries. It produces a bitter sap that can be tapped from the leaves. The sap has traditionally been used as a purgative and is said to have anti-inflammatory, anti-arthritic and bitter tonic properties (van Wyk, 2008). The sap is collected and dried to produce a dark brown solid substance known as bitter aloes or Cape Aloe lumps. While a portion of the annual production is sold and used locally, a larger portion is exported to various countries in Europe (van Wyk *et al*., 1995).

The leaf gel of *A. ferox* contains polysaccharides as well as various phenolic compounds. The gel has been used as an oral treatment for malaria, cancer, infections, and inflammation, as well as a topical treatment for various skin ailments including eczema. The leaf extracts contain enzymes that have been proven to relieve pain and decrease inflammation (Chen *et al.*, 2012; Cock, 2015). Jia and colleagues (2008) studied the effects of *A. ferox* whole-leaf juice on wound healing using rat models. The results indicated that the *A. ferox* whole-leaf juice increased the rate of wound healing with shorter periods for epithelialization to occur.

1.4 Actinobacteria

Actinobacteria were once considered to be the intermediate form between fungi and bacteria but these comparisons, specifically to fungi, were merely superficial. Actinobacteria now form one of the largest phyla within the Bacteria domain (Ait Barka *et al.*, 2015). They are filamentous, aerobic organisms with a high guanine and cytosine (G+C) content in their genomic DNA. Actinobacteria can be found in a myriad of habitats including terrestrial and

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aquatic environments (Trabelsi *et al.*, 2016). They are known to have very diverse morphologies which are still used in phenotypic characterisation of species. They are also known to produce a wide variety of secondary metabolites and enzymes making them beneficial in many fields including the pharmaceutical as well as the paper and textile industry (Macagnan *et al.*, 2006; Ventura *et al.*, 2007; Duraipandiyan *et al.*, 2010).

1.4.1 Habitats of Actinobacteria

Actinobacteria are acknowledged as one of the most prevalent microbial communities in soil (Basilio *et al.*, 2003; Solans *et al.*, 2016). While terrestrial environments are the main habitats for actinobacteria, they have been increasingly isolated from aquatic environments. In 1981, a study done by Cross demonstrated the prospect of isolating actinobacteria from freshwater locations, with the main species present being *Streptomyces, Micromonospora, Thermoactinomyces,* and *Actinoplanes* (Cross, 1981). However, there is still debate as to whether the actinobacteria isolated from this environment are indigenous or are rather due to wash in from neighbouring terrestrial habitats (Baskaran and Mohan, 2011).

Along with these habitats, actinobacteria have also been isolated from plants and gastrointestinal tracts of animals where they are either pathogens or commensals. Macagnan and associates (2006), isolated 336 endospore-forming bacteria, including actinobacteria, from the surface of cacao pods in order to test for activity against *Phytophthora palmivora* and *Crinipellis perniciosa.* They found that five of the actinobacterial isolates, identified as *Streptomyces* species had promising activity against *C. perniciosa*, the causative agent of witches' broom disease, under controlled conditions. Unfortunately, no activity was found against *P. palmivora,* which causes black pod disease in cacao.

Wang *et al.* (2018) and Huang *et al*. (2019) took to a more unique source for the isolation of actinobacteria. Wang and co-workers took an interest in the Tibetan antelope and its ability to adapt to the unique environment of the Qinghai-Tibetan Plateau. This led them to analyse the faeces of the antelope which resulted in the isolation of a novel strain, *Nocardioides houyundeii.* Huang and colleagues studied the faeces of *Equus kiang,* known as Tibetan wild ass, from the same region. They were also able to isolate a novel strain, *Nocardioides yefusunii*. While no activity tests have been done or published as of yet, chemotaxonomy results are available.

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1.4.2 Terrestrial environment

Terrestrial environments, such as soil, are one of the most important and highly populated habitats for actinobacteria. The actinobacteria isolated from these regions are known to play significant roles in soil ecology and are able to produce an assortment of interesting antimicrobial compounds. *Streptomyces* species, which comprise 50% of the population, are important in soil ecology due to their role in the mobilization of nutrients, nitrogen fixation, phosphorus solubilisation and the degradation of polymers such as chitin. They are also known to be a major source of many of the antibiotics currently available (Oskay *et al*., 2004; Thakur *et al.,* 2007; Stevenson and Hallsworth, 2014)

Despite the fact that *Streptomyces s*pecies are the most frequently isolated actinobacterial taxa in terrestrial environments, it is still worth looking at soil for novel actinobacteria. Biswas and colleagues isolated a novel streptomycete which they named *Streptomyces euryhalinus* (Biswas *et al.*, 2017). Trabelsi and associates (2016) isolated 54 actinomycetes that produced bioactive compounds from four types of rhizosperic soil in Tunisia. The isolates included *Actinomadura, Micromonospora, Nocardia, Pseudonocardia,* and *Streptomyces* species. A novel strain, named *Nocardioides solisilvae,* was isolated from forest soil in India by Sultanpuram, Mothe and Mohammed (2015).

Nithya *et al.* (2015) isolated actinobacteria from ten desert soil samples, with each sample varying in the abundance and diversity of actinobacteria present. The isolates were screened for antimicrobial activity, and it was found that they exhibited varying degrees of activity against bacterial and yeast-like pathogens. Schneider and colleagues (2007) isolated a *Nocardia* species from mangrove soil in Malaysia. The isolate was shown to produced two new cytotoxic metabolites which inhibits human cancer cell lines, including breast carcinoma and gastric adenocarcinoma.

Cheah and associates (2015) explored volcanic soil in Antarctica, a more extreme environment with a temperature of 94 °C, for actinobacteria. They isolated 24 actinobacteria which were characterized and tested for antimicrobial activity. Bioactive isolates were identified as *Terrabacter lapilli, Leifsonia soli,* and *Gordonia terrae,* and these strains were found to have activity against *Salmonella typhimurium* and *Salmonella paratyphi* A. Lee and associates (2012) isolated 39 strains of actinobacteria from four locations in the Barrientos Island. Fifteen of the isolates exhibited activity against pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA)*, Candida albicans,* and *Pseudomonas*

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aeruginosa. The isolates were found to represent a variety of genera including *Arthrobacter, Brevibacterium, Dermacoccus, Demetria, Gordonia, Janibacter, Kocuria, Lapillicoccus, Leifsonia, Microbacterium, Micromonospora, Nocardioides*, and *Rhodococcus.*

1.4.3 Plant associations

In recent years, the epiphytic and endophytic environments of higher plants have been identified as an emerging source of biologically active organisms, with endophytic actinobacteria being referred to as "chemical factories" within plants (Kaewkla and Franco, 2013; Tanvir *et al.*, 2016). Endophytes produce secondary metabolites that can directly and indirectly aid in plant growth promotion, as well as be exploited in a number of industries (Golinska *et al.*, 2015). The strains isolated are dependent on several factors including geographical location and the host plants. Since endophytic environments remain largely unexplored, and they represent significant sources for the isolation of rare endophytic actinobacteria from plants that are endemic to a region (Golinska *et al.*, 2015; Tanvir *et al.*, 2016).

Numerous studies have reported the isolation of actinobacteria from medicinal plants. Zhao and colleagues (2011) isolated *Micromonospora, Nonomuraea, Oerskovia, Promicromonospora,* and *Rhodococcus* species from medicinal plants growing in Panxi plateau, China. Sheng Qin *et al.*, (2009) isolated a variety of rare actinobacteria including *Actinocorallia, Blastococcus, Dactylosporangium, Dietzia, Jiangella, Oerskovia Promicromonospora,* and *Saccharopolyspora* species from a rain forest in China. Some of the more common endophytic actinobacteria that have been isolated are represented in Table 1.1.

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Table 1.1: A list of the most abundant actinobacteria found in various plant species.

Plant species	Actinomycetes taxa	Reference
***Alpinia galanga* (Galangal)**	*Streptomyces, Nocardia, Microbispora, Micromonospora*	Taechowisan *et al*. (2008)
***Maytenus Austroyunnanensis* (Maytenus)**	*Saccharopolyspora, Actinomadura*	Qin *et al.* (2008, 2009)
***Azadirachta indica* (Neem)**	*Streptomyces, Streptosporangium, Microbispora, Streptoverticillium, Saccharomonospora, Nocardia*	Verma *et al.* (2009)
***Aquilaria crassna* (Agar wood)**	*Streptomyces, Nonomuraea, Actinomadura, Pseudonocardia, Nocardia*	Nimnoi, *et al.* (2010)
***Zea mays* (Maize)**	*Microbispora, Streptomyces, Streptosporangium*	de Araújo *et al.* (2000)
***Acacia auriculiformis* (Earpod wattle)**	*Actinoallomurus*	Thamchaipenet *et al.* (2010)

Abdalla and McGaw (2018) discussed the potential of South African plants and their traditional uses in terms of discovery of promising endophytic microbes and their secondary metabolites. Due to the fact that endophytic microbes and their host plants form intimate associations there may be an overlap in the metabolites produced by each of them. It is mentioned that the bioactive compounds produced by these microorganisms may be due to their own development, or it might be a result of their host's genetic material recombining with their own. This intergeneric genetic exchange could be the reason why symbiotic endophytes and their hosts are able to produce identical secondary metabolites.

This hypothesis is validated by studies such as one performed by Strobel and associates (1996). Taxol, an anticancer drug initially isolated from *Taxus brevifolis* (Pacific yew) (Wani *et al.*, 1971), has been isolated from other *Taxus* species in miniscule quantities. Strobel and co-workers studied the microbes associated with yew tree, specifically *T. wallichiana.* During their research, they isolated *Pestalotiopsis microspora*, an endophytic fungus that

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colonizes *T. wallichiana,* and found that it produces taxol that is identical to that produced by the host plant. The production of taxol by an endophytic fungus may account for the fact that its production is limited to only one *Taxus* species.

Numerous antibiotics produced by endophytic actinobacteria isolated from medicinal plants have revealed an extensive range of bioactivities against viruses, fungi, and bacteria. This showcases the promising and broad-spectrum antimicrobial potential of the secondary metabolites obtained from endophytic actinobacteria (Golinska *et al.*, 2015). The abundant ethno-medicinal culture in South Africa, as well as the large diversity of plant species, their environment, biology and bioactivities, may provide an unexplored source of potentially novel endophytes (Abdalla and Mcgaw, 2018).

1.5 Life cycle

The vast majority of the actinobacteria that have been isolated are soil dwelling saprophytes that are semi-dormant spores for most of their lifecycle. The number and varieties of actinobacteria found in soil are determined by the soil type, pH, temperature, aeration, cultivation, as well as organic matter and moisture content (Arifuzzaman *et al.*, 2010). Under favourable conditions, the spores germinate and grow by forming branching mycelia that propagate into the substrate. During unfavourable condition, however, the colonies adapt and form hydrophobic aerial mycelia which are able to separate and form resistant spores (Kieser *et al.*, 2000; Schrempf, 2008; Flärdh and Buttner, 2009).

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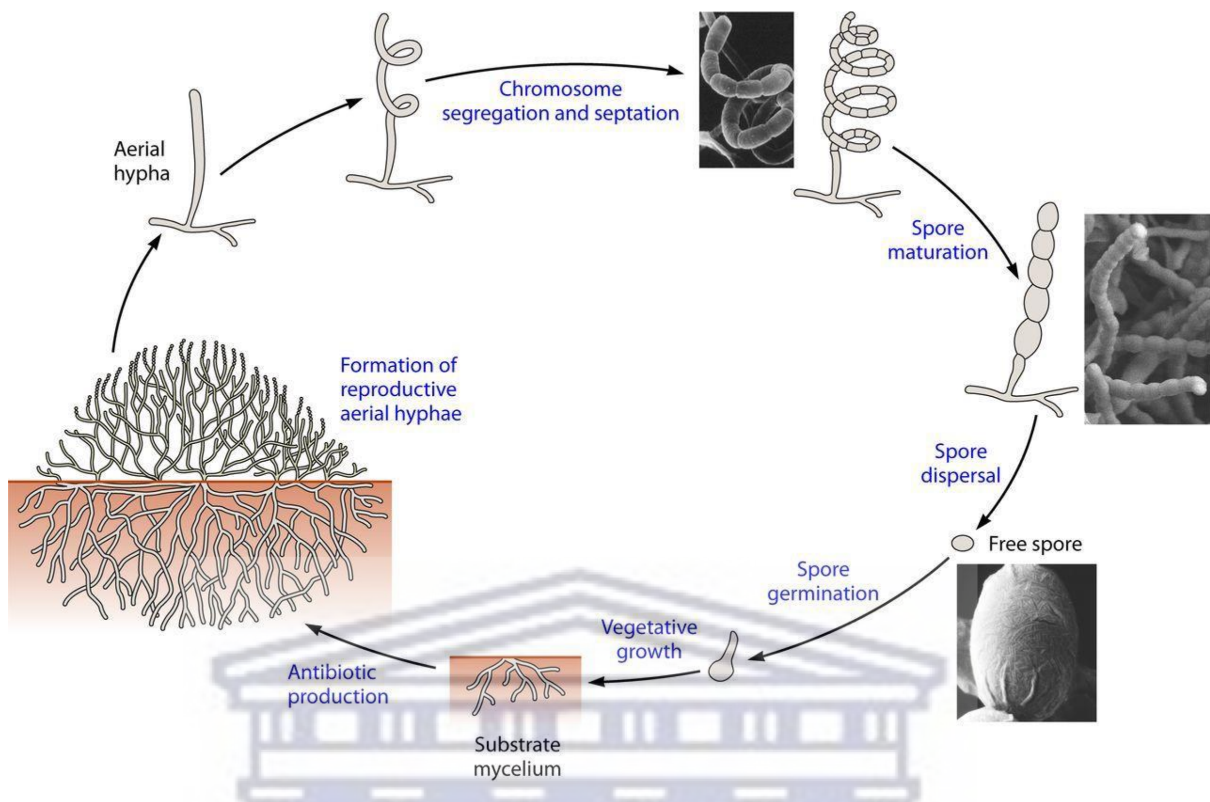

Figure 1.2: A schematic representation of the life cycle of sporulating actinobacteria (Ait Barka *et al.*, 2015).

1.6 Taxonomy

1.6.1 Morphological characteristics

Actinobacteria are morphologically diverse with their morphologies ranging from coccoid or rod-coccoid to fragmenting hyphal forms or highly differentiated branched mycelium (Ventura *et al.*, 2007). They often appear as compact, leathery, conical mounds with aerial mycelia when cultured. The cultural characteristics of actinobacteria play a vital role in the initial identification of the organisms. Shirling and Gottlied (1966) developed methods for observing and distinguishing between *Streptomyces* species. This included cultivating the isolates on various media stated in the International *Streptomyces* Project (ISP) such as yeast extract- malt extract agar, oatmeal agar, inorganic salts-starch agar, and glycerol-asparagine agar. As the cultures mature, they usually develop a significant amount of spore mass. This allows for the observation of spore morphology and colour, the presence or absence of any mycelium, and the production of diffusible pigment production (Shirling and Gottlieb, 1966).

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One of the most significant characteristics used to differentiate between species is the presence or absence of the aerial and substrate mycelium.

The aerial mycelium are hyphae that develop on the substrate mycelium and grow upwards. Generally, they are thicker and more fibrous than the substrate mycelia, giving the actinobacterial colony a velvety, fluffy, or powdery look (Li *et al.*, 2016). The diversity of the aerial mycelium is significant enough for it to be used to separate isolates into groups with similar morphologies under fixed conditions. The main function of the substrate mycelium is to absorb nutrients and therefore, they either grow on the surface or into the growth medium. Some substrate mycelia have hyphae which have the ability to produce either water- or fat-soluble pigments. These pigments are responsible for causing the medium to change colour and for the colony to inherit the colour of the hyphae, respectively. Common mycelia pigment colours include white, orange, red, yellow, pink, black, brown, and green (Ranjani *et al.*, 2016). In 1958, seven subdivisions of *Streptomyces* species were proposed based on the morphology of their mycelium. Those subdivisions are represented in Table 1.2.

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Table 1.2: The seven morphological sections proposed by Pridham *et al.* (1958).

Figure	Section	Description
	Rectus- Flexibilis (RF)	Straight, flexuous, or fascicled sporophores.
	Retinaculum-Apertum (RA)	Sporophores present as hooks, open loops, or primitive spirals.
	Spira (S)	Short, gnarled, or compact spirals or extended long and open spirals.
	Monoverticillus (MV)	Sporophores as primary verticils or whorls attached to long, straight branches; no spirals.
	Monoverticillus-Spira (MV-S).	Sporophores as primary verticils or whorls attached to long, straight branches; elements of verticils or whorls spiralled.
	Biverticillus (BIV)	Streptomycetes with sporophores as compound verticils or whorls attached to long, straight branches.
	Biverticillus-Spira (BIV-S).	Streptomycetes with sporophores as compound verticils or whorls attached to long, straight branches; elements of secondary verticils or whorls spiralled.

1.6.2 Physiological identification

As previously mentioned, actinobacteria are primarily saprophytic soil dwelling microorganisms that spend a large portion of their life cycle as spores. Sporulation plays a role in their survival in dry, nutrient deficient environments (Cao *et al*., 2005; Sathya *et al*., 2017). While sporulation plays a role in desiccation tolerance, some nonsporulating

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actinobacteria such as *Rhodococcus jostii* are also able to endure desiccation (LeBlanc *et al.*, 2008).

Generally, soil actinobacteria grow optimally in neutral or slightly alkaline environments with the optimal pH range being between 5.0 and 9.0. However, studies have been performed to confirm the existence of extremophilic actinobacteria. Acidophilic actinobacteria are able to grow in the 3.5 to 6.5 pH range with the optimum pH being 4.5 to 5.5. Mesophilic actinobacteria have an optimal growth temperature between 20°C to 42°C and among them are thermo-tolerant species, which are able to survive at 50°C. The moderately thermophilic strains grow optimally at 45-55 °C, while the strictly thermophilic have an optimum of 55-60 °C (Khan and Williams, 1975; Basilio *et al.*, 2003; Ait Barka *et al.*, 2015).

1.6.3 Nucleic acid analysis

Traditionally classification of actinobacteria was based on their morphological, physiological, and biochemical characteristics. However, these methods are inadequate, as they are time consuming, lack sensitivity and often are not reproducible. Phenotypic characterisation has been challenged by molecular taxonomic data, especially with the advent of genome sequencing which led to a rapid progression in the field. This has resulted in the reclassification of some species based on their molecular data. Therefore, polyphasic taxonomy is an identification approach ideal for actinobacteria as it takes all available phenotypic and genotypic data into account when classifying microorganisms (Cleenwerck and De Vos, 2008; Zhi *et al.*, 2009; Verma *et al.*, 2013; Singh *et al.*, 2016). In addition, the employment of molecular techniques is also beneficial for the identification of actinobacteria that have yet to be cultured (Stach *et al.*, 2003). Currently, in order to validly publish a new species description, sequencing of the 16S rRNA gene and DNA-DNA hybridization (in some incidences) is compulsory.

The 16S rRNA gene is important in determining relationships between organisms and is the most common housekeeping gene studied for bacterial phylogeny and taxonomy. This is due to the fact that it is large enough to ascertain informational data, it is a common gene in most bacteria, and the function of the gene remains constant. It is therefore safe to assume that changes in the sequence of the gene can be used to reliably determine evolutionary timelines (Wang *et al.,* 1996; Janda and Abbott, 2007). Based on genomic taxonomy classification of

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the Actinobacteria phylum, two orders, ten families, and 17 genera were proposed, as well as the genera reclassification of over 100 species (Nouioui *et al.*, 2018).

1.7 Rare actinobacteria

As mentioned previously, *Streptomyces* has been reported to be the most abundant genus isolated from soil due to conventional isolation techniques favouring their selection (Seong *et al.,* 2001). Actinobacteria, particularly spore-forming actinobacteria, represent the most biotechnologically and economically valuable prokaryotes, synthesizing more than half of the bioactive compounds present in the *Antibiotic Literature Database* (Lazzarini *et al.*, 2000). However, the estimated rediscovery rate of bioactive compounds produced by microbes currently in culture is 95% (Fenical *et al.*, 1999).

It has recently been demonstrated that the rare actinobacteria species, which are more challenging to isolate and cultivate, likely represent unique sources of novel biologically active compounds (Baltz, 2006). It has been hypothesised that to find these rare species it is important to explore new microbial habitats. One biologically important niche that has previously been overlooked is higher plants, specifically their inner tissue. Studies have shown that actinobacteria are capable of forming intimate associations with host plants and ultimately colonizing their inner tissue. *Streptomyces scabies* and *Frankia* species serve as examples of such plant symbionts as they can infiltrate their host and establish either endophytic or pathogenic associations (Benson and Silvester, 1993; Doumbou *et al.*, 1998).

Endophytes are beneficial to their host plants in many ways including being insect- and pest repellents, producing growth promoting metabolites, and providing antimicrobials against plant pathogens (Staniek *et al.*, 2008; Rai *et al.*, 2014). These endophytes also have immense potential to produce novel secondary metabolites, which can be exploited in many industries (Golinska *et al.*, 2015). It is suspected that a higher diversity of endophytes could be isolated from endemic plants unique to specific habitats (Kaewkla and Franco, 2013). Thus, unexplored habitats, especially rare niches, are beneficial to the discovery of novel bioactive compounds (Vollmar *et al.*, 2009). It is important to implement improved methodologies to isolate these rare actinobacteria in order to avoid repetitive isolation of known strains and compounds (Takahashi and Omura, 2003; Bérdy, 2005; Singh *et al.*, 2016).

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1.8 The role of actinobacteria in nature and industry

Actinobacteria are known to produce a variety of bioactive secondary metabolites important to a wide range of industries including the industrial, agricultural and medical fields. They are isolated from numerous natural sources, including rhizopheric soil and plant tissue with these environments greatly influencing their biological functions and secondary metabolite production (Ait Barka *et al.*, 2015; Wohlleben *et al.*, 2017).

1.8.1 Biological role in plant and soil

The majority of the actinobacteria isolated are saprophytic with only a few being pathogenic to plants. Pathogens in the *Streptomyces* and *Rhodococcus* genera have a wide range of hosts from model plants to commercial crops. *Streptomyces* strains such as *S. scabies, S. caviscabies, S. acidiscabies,* and *S. turgidiscabies* are known to cause scab diseases in potatoes characterized by corky tuber abrasions (Miyajima *et al.*, 1998; Hogenhout and Loria, 2008; Huguet-Tapia *et al.*, 2011). *Rhodococcus* strains such as *R. fascians* are epiphytes that are also able to form endophytic populations. They cause the formation of leafy galls which also inhibit the growth of host plants (Koen Goethals *et al.*, 2001).

Actinobacteria play a role in a variety of important functions such as the degradation of a multitude of organic compounds including cellulose, organic acids, and polysaccharide. They are responsible for the decomposition of humus in soil and for the earthy smell of freshly ploughed soils. Their ability to metabolize complex organic compounds makes them beneficial in their role to remove xenobiotic compounds from soil (Ranjani *et al.*, 2016; Alvarez *et al.*, 2017). Solans and colleagues isolated 32 saprophytic actinobacteria from rhizospheric soil of *Lotus tenuis* in Argentina. Eight of these isolates were identified to have biocontrol-related activities including the production of hydrogen cyanide, lytic extracellular enzymes, siderophores, and antifungal activity (Solans *et al.*, 2016). Actinobacteria are able to improve nutrient acquisition in plants, as well as stimulate hormone production, thus playing a role plant growth promotion (PGP) (Palaniyandi *et al.*, 2013).

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1.8.1.1 Plant growth promotion

Bacteria that play a role in plant growth promotion can do so by forming symbiotic relationships with or by colonising the inner tissue of plants (Glick, 2012). Actinobacteria can contribute to PGP using either direct or indirect methods, which can be seen in Figure 1.3. Some of the direct methods they might employ include phosphate solubilisation, nitrogen fixation, and the production of plant growth regulators and siderophores (Palaniyandi *et al.*, 2013). It has also been reported that they assuage stresses, both biotic and abiotic, in plants. Due to this fact, and their ability to produce potent secondary metabolites and enzymes, they are considered to be cost-efficient, eco-friendly, and efficient candidates for biosorption, which aids in environmental stress tolerance (Bankar and Nagaraja, 2018).

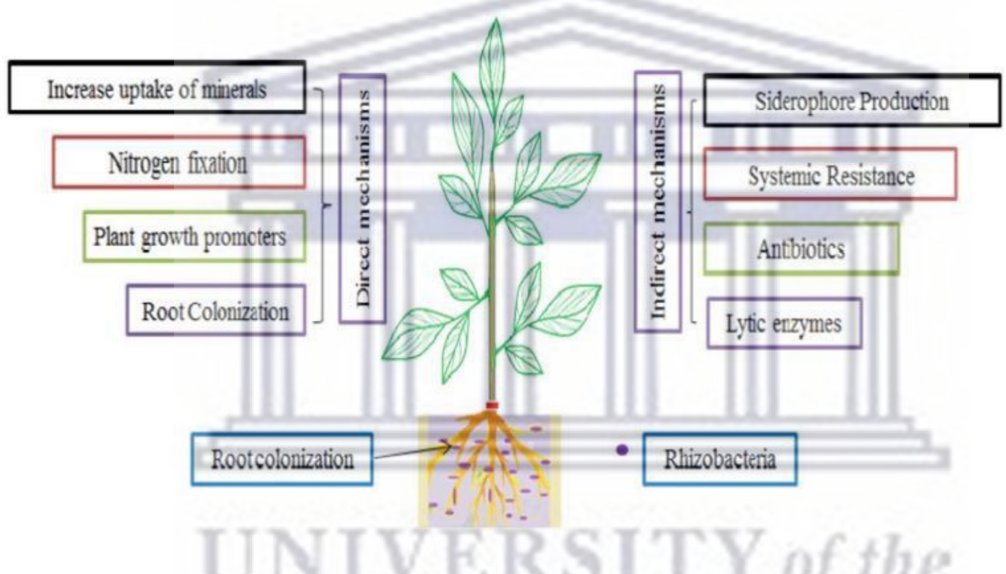

Figure 1.3: A schematic representation of the direct and indirect mechanisms for plant growth promotion. (Adapted from Ngoma *et al.* (2012)).

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Getha and co-workers (2005) characterized *Streptomyces* strain g10, which exhibited antifungal activity against *Fusarium oxysporum* f. sp. *cubense,* the causative agent of wilting, chlorosis and necrosis in leaves. They found that treating banana plantlets with the isolate significantly reduced rhizome discoloration, wilt severity, and disease severity in comparison to untreated plantlets. The strain employs a more indirect method of PGP namely antibiotic production which contributes to the reduction of disease symptoms by controlling the plant pathogens and their subsequent diseases (Arseneault and Filion, 2017). Other indirect methods include induced systemic resistance, competing with pathogens for nutrients, and the production of siderophores (Ngoma *et al.,* 2012).

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Actinobacteria are producers of siderophores which aid in the uptake of iron by binding to Fe^{3+} present in the environment. Siderophores form a family of at least 500 compounds based on their diverse chemical structure, with the most prevalent structural groups including catechols and hydroxamates (Wang *et al.*, 2014). Catechols include streptobactin, originally isolated from *Streptomyces* species (Matsuo *et al.*, 2011), while hydroxamates include desferrioxamine produced by *Streptomyces coelicolor* and *Amycolatopsis* species (Traxler *et al.*, 2013). Siderophoric compounds produced by some plant growth promoting actinobacteria work by depriving pathogenic fungi of iron, while others pull iron from heterologous siderophores formed by cohabiting microbes (Lodewyckx *et al.*, 2002).

1.8.2 Importance in industry and medicine

1.8.2.1 Antimicrobials

Pathogenic micro-organisms are becoming increasingly resistant to existing antibiotics (Arifuzzaman *et al.*, 2010), and new antibiotics are needed for the treatment of drug resistant infections. There is a global search for novel antimicrobial compounds that are potent, sustainable, and broad-spectrum from a number of sources including microbes (Bérdy, 2005; Hayakawa, 2008; Praveen *et al.*, 2008). Actinobacteria remain fruitful producers of antimicrobial agents with a large number of these compounds being discovered due to the screening of their natural habitats (Duraipandiyan *et al.*, 2010; Gallagher *et al.*, 2010).

A wide variety of actinobacteria are capable of producing secondary metabolites with biological activities such as antifungal, antibiotic, antiviral, anticancer, enzyme, and other important compounds (Arifuzzaman *et al.*, 2010; Baskaran *et al.*, 2011). Nearly 80% of the antibiotics available today have been isolated from actinobacteria, particularly from *Streptomyces* and *Micromonospora* species. The re-isolation of these species has led to a limited pool of antimicrobial compounds. Therefore, when screening for novel secondary metabolites, the focus has shifted to include actinobacterial species that are more difficult to isolate and culture, in addition to those inhabiting more extreme environments (Blackall *et al.*, 1989; Edwards, 1993; Williams *et al.*, 1993; Lazzarini *et al.*, 2000)

In the last five decades, of the estimated 12 000 antibiotics discovered 70% were produced by actinobacteria, with the remaining 30% being produced by non-actinobacteria and filamentous fungi. There are a number of structural classes of antibiotics derived from

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actinobacteria including ansamycins (e.g., rifampin), β-lactams (cephalosporins), anthracyclines (e.g., doxorubicin), aminoglycosides (e.g., streptomycin and kanamycin), macrolides (e.g., erythromycin), and tetracyclines (Laskaris *et al.*, 2010). Table 1.3 shows some additional classes as well as representative drugs and the species that produce them.

Table 1.3: Classes of antibiotics produced by Actinobacteria with representative drugs. (Adapted from Hutchings *et al.* (2019)).

Class	Example	Producing Actinobacteria
Aminoglycosides	Kanamycin A	*Streptomyces kanamyceticus*
Ansamycins	Rifamycin SV	*Amycolatopsis rifamycinica*
Cycloserines	Seromycin	*Streptomyces orchidaceus*
Glycopeptides	Vancomycin	*Amycolatopsis orientalis*
Lipopeptides	Daptomycin	*Streptomyces roseosporus*
Macrolides	Erythromycin	*Saccharopolyspora erythraea*
Tetracyclines	Tetracycline	*Streptomyces aureofaciens*
Tuberactinomycins	Viomycin	*Streptomyces puniceus*

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1.8.2.2 Enzymes

Actinobacteria from both terrestrial and marine environments produce multiple biologically active enzymes that are beneficial in a variety of industries. These enzymes include cellulases, pectinases, proteases, xylanases, and amylases, which aid in extracellular digestion. Amylases, in particular, degrade starch and are thus of significance in industries such as the paper and textile industry, food industry, and fermentation (Gupta *et al.*, 2003). Actinobacteria also produce celluloses and lipases which can be used in the detergent industries, diagnostic settings, in addition to food and pharmaceutical industries (Schmid and Verger, 1998). A number of actinobacteria, particularly those isolated from soil, have been shown to be significant sources of L-asparaginase which has been used in the food industry, as well as a pharmaceutical for the treatment of cancer and tumours (Izadpanah *et al.*, 2018).

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1.9 Rationale for the research project

With the increased emergence of antibiotic resistant pathogens, there is an urgent need to find novel antibiotics. While actinobacteria have been exploited for their production of secondary metabolites for decades, they are likely to remain a source of novel antimicrobial compounds. Based on available research it is clear that while soil has been extensively exploited, its potential as a source of novel actinobacterial species should not be overlooked. However, due to the constant re-isolation of known *Streptomyces* species, and subsequent limited compound pool, it is imperative that we find novel sources for the isolation of rare actinobacteria.

Looking at medicinal plants as a source of novel organisms could be of great importance. This increases the possibility of isolating rare actinobacterial species due to their unique environments. These organisms in turn have the potential to produce unique bioactive compounds which could be exploited in numerous industries including the pharmaceutical industry. The research currently available on actinobacteria isolated from medicinal plants supports the premise that they hold great potential, and it would be irresponsible to not explore these environments.

1.10 Research aims and objectives

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The overarching aim of this study is to determine the biological and enzymatic activity of actinobacteria associated with the indigenous medicinal plant *Aloe ferox*. This will be accomplished by employing isolation techniques to encourage the isolation of novel species. A variety of screening techniques will be used to detect biological activity.

The main research objectives of this study include:

- Isolation and characterisation of actinobacteria isolated from *A. ferox.*
- Bioactivity screening for antimicrobial as well as enzymatic activity of isolates.
- Metagenomic analysis of soil and leaf samples.

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Chapter 2: Methodology

Unless stated otherwise, all media used in this study was supplied by Sigma Aldrich, with analytical grade solutions supplied by Labchem.

2.1 Sampling

Plant and soil samples were collected in Albertinia, South Africa (34.2118° S, 21.5749° E) from a plantation owned by Organic Aloe in April 2016. All the plants located within the sampling site had originally grown on various farms throughout Albertinia and had been moved as established plants. Two sample sites were located at the top of a hill, and two sites were located at the base of a hill and were referred to as site T and B, respectively. Site T included plants that were originally grown in more mountainous regions, while the site B had plants grown on a farm near the town. An additional site, referred to as W, was located on the side of the road and used to sample "wild" plants which were planted by the original Aloe farmers in the area making them significantly older (>50 years) than the other plants sampled. Two different plants were sampled at each point. Leaf samples were collected by using a pocketknife, while soil samples were collected using a graduated spade which was sterilised using 70% ethanol in between each sample collection. A total of six leaf and six soil samples were placed in their respective sterile plastic bags and stored at 4°C until processing.

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2.2 Isolations, cultivation, and storage

The isolation of actinobacteria was done using a variety of isolation media listed in Table 2.1. All media was supplemented with cycloheximide (50 µg/ml) dissolved in 100 % methanol. Naladixic acid sodium salt (50 µg/ml) was added to the HV and CHV media.

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Table 2.1: Various isolation media used and their respective supplements/additives.

Media	Supplements	Reference
Actinomycete isolation agar (AI)	Glucose or glycerol (Glu/Gly)	(Atlas, 2010)
Humic Acid Vitamin (HV)		(Zhang, 2011)
Complex HV (CHV)	Soil extract	(Zhang, 2011)
Starch-casein-nitrate (SCN)		(Atlas, 2010)
Yeast extract - extract malt (YEME)	Plant extract (15 g/L)	(Shirling and Gottlieb, 1966)
Phytagel	Plant extract (15 g/L)	

Plant extract was prepared by blending the aloe leaves to form a liquid pulp which was then autoclaved before being added to sterile media. Soil extract was prepared by weighing out 100 g of soil, avoiding stones where possible, and adding it to 1 L of distilled water before autoclaving. Once cooled (while working in a laminar flow) the soil was allowed to settle, and the water was transferred to a sterile 1 L Schott bottle ensuring that the soil was not disturbed. This was then used to prepare the appropriate media.

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Various isolation techniques were used to isolate epiphytes, endophytes, and rhizospheric organisms. In order to isolate the epiphytes, sterile swabs were moistened in sterile distilled water and used to swab the surface of the leaves before surface sterilization. The swabs were streaked onto the various isolation media. Endophytes were isolated after surface sterilization. This was done by first thoroughly washing the leaves under running tap water followed by distilled water containing a few drops (30 – 50 µl) of Tween20. The leaves were left to air-dry on the bench, cut into two or three pieces depending on their size, and put into 2 L beakers. These beakers were moved to the laminar flow to complete the final steps of sterilization. The leaves were sequentially immersed in 70 % ethanol for 3 minutes followed by a 3 % sodium hypochlorite solution for 2 minutes. This was followed by three consecutive washes in distilled water with 100 µl of the third wash being plated onto LB agar plates. The control plates were incubated for 1-2 days at 37°C to ensure that the sterilization procedure was successful. The exposed ends of each leaf were removed and discarded while the inner

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tissue was cut into pieces. These pieces were placed in a sterile 50 ml Greiner tube containing 25 ml of 12.5 mM potassium phosphate buffer (pH 7.0). This was manually homogenised using a sterile glass rod and 100 µl aliquots were plated onto various isolation media using the standard spread plate technique (Young, 1979).

For the isolation of rhizospheric organisms, a microwave pre-treatment was conducted to reduce the numbers of contaminating organisms. Rhizopheric organisms were isolated by adding 1 g of soil sample to 10 ml of sterile distilled water. The samples were vortexed for a minute and left to stand for an additional minute. The soil suspensions were poured into a sterile glass plate and heated in a microwave at maximum power for five seconds. Ten-fold dilutions, from 10^{-2} to 10^{-5}, were prepared using sterile water and 100 µl aliquots of each dilution were plated on the various isolation media using the spread plate technique.

All plates were incubated at 28°C for 14 days, except for plates containing endophytes which were incubated for at least a month. After the incubation periods, isolates were selected, based on their morphology, using sterile toothpicks, and plated onto fresh media without antibiotics. The plates were incubated at 28°C and checked every few days. Isolates were restreaked until pure isolates were obtained. These isolates were cultured in 15-20 ml of YEME broth at room temperature for 5 days on a shaker set to 150 rpm. The cultures were Gram stained to confirm purity before making glycerol stocks (20 % final concentration). Cell pellets were obtained by centrifuging 1 ml of the culture in sterile Eppendorf tubes for one minute at 10 000 rpm and discarding the supernatant. The procedure was repeated until ~200 µl cell mass was obtained. Both the pellets and the glycerol stocks were stored at -20°C until needed.

2.3 Morphological characterisation

Characterisation of isolates was performed using the methods defined in the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). ISP 4 was used to observe the pigmentation of both the substrate and aerial mycelium. ISP 5 was used to screen for the production of diffusible pigment, as well as the effect of pH on pigment production. This was done by excising three 1 cm by 1 cm agar blocks from the respective ISP 5 agar plates using

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a sterile scalpel. Approximately 50 µl of 50 mM NaOH and 50 mM HCl were added to separate blocks, and dH_2O was added to the third block as a negative control. ISP 6 and ISP 7 were used to determine melanin production.

ISP 9 was used to determine carbon source utilisation. The media was supplemented with the selected filter sterilised carbon sources, including L-asparagine and aloe pulp, to a final concentration of 1 %. ISP 9 without an added carbon source was used as a negative control. The experiment was performed by growing the isolates in 20 ml of YEME liquid culture for 10 days at 28°C in a shaking incubator. After incubation, samples were transferred to sterile 50 ml Falcon tubes and centrifuged at 4 000 rpm for 10 minutes. The supernatants were discarded, and the pellets were washed by adding 20 ml of sterile dH_2O to each Falcon tube, inverting, and centrifuging at 4 000 rpm for 10 minutes. The supernatants were decanted, and the wash step was repeated three times. The pellets were then resuspended in 500 µl sterile dH_2O and 20-50 µl of the washed cell suspension was added to the center of the plate and streaked in an “X” formation, as seen in Figure 2.1, using a sterile inoculating loop. Plates were incubated for 14 days at 28°C before observation.

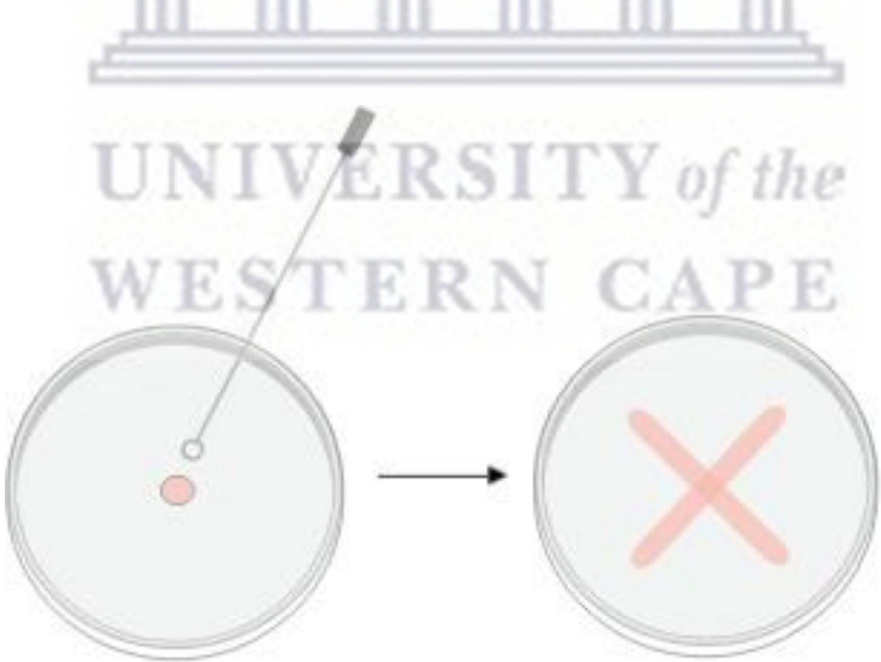

Figure 2.1: An illustration of the streaking pattern used for ISP 9 screening (Created with BioRender.com).

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2.4 DNA identification

Genomic DNA was extracted using a modified version of the method described by Wang *et al.*1996. Cell pellets were lysed by adding 500 µl of lysis buffer (25 mM Tris-HCl (pH 8.0); 10 mM EDTA; 50 mM glucose; 25 mg lysozyme and proteinase K (20 mg/ml)) and incubating overnight at 37°C. SDS was added to a final concentration of 1 %, vortexed, and incubated for 30 minutes at 65°C in a water bath. An equal volume of phenol was added to the samples and the tubes were gently inverted before centrifuging at 10 000 rpm for 1 minute. The top aqueous layer was carefully transferred to a sterile Eppendorf tube ensuring that the protein interface was not disturbed. The phenol step was repeated if the aqueous phase appeared murky. An equal volume of chloroform: *iso*-amyl alcohol (24:1; v/v) was added, the tubes were inverted and centrifuged at 10 000 rpm for 1 minute as before. The DNA was precipitated by adding an equal volume of ice-cold isopropanol to the aqueous phase and centrifuging at 10 000 rpm for 5 minutes. The supernatant was discarded, and the pellets were left to air-dry before being resuspended in 100 µl of 1X TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) and stored at 4°C until further use.

Agarose gel electrophoresis was performed to ensure genomic DNA was extracted and a NanoDrop ND-1000 was used to determine the purity and concentration of the DNA. A 1.8 % agarose gel was prepared with 0.5 X TAE containing ethidium bromide (0.5 µg/ml) and the gel was electrophoresed in 0.5 X TAE for 1 hour at 90 V. The size of the bands was determined by including a lambda PstI molecular weight marker and visualising the gels using an Alpha imager HP UV transilluminator.

2.5 16S rRNA gene amplification

In order to amplify the 16S rRNA gene, the universal primers F1 (5’-AGAGTTTGATCTGGCTCAG-3’) and R5 (5’- ACGGTACCTTGTTACGACTT-3’) were used to generate the amplicons (Cook and Meyers, 2003). The primer set E9F (5’-GAGTTTGATCCTGGCTCAG -3’) (Farrelly *et al.*, 1995) and U1510R (5′-GGTTACCTTGTTACACTT- 3’) (Reysenbach *et al.*, 1995) were used as an alternative primer set for selected samples.

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The PCR was set up using Dream*Taq* DNA Polymerases, with each 50 µl reaction containing a final concentration of 1 x Dream*Taq* Buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1 U of Dream*Taq*, and 1 µg of template DNA. Due to the inherent difficulties amplifying high GC DNA, alternative polymerase systems were tested, including Phusion and KAPA2G Robust.

A 20 µl PCR reaction using Phusion High-Fidelity DNA Polymerase contained a final concentration of 1 x Phusion GC Buffer, 200 µM dNTPs, 0.5 µM of each primer, 0.4 U of polymerase, and <250 ng of template DNA. The optional 3% DMSO was also added to the master mix. A 25 µl PCR reaction was setup using KAPA2G Robust which contained a final concentration of 1 x KAPA2G Buffer B, 0.2 mM dNTPs, 0.5 µM of each primer, 0.5 U of polymerase, 1 x KAPA Enhancer 1 and 10 ng of template DNA.

A Bio Rad T100 thermal cycler was used for PCR amplification and the parameters used for each polymerase are listed in Table 2.2.

Table 2.2: The PCR cycling conditions used for three polymerases tested in this study.

	Dream*Taq*		**Phusion High-fidelity**		**KAPA2G Robust**	
Initial denaturation	95°C for 3 minutes		98°C for 30 seconds		95°C for 2 minutes	
Denaturation	95°C for 30 seconds	34 Cycles	98°C for 10 seconds	30 Cycles	95°C for 30 seconds	34 Cycles
Annealing	54°C for 30 seconds		56°C for 25 seconds		56°C for 30 seconds	
Elongation	72°C for 2 minutes		72°C for 30 seconds		72°C for 1 minute	
Final elongation	72°C for 5 minutes		72°C for 10 minutes		72°C for 5 minutes	

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The resulting PCR products were electrophoresed in a 1 % gel and visualised as described above (section 2.4). The amplicons were purified using a NucleoSpin gel and PCR Clean-up kit (Machery-Nagel) according to the manufacturer's instructions and sequenced by the Central Analytical Facility, University of Stellenbosch. The sequence data was edited using BioEdit (Hall, 1999) and species were identified through EzBioCloud (https://www.ezbiocloud.net/), as well as BLASTN (https://blast.ncbi.nlm.nih.gov).

2.6 Antimicrobial screening

The isolates were selected for antimicrobial testing based on their distinctive morphological appearance and pigment production. Antimicrobial screening was performed using the perpendicular streaking method as a preliminary screening technique followed by the agar overlay technique. Three different media including Starch-casein nitrate (SCN), Yeast extract-malt extract (YEME), and ISP 3 media were used. The six pathogens used for screening were standard susceptibility testing strains and/or clinical isolates: *Acinetobacter baumannii* ATCC 19606, *Candida Albicans* ATCC 24433, *Enterococcus faecalis* van A ATCC 51299, *Escherichia coli* ATCC 25922, *Mycobacterium aurum* A+ (clinical isolate, kindly provided by Dr Paul Meyers, UCT), and *Staphylococcus aureus* ATCC 29213. Prior to plating all isolates were grown overnight in the media and temperature specified in Table 2.3, shaking at 250 rpm.

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Table 2.3: The conditions, including media and temperature, in which the selected test strains were grown.

TEST STRAIN	MEDIA	TEMPERATURE (°C)
Acinetobacter baumannii	Trypticase soy (TSA or TSB)	28
Candida albicans	Yeast extract peptone dextrose (YPD)	37
Enterococcus faecalis	Brain heart infusion (BHI)	37
Escherichia coli	Trypticase soy (TSA or TSB)	37
Mycobacterium aurum	Luria-Bertani (LB)	37
Staphylococcus aureus	Nutrient agar (NA)	37

For the perpendicular streak test the isolates were inoculated as a single streak down the center of an agar plate, one isolate per plate. The plates were incubated at 28°C for 11 days. Following the incubation, 10 µl of each test organism (with OD_{600} at 0.5) was inoculated perpendicularly to the isolate, as seen in Figure 2.2, and the plates were further incubated at 37°C for two days. The distance between the isolate and test strain was checked on both days and an arbitrary grading system was used to select active isolates.

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Figure 2.2: Template used for antimicrobial screening using the perpendicular streaking method (Created with BioRender.com).

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Isolates were selected for further testing via agar overlay method based on the findings of the preliminary screening. The isolates were stab inoculated onto each media using sterile toothpicks and incubated at 28°C for 11 days. The overlays were performed following incubation by inoculating each test strain into 0.8 % of their respective agar. The amount of culture added per 10 ml sloppy agar was calculated using the following formula: OD_{600} x Xμl = 2 for *C. albicans,* OD_{600} x X μl = 4 for *E. coli*, and OD_{600} x X μl = 180 for the remaining test strains; where OD_{600} is the optical density at 600 nm and X μl is the amount of culture added. The appropriate amount of culture was inoculated into the molten sloppy agar, vigorously vortexed and poured carefully over the stab inoculated actinobacterial isolates ensuring the entire surface of the plate was covered. The plates were incubated at 37°C for two days, checking for zones of inhibition on each day. *A. baumannii* plates were incubated at 28°C.

2.7 Enzyme activity

Isolates were screened for enzymatic activity including amylase, cellulase, xylanase, and pectinase activity. The media used was Bennett's glucose medium (Kitouni *et al.*, 2005), as well as pectin hydrolysis medium. The Bennett's media was supplemented with either 1 % (final concentrations) sterile soluble starch, 0.3 % CMC cellulose, or 0.4 % beechwood xylan. All plates were incubated for 7 days at 28°C. Following incubation, starch and xylan plates were flooded with Gram's iodine. All plates were checked immediately for zones of clearing and xylan plates were checked again after 15-20 minutes. Cellulose plates were flooded with 0.1 % Congo red and left for 15 minutes before decolorizing using 1M NaCl. The plates were checked for clear zones after decolorizing and then left at room temperature overnight and checked again. Pectin plates were flooded with 1 % CTAB and checked for precipitation after 30-40 minutes.

Isolates were also checked for siderophores activity on Chromeazeurol S (CAS) media plates (Schwyn and Neilands, 1987). This was done by stab inoculating isolates onto the agar and incubating at 28°C for 14 days. The plates were checked for a zone of clearing on the 7th and 14th day of incubation.

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2.8 Antimicrobial solvent extractions

The isolates were grown in 20 ml of either YEME or SCN broth at 28°C in a shaking incubator for 3 days. The starter cultures were transferred to 80 ml of the same broth and incubated for a further 14 days under the same conditions. Following incubation, the cultures were filtered into a sterile 250 ml Schott bottle using coffee filters. The filtered culture was then divided by transferring half of the culture, roughly 50 ml, into another sterile 250 ml Schott bottle. The culture filtrate was extracted with an equal volume of either ethyl acetate or chloroform, respectively. The cell mass collected on the coffee filters were transferred to a sterile flask containing 15ml of methanol using a sterile spatula. The flasks and bottles were incubated on a shaker at room temperature for 1-2 hours.

After the incubation period the contents of the flasks were carefully transferred to a separation funnel and allowed to settle for 5 minutes in order for phase separation to occur. The organic phase was collected in a sterile glass petri dish while the aqueous phase was discarded. These extracts were then left in a fume hood for 48 hours to allow the solvent to evaporate. The resulting extract was resuspended in 3 ml of the respective solvent and then transferred to a McCartney tube. This was then allowed to evaporate for a further 24 hours and the resulting extracts were stored at 4°C until further use.

At the time of testing the extracts were re-dissolved using their respective solvents so that they were 50 times more concentrated than the initial volume. Extracts were tested using the Kirby-Bauer disk diffusion method of activity screening. The disks used for the experiment were made using 6 mm filter paper and then sterilised by autoclaving. Disks were pre-treated with 5% DMSO and allowed to dry before adding 50 µl of the extracts. These disks were allowed to dry fully overnight before plating.

Test strains were grown overnight as stated in the antimicrobial screening section (section 6) to an OD_{600} of 0.5. The test strains were spread plated onto their respective media and allowed to dry. The prepared Kirby-Bauer disks were applied to the inoculated plates using sterile tweezers. Each plate contained a disk for MetOH, EtAc, and $ChCl_3$ extracts as well as a positive control for the pathogen. Positive controls included vancomycin for *E. coli* and *C. albicans*, polymyxin B for *E. faecalis*, and ampicillin for *A. baumannii.* Negative control disks were also made in a similar manner by performing extractions on uninoculated media.

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These disks were plated on a separate plate containing the pathogens. These plates were incubated at 37°C for 24 hours before the results were observed.

The well diffusion assay was performed as an alternative testing method. After plating the test strains and allowing them to dry as stated above, wells were made in the agar using a sterile p1000 tip. The ends of the tips were cut in order to ensure a consistent 0.5 mm diameter of the wells and autoclaved before use. In order to screen the extracts, 100 µl of extract was added to their respective wells and allowed to dry prior to being incubated at 37°C for 24 hours before the results were observed.

2.9 Thin layer chromatography (TLC) and bioautography

TLC was performed on extracts that had activity against *E. coli.* The TLC Silica Gel sheets (Merck) were prepared by cutting them into 7×10 cm sections. A 1 cm origin line was drawn at the top and bottom of the sheet with a 0.5 cm border on either side. At the origin line, 50- 100 µl of extract was added and allowed to dry. A chamber was prepared by using EtAc as a mobile phase, adding it to a glass beaker, and sealing it with foil. This was left in the fume hood for 30 minutes to create a saturated environment. Following this period, the TCL plate was carefully added to the beaker, ensuring that the solvent does not touch the origin line.

For the bioautography, an *E. coli* culture was grown overnight to an OD_{600} of 0.5 in LB. The TLC plates were placed into a sealable container lined with moist tissue paper. A pipette was used to overlay the plates with the overnight culture while tilting the container to ensure the entire plate was covered. The sealed container was incubated at 37°C overnight. Following incubation, the plates were stained with MTT (0.25%) using the above-mentioned overlay technique. The plates were returned to the incubator for 3-4 hours and checked every hour for clear zones.

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2.10 Metagenomic DNA extraction and amplification

Metagenomic DNA was isolated from both plant and soil samples in order to potentially identify unculturable actinobacteria. The metagenomic DNA from soil was isolated using the MoBio Power Soil DNA Isolation Kit, as per manufacturer's instructions, while a modified version of Li and associates (2007) was used to isolate the plant metagenomic DNA. Frozen sterilized leaf samples were cut into small pieces, weighed, and added to a mortar with PVP40 powder (0.1 g per 1 g leaves). The samples were ground up with the help of liquid nitrogen and then transferred to a 2 ml Eppendorf tube. 1 ml of Extraction Buffer I (100 mM Tris-Cl (pH 8.0), 50 mM EDTA, 500 mM NaCl, and 2% 2-Mercaptoethanol (added just prior to use)) was added to each tube and left at room temperature for 10 minutes before being centrifuged at 8 000 rpm for 10 minutes at 4°C. The supernatant was discarded and 1 ml of preheated Extraction Buffer II (2% CTAB, 1.4 M NaCl, 100 mM Tris-Cl (pH 8.0), 0.5 M EDTA (pH 8.0), and 2% 2-Mercaptoethanol (added just prior to use)) was added to each tube and incubated for an hour at 65°C, with occasional gentle mixing. The samples were then centrifuged at 8 000 rpm for 10 minutes 4°C, and the resulting supernatant was transferred to a clean 2 ml Eppendorf. An equal volume of chloroform: *iso*-amyl alcohol (24:1; v/v) was added to the tubes which were then inverted for a minute followed by a 10-minute incubation at room temperature. The tubes were centrifuge at 10 000 rpm for 10 minutes at 4°C and the aqueous phase was transferred to a clean 2 ml Eppendorf tube repeating the C: I step. A tenth of the sample volume of 2M potassium acetate (pH 5.2) was added to each tube and the upper aqueous phase was transferred to a clean 2 ml Eppendorf. Two thirds of the sample volume of ice-cold isopropanol was added before incubating the tubes at -20°C for ~30 minutes. Tubes were centrifuge at 12 000 rpm for 10 minutes at 4°C, the supernatant discarded, and the pellet washed once or twice with 70% EtOH before allowing it to air dry. The pellet was resuspended in 70 µl sterilized double distilled water.

The DNA isolated from plant and soil samples were successfully amplified using Phusion polymerase and Robust polymerase, respectively. The PCR products were electrophoresed on a 1% agarose gel and a gel clean-up was performed using the NucleoSpin gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions and sequenced by the Central Analytical Facility, University of Stellenbosch. The sequence data was edited using Bio Edit (Hall, 1999) and identified through EzBioCloud (https://www.ezbiocloud.net/) as well as BLASTn (https://blast.ncbi.nlm.nih.gov).

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2.11 Cloning of metagenomic DNA

A gel clean-up was performed on the PCR products using the NucleoSpin gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. The Thermo Scientific CloneJet PCR Cloning Kit was used to clone the resulting products. The ligation procedure was performed as per the manufacturer's instructions and the products were used immediately for transformation using electro-competent as well as chemically competent cells.

2.11.1 Preparation of electro-competent cells and electroporation

Electro-competent cells were prepared using an overnight culture of *E. coli* DH5-α cells grown in LB. 500 µl of the culture was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of LB. The flasks were incubated at 37°C in a shaking incubator until an OD between 0.6 and 0.8 was reached, at which point the flasks were put on ice to halt growth. The 50 ml culture was transferred into two 50 ml Falcon tubes. These were then centrifuged at 5 000 rpm for 5 minutes at 4°C and the resulting supernatant was discarded. The pellet was resuspended and washed in 40 ml of 10% glycerol followed by centrifugation at 5 000 rpm for 5 minutes at 4°C. The supernatant was discarded, and two more wash steps were performed. The pellets were resuspended in 500 µl of 10 % glycerol. 50 µl aliquots of the cells were transferred to 2 ml Cryogenic tubes and stored at -80°C until needed.

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The BioRad Gene Pulser was used to perform electroporation. Electroporation cuvettes as well as microfuge tubes were pre-chilled on ice while allowing the electro-competent cells to thaw on ice. 50 µl of the cells were added to the microfuge tubes followed by 2 µl of DNA. The contents were gently mixed and transferred to the chilled cuvettes, ensuring no bubbles were present. The cuvettes were electroporated using the *E. coli* setting. Immediately after electroporation 1 ml of LB media was added to the cuvette. The contents were then transferred to a 2 ml Eppendorf tube and incubated at 37°C in a shaking incubator for a one-hour recovery. Following recovery, 1:10 and 1:100 dilutions were performed on the transformations and each dilution was plated in triplicate on LB plates containing a final concentration of 100 µg/ml ampicillin, 0.5 mM IPTG, and 80 µg/ml X-gal and incubated overnight at 37°C.

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The transformation was optimized by making use of SOC media for the recovery of cells as well as heating the recovery media and agar plates prior to use.

2.11.2 Preparation and transformation of chemically competent cells

Glycerol stocks of *E. coli* DH5-α cells were plated on LB agar plated and incubated overnight at 37°C. A single colony was picked from the plate, inoculated into a McCartney flask containing 10 ml of LB, and incubated in a shaking incubator overnight at 37°C. Once an OD_{600} between 0.35 and 0.4 was reached, cultures were incubated on ice for 30 minutes. All reagents and tubes used for the cell preparation were also incubated on ice prior to use. The overnight culture was transferred to a 50 ml Falcon tube and centrifuged at 3 000 *x g* for 15 minutes at 4°C. The supernatant was removed, and the pellet was resuspended in 20 ml of 100 mM $MgCl_2$ and centrifuged at 2 000 *x g* for 15 minutes at 4°C. The supernatant was removed, and the pellet was resuspended in 40 ml of 100 mM $CaCl_2$ and incubated on ice for 20 minutes. The samples were centrifuged at 2 000 *x g* for 15 minutes at 4°C, the supernatant discarded, and the pellet was resuspended in 10 ml of a buffer containing 85 mM $CaCl_2$ and 15% glycerol. This was centrifuged at 1 000 *x g* for 15 minutes at 4°C and the resulting pellet was resuspended in 2 ml of the previous buffer before preparing 50 µl aliquots in 2 ml Cryogenic tubes which were stored at -80°C until further use.

The heat shock method was used to transform the ligation reactions. 50 µl of the competent cells were thawed on ice before adding 5 µl of the ligation reaction and incubating the reaction on ice for 30 minutes. The cells were heat shocked for 40 seconds at 42°C and then immediately put on ice for 2 minutes. 950 µl of LB was added to cells and incubated in a shaking incubator for an hour at 37°C. The transformations were plated of LB agar plates containing 100 µg/ml ampicillin and incubated at 37°C overnight.

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2.12 Colony PCR and sequencing

Transformation plates were checked after incubation and single colonies were picked for colony PCR. A 25 µl PCR was setup using KAPA2G Robust which contained a final concentration of 1 x KAPA2G Buffer B, 1 x KAPA Enhancer 1, 0.2 mM dNTPs, 0.5 µM of each primer (E9F and U1510R), and 0.5 U of polymerase. The single colonies were picked using a sterile pipette tip, ensuring not to touch the agar, and resuspended in the PCR mix. Additionally, a separate PCR was set up with colonies that were heat shocked prior to amplification. This was done by inoculating a colony into 10 µl of sterile water, heating the mixture at 95°C for 3 minutes and centrifuging the sample at 11 500 rpm for 2 minutes. 1 µl of this was used as DNA template in the PCR. The parameters for the PCR were as described in Table 2.2.

The PCR products were electrophoresed on a 1% agarose gel and a gel clean-up was performed using the Qiagen NucleoSpin gel and PCR Clean-up kit, according to the manufacturer's instructions, and sequenced by the Central Analytical Facility, University of Stellenbosch. The sequence data was edited using BioEdit (Hall, 1999) and identified through EzBioCloud (https://www.ezbiocloud.net/) as well as BLASTn (https://blast.ncbi.nlm.nih.gov).

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Chapter 3: Results and discussion – Isolation and identification of isolates

3.1 Isolations, cultivation, and storage

The diverse habitats provided by plants (endo-, rhizo- and phylo-sphere), each with its own unique conditions, support colonisation by a wide variety of different microorganisms. Growth and colonisation are dependent on the micro-environment and the conditions provided by it. This includes the temperature, pH, and the nutrients available (Bisen *et al.*, 2012).

In the present study epiphytic and rhizopheric filamentous actinobacteria were successfully isolated from leaf and rhizospheric soil samples. Endophytic actinobacteria are difficult to cultivate and while aloe extract was added to the isolation media to encourage their isolation only three were isolated. Unfortunately, these isolates did not survive subsequent subculturing and were therefore excluded from further analysis. This finding was not unexpected as it has been reported elsewhere that some endophytic microorganisms cannot be maintained for extended periods away from the host plant (Abdalla and Mcgaw, 2018). Using the various isolation techniques and media mentioned, a total of 879 isolates were initially obtained. Tables 3.1 and 3.2 indicate the number of isolates obtained from each sample and the number of isolates obtained on the different isolation media, respectively. Only 61 isolates that displayed actinobacterial characteristics or had any uniquely interesting characteristics (colony morphology, unusual combination of mycelium colours etc.) were selected for antimicrobial activity screening.

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Table 3.1: Number of isolates obtained from specific isolation sites.

Sample	*Epiphytes*	*Rhizophytes*	*Endophytes*
TP1	4	33	0
TP2	24	429	0
BP1	0	41	2
BP2	11	94	0
WP1	2	25	1
WP2	11	200	2
TOTAL	52	822	5

TP- top plant; BP – bottom plant; WP – wild plant.

Table 3.2: Number of isolates obtained from specific isolation media.

Isolation media	Number of isolates
SCN	341
YEME + plant extract	205
AI + Gly	144
AI + Glu	77
Phytagel + plant extract	25
HV	87
CHV	3

SCN- starch-casein-nitrate; YEME- yeast-malt extract; AI- actinomycete isolation; Gly- glycerol; Glu- glucose; HV- humic acid vitamin; CHV- complex humic acid vitamin.

Table 3.1 indicates the total number of isolates obtained from each sampling site with plant two from the top sample site (TP2) having the highest number of isolates. This finding is interesting as plants from the top site were originally from a mountainous region outside Albertinia, and as such would have been exposed to more extreme environmental conditions, particularly extreme temperature fluctuations and soils with lower nutrients. There is also a significant difference in the number of isolates obtained from soil samples surrounding the

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second plant from the top sampling site (TP2) and the “wild” sampling site (WP2) respectively, in comparison to the soil surrounding the first plants at those sites.

The results in Table 3.2 indicate the total number of isolates per isolation media. It is observed that SCN was the preferred media for the growth of actinobacteria, closely followed by the YEME media supplemented with plant extract. The addition of plant extract was to account for the actinobacteria that required plant compounds to flourish. Actinomycete Isolation agar was made with either glycerol or glucose as a carbon source. It is observed that the media containing glycerol has double the number of isolates when compared to the media with glucose. This could suggest that actinobacteria were inhibited by glucose (catabolite repression), which may also account for the fact that SCN (which doesn’t contain glucose) was the preferred isolation medium. HV media which is supplemented with soil extract was also used to aid in the isolation of rare actinobacteria by mimicking the natural environment of these isolates.

3.2 DNA identification and 16S rRNA gene amplification

16S rRNA genes were successfully amplified for all ten of the selected isolates. The amplicons were sequenced using Sanger sequencing, the resulting chromatograms were edited with BioEdit, and the strains were identified through BLASTn analysis and EzBioCloud. Phylogenetic analysis results obtained from EzBioCloud are presented in Table 3.3 with only the top two closest relatives (type strains) for each of the isolates being included. These results were also used in the construction of phylogenetic trees as seen in Figures 3.1 and 3.2.

As expected for soil samples, most of the isolates were identified as *Streptomyces* species (Arifuzzaman *et al.*, 2010). Isolate 507 was identified as *Amycolatopsis nivea* which was interesting since the genus *Amycolatopsis* is considered a “rare” genus. The isolate was also identified at a similarity of 98.74% indicating that it is likely a novel *Amycolatopsis* species and the name *Amycolatopsis aloensis*^{T} is proposed. Isolate 257 was identified as *Stenotrophomonas bentonitica* (99.75% similarity). This is a Gram-negative bacterium that has previously been isolated from bentonite formations in Spain (Sánchez-Castro *et al.*, 2017). Since isolates were frequently Gram stained and isolate 257 was morphologically identified as an actinobacteria, it is assumed that the isolate was contaminated but it is difficult to determine at which point in the study this occurred.

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When looking at Table 3.3, it is observed that all *Streptomyces* strains have high similarities to published species. This speaks to how limited 16S rRNA gene identification is within the *Streptomyces* genus and it is therefore suggested that future studies include other housekeeping genes for species delineation. A few isolates were identified as being most similar to the same species. This, however, is normal for isolates taken from the same sample as the abundance and diversity of bacteria isolated is dependent on the environmental conditions (Arifuzzaman *et al.*, 2010). Isolates similar to the same species include isolates 560 and 611, both isolated from sample TP2, as well as 789 (from sample WP2) which had near identical 16S genes, with a few codon differences, and were all most similar to *Streptomyces pratensis* (99.87, 99.76, and 99.74% similarity, respectively). Isolates 579 and 590, also from sample TP 2, were both identified as being most closely related to *Streptomyces ambofaciens* (99.75, and 99.10% similarity respectively). Based on these results, it is expected that these isolates would be grouped together in the phylogenetic tree, but this is not the case for isolates 560, 611, and 789 as seen in Figure 3.1. These isolates were all separated from each other but it can be seen that isolate 560 was grouped with isolate 9, most closely related to *Streptomyces microflavus* (99.88% similarity). It is noted that isolates 560 and 789 were also related to *S. microflavus* with 99.62% similarity. It should be noted that the bootstrap values for many of the minor branches is low (under 40) so one should be conscious when drawing conclusions. This is likely due to the fact that all the sequences included in the tree were very similar.

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Table 3.3: 16S rRNA gene identification as seen on EzBioCloud.

Isolate	*Closest relative*	*Identity (%)*	*Accession number*
1	*Streptomyces afghaniensis*	*99.75*	*AB184847*
	Streptomyces africanus	*99.62*	*MUKA01000156*
9	*Streptomyces microflavus*	*99.88*	*AB184284*
	Streptomyces griseus	*99.88*	*M76388*
257	*Stenotrophomonas bentonitica*	*99.75*	*LT622838*
	Stenotrophomonas rhizophila	*99.13*	*CP007597*
507	*Amycolatopsis nivea*	*98.74*	*KP232907*
	Amycolatopsis rubida	*97.73*	*Jgi.1085901*
560	*Streptomyces pratensis*	*99.87*	*JQ 806215*
	Streptomyces microflavus	*99.62*	*AB184284*
563	*Streptomyces fractus*	*99.36*	*FJ857947*
	Streptomyces endophyticus	*99.23*	*GU367154*
579	*Streptomyces ambofaciens*	*99.75*	*CP012382*
	Streptomyces iakyrus	*99.49*	*JNXI01000062*
590	*Streptomyces ambofaciens*	*99.10*	*CP012382*
	Streptomyces iakyrus	*98.85*	*JNXI01000062*
611	*Streptomyces pratensis*	*99.76*	*JQ806215*
	Streptomyces badius	*99.52*	*AY999783*
789	*Streptomyces pratensis*	*99.74*	*JQ 806215*
	Streptomyces microflavus	*99.62*	*AB184284*

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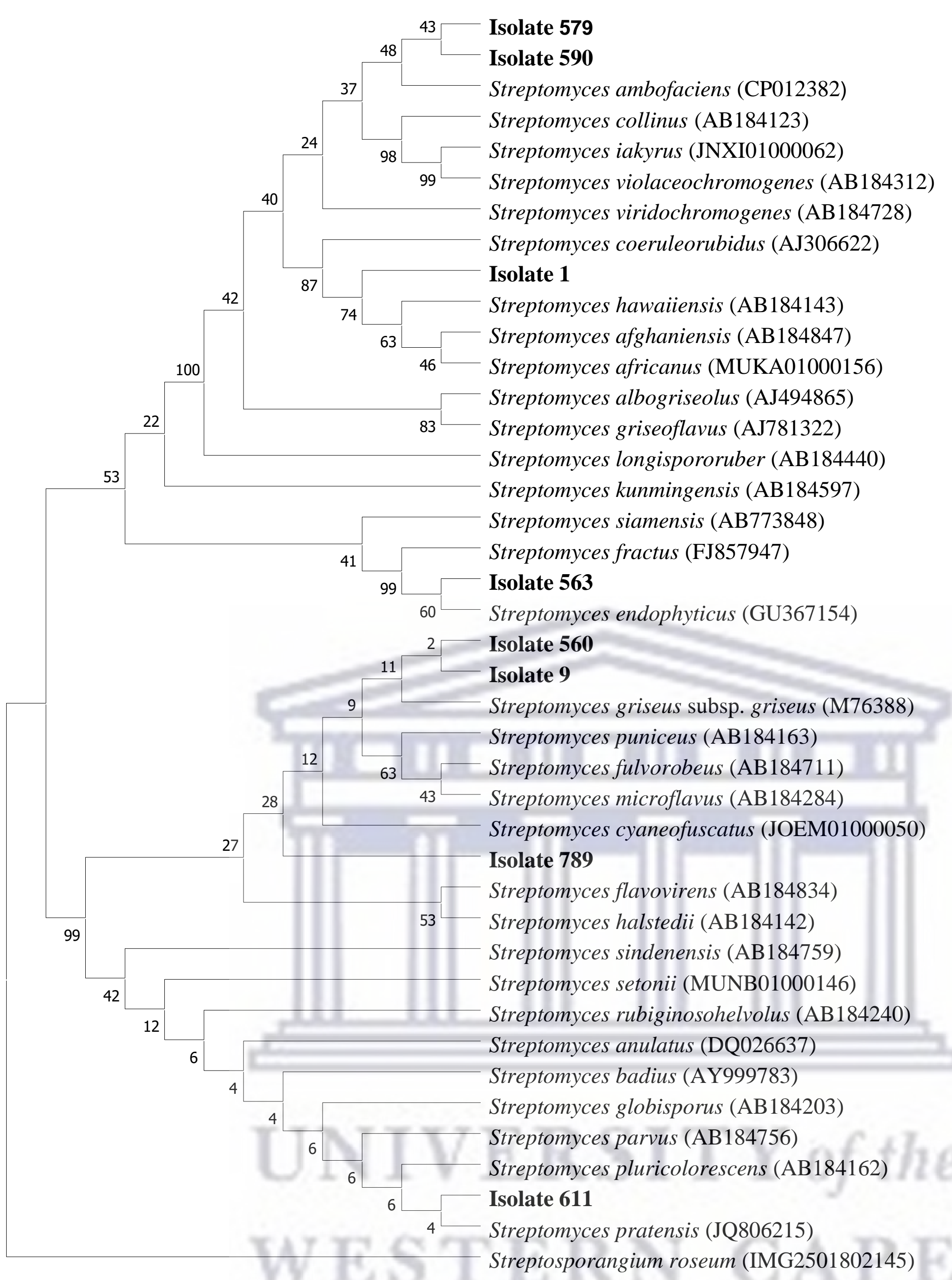

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Figure 3.1: Neighbour joining phylogenetic tree showing the relationship between all isolates identified as *Streptomyces* species.

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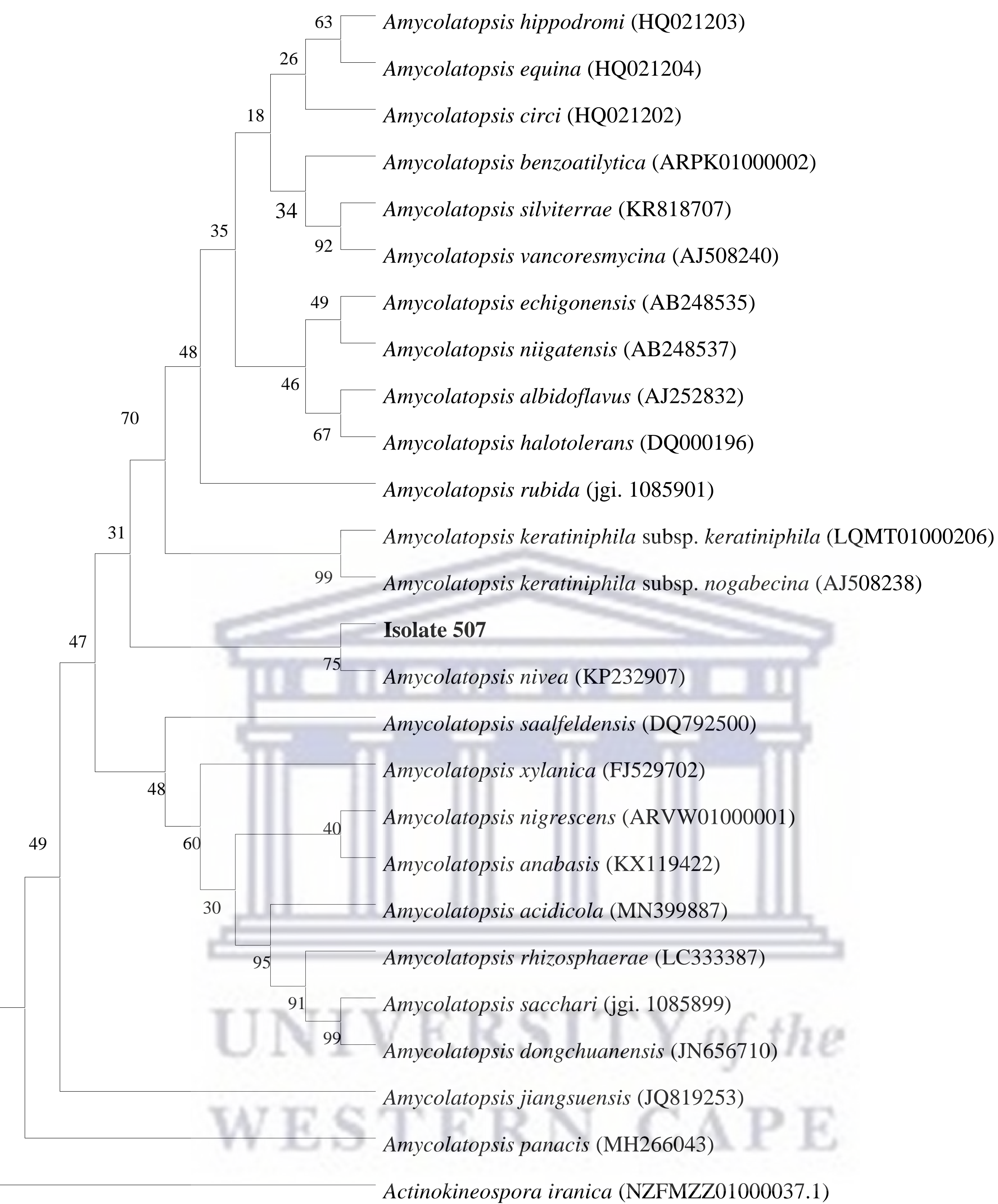

Figure 3.2: Neighbour joining phylogenetic tree showing the relationship between isolate 507 and the related *Amycolatopsis* species.

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A review of literature revealed that for the most part the closest relatives were previously isolated from soil, terrestrial and marine samples. This includes *Streptomyces afghaniensis* isolated from marine soil in India (Vijayakumar *et al.*, 2012), *Streptomyces microflavus* isolated from a soil sample taken in Tunisia (Smaoui *et al.*, 2012), *Amycolatopsis nivea* isolated from a sediment sample in China (Niu *et al.*, 2020), *Streptomyces pratensis* isolated from soil (Rong *et al.*, 2013), and *Streptomyces ambofaciens* isolated from soil (Thibessard *et al.*, 2015). It can be noted that the properties of terrestrial soil typically differ from those of sediments and marine soil. Interestingly, isolate 563 was identified as being closely related to *Streptomyces fractus* which has been previously isolated from the gut of a termite found in the Tygerberg Nature Reserve, Cape Town. This strain, *S. fractus*, is also closely related to *Streptomyces endophyticus* which was previously isolated from the roots of *Artemisia annua* L., a medicinal plant found in China (Li *et al.*, 2013; Rohland and Meyers, 2015).

The identified species were also all known antibiotic producers which validates the possibility of new antimicrobial compounds being isolated from actinobacteria associated with medicinal plant. Based on preliminary phenotypic and phylogenetic results, further characterisation of several of the isolates, especially 507, is warranted.

3.3 Morphological characterisation

Morphological and phenotypic characterisation plays an important role in the identification of actinobacteria. Isolates were cultured on various ISP media including ISP 4, 5, 6, 7, and 9 (Tables 3.4 to 3.6).

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Table 3.4: Determining the mycelia and diffusible pigment colour on ISP4. Comparison to the British Standards colour chart.

	ISP4		
Isolate	AERIAL	SUBSTRATE	PIGMENT
1	Shell pink; International orange/signal red; fluffy light pale blue	Dark salmon/International orange	Dark salmon pink
9	Camo beige/Dark camo brown	Camo beige/Biscuit	Grey toned vellum
257	Light French grey	Light service brown with light stone trim	Dark camo desert sand
507	White	Vellum	Grey toned vellum
560	Light French grey/Light aircraft grey	Pale cream/Manilla	Dark vellum
563	Light French grey/Light aircraft grey	Pale cream/Manilla	Dark vellum
579	Camo beige/greyish light violet	Light maroon	Light camo desert sand
590	-	-	-
611	Light camo beige	(Light) dark earth/greyish camo beige	Camo beige
789	French grey with camo beige	Camo beige/Slate	Light spruce green

As seen in Table 3.4, isolate 590 had no recorded results as it was no longer able to grow. Isolate 1 interestingly produced three shades/types of aerial mycelium including shell pink, international orange-signal red, and fluffy light pale blue. This unusual colour combination can be seen in Figure 3.3 where the pale blue appears to be the spore mass of the isolate. When comparing these results to that of its closest relative, *S. afghaniensis* and *S. africanus*, these isolates are not phenotypically similar. Vijayakumar and co-workers (2012) characterized the type of strain of *S. afghaniensis* and reported that their strain produced white aerial mycelium with "colourless" substrate mycelium on ISP 4. On the other hand, while Meyers *et al.*, 2004 reported that *S. africanus* produced blue aerial mycelium, the substrate mycelium was yellow not orange.

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Figure 3.3: Isolate 1 grown on ISP 4 media, the dark salmon pink substrate and pale blue aerial mycelium are visible.

Isolate 9 produced camo beige and biscuit coloured aerial and substrate mycelium on ISP 4 which differs from *S. microflavus* isolated by Smaoui and associates (2012). According to their study, *S. microflavus* produced white aerial mycelium with a yellowish white substrate mycelium when grown on ISP 4. Isolate 507 produced white aerial and vellum substrate mycelium which is comparable to the strain of *A. nivea* identified by Niu and colleagues (2020) which produced white aerial hyphae with light yellow substrate mycelium. This colour combination is however not uncommon among *Amycolatopsis* strains.

While isolates 560, 611, and 789 were all identified as being most similar to *S. pratensis*, the results obtained on ISP 4 media, as seen in Table 3.4, indicate that they are not phenotypically identical to each other, although there are morphological similarities between these isolates. Isolate 579 and 590 were both identified as being most similar to *S. ambofaciens* but due to isolate 590 not being able to grow on ISP 4 the morphological features of the two strains could not be compared.

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Figure 3.4: Isolate 560 (A), 611(B), and 789(C) grown on ISP 4 media. Morphological similarities can be seen between these isolates including the fluffy texture of the colonies, and similar pigmentation.

Table 3.5: ISP 5 results as determined by the British Standards colour chart testing the pigment reaction to different pH levels.

	ISP5			
Isolate	PIGMENT	H_2O	HCl	NaOH
1	Bright salmon/international orange	-	-	Cherry/crimson/ruby
9	Light stone	-	-	-
257	Light beige with slight pink	-	-	Pale roundel red
507	Vellum	-	-	-
560	Vellum/Manilla		More vellum	-
563	-	-	-	-
579	Terracotta/Venetian red		Crimson	Deep maroon
590	-		-	-
611	Salmon pink/shell pink		Vellum	-
789	Vellum	-	-	-

Hyphens (-) indicates no changes occurred.

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Table 3.5 shows the results for growth on ISP 5 which is primarily used to screen for the production of diffusible pigments, as well as the pH sensitivity of the pigments. Isolates 563 and 590 had difficulty growing on this media (possibly due to an inability to utilise the available C or N sources, glycerol and asparagine, respectively) and therefore no results are recorded for these strains. The results for isolate 1 indicated a sensitivity to basic pH changes as the addition of NaOH caused a colour change in the pigment produced.

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The diffusible pigments produced by isolates 560 and 611 were sensitive to acids as suggested by the change in pigment colour when HCl was added. Isolate 257 was sensitive to alkaline conditions as suggested by the change in pigment colour when NaOH was added. Isolate 579 was sensitive to both HCl and NaOH.

Table 3.6: ISP 6 and 7 testing the melanin production, and ISP 9 results showing the carbon source reaction.

	ISP6	ISP7	ISP9		
Isolate			Control	Aloe	Arabinose
1	✓	pinkish tint ✓	✓*	→	+++
9	×	✓	✓	→	+++
257	✓	×	✓*	→	+++
507	no growth	×	✓	→	+++
560	✓	✓	✓	→	+++
563	no growth	no growth		contaminated	
579	no growth	no growth	✓	→	→
590	no growth	no growth		no growth	
611	✓	✓	✓	→/ (+)	++/+++
789	✓	✓	✓	→	→

*Indicates very slight growth; +: poor, ++: moderate, +++: good growth in comparison to the controls. Arrows indicate no change in growth compared to the controls.

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Table 3.6 shows the results of growth on ISP 6,7, and 9 media. ISP 6 and 7 were used to test for melanin production, with the main difference between the two media being that isolates are required to synthesize the precursor tyrosine in ISP 6, while tyrosine is supplied in ISP 7. As seen in the table, some isolates had difficulty growing on these media and therefore have no results. Isolates 1, 560, 611, and 789 were able to produce melanin on both media, while isolate 9 only produced on ISP 7 which would imply this strain is not able to synthesise the precursor. Isolate 1 produced a pigment with a pinkish tint on ISP7 which is unusual as melanin typically has a brown or blue-black sheen (Shirling and Gottlieb, 1966). ISP 9 was used to test for the utilization of a specific carbon source (L-arabinose) which is a key polysaccharide in plants (Rautengarten *et al.*, 2017). L-arabinose was chosen for its presence in *Aloe ferox* gel as determined by a study conducted by O'Brien *et al.*, 2011. The controls for ISP 9 included media without a carbon source for a negative control and plant extract as a positive control. However, future studies should include the use of a positive control containing a pure single carbon source such as glucose.

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Isolate 1 produced melanin on both ISP 6 and 7 which differs from the second closest relative, *S. africanus*, which did not produce melanin or diffusible pigment on any media (Meyers *et al.*, 2004). Isolate 9 was negative on ISP 6, similar to the closest relative *S. microflavus,* but positive for ISP 7 unlike the closest relative ((Smaoui *et al.*, 2012). Isolate 507 was unable to grow on ISP 6 and did not produce melanin on ISP 7 similar to its closest relative. Isolates 560, 611, and 789 were positive on both ISP 6 and 7. These isolates were all closely related to *Streptomyces pratensis,* which produced a positive result on tyrosine agar (ISP 7) (Tork *et al.*, 2018). Isolates 563, 579, and 590 were all unable to grow on ISP 6 and 7.

All isolates, except isolates 563 and 590, were able to grow on the ISP 9 plates. The isolates had minimal to no growth changes when the media was supplemented with aloe sap as the carbon source. However, when L-arabinose was used as the carbon source, a significant growth increase was seen for most of the isolates indicating most isolates were able to grow on one of the primary carbon sources present in aloe leaves. Isolates 579 and 789 did not display a growth increase on either media. The type strain of *S. microflavus* identified by Smaoui and associates, as well as a strain of *A. nivea* identified by (Niu *et al.*, 2020), was able to utilize L-arabinose, glucose, and glycerol as sole carbon sources similar to their closest relatives isolate 9 and 507, respectively.

Along with the ISP tests, the optimal growth temperature and pH were tested on Bennetts media, represented in Table 3.7.

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Table 3.7: Optimal growth temperature and pH levels as seen on Bennetts media.

			pH7				pH 4.3	pH 5	pH 9	pH 11	pH 13
Isolate	4°C	10°C	15°C	28°C	37°C	55°C					
9		+++	+++	+++	+++		+	+++	+++	+++	+++
257		++	++	++	++		-	++	++	+++	+++
507		+	+++	+++	+++		+++	+++	+++	+++	+++
560		++(+)	+++	+++	++		-	-	+++	++	+++
563		+++	+++	+++	+++		-	-	+++	+++	+
579		+++	++	+++	++		-	++	+++	+++	+++
611		++	+	++	+		-	+	+++	+++	+++
789		+++	+++	+++	+++		-	-	++(+)	+++	+++

* Growth is indicated as +: poor, ++: moderate, +++: good**,** -: no growth

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Isolates 1 and 590 have no results as they stopped growing. Isolate 789 was interesting at 37°C as it created a sort of gradient in colour as seen in Figure 3.5. As expected, the strains were mesophilic and grew optimally from 15-37°C, no growth was detected at 4°C, with a few strains growing relatively strongly at 10°C (isolates 9, 563, 579 and 789). The isolates were able to grow in alkaline conditions with the optimum pH being between 7.0 and 13.0 (pH 11.0 for isolate 563). Most isolates were unable to grow in acidic conditions except for isolate 507 which had strong growth at pH 4.3 and 5 and isolate 9 had strong growth at pH 5. This finding is interesting as the soil used for isolation was slightly acidic.

Figure 3.5: Isolate 789 grown on Bennetts media at 37°C. No pigment production is seen around the "lawn" of vellum to light beige colonies, while a dark crimson to brown pigment can be seen around the less condensed colonies that appeared to be light camouflage red.

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3.4 Metagenomic DNA extraction and amplification

Metagenomic DNA extractions were performed on plant and soil samples. The DNA was electrophoresed on a 1.8% gel to confirm that the extracted DNA was microbial in origin and not plant DNA. It also confirmed that the DNA was not degraded and did not contain inhibitors which would prevent PCR. While the extractions were successful for soil samples, as determined by the presence of high molecular weight DNA as seen in Figure 3.6 (lanes 7, 8, 10, 11, 13, and 14), DNA extraction from plant samples was more difficult and no metagenomic DNA was detected on the gels (lanes 2-4). Following the extractions, the DNA was subjected

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to 16S rRNA gene amplification which was successful for soil samples but not for plant samples. The 16S rRNA metagenomic results of the soil samples can be seen in Figure 3.7.

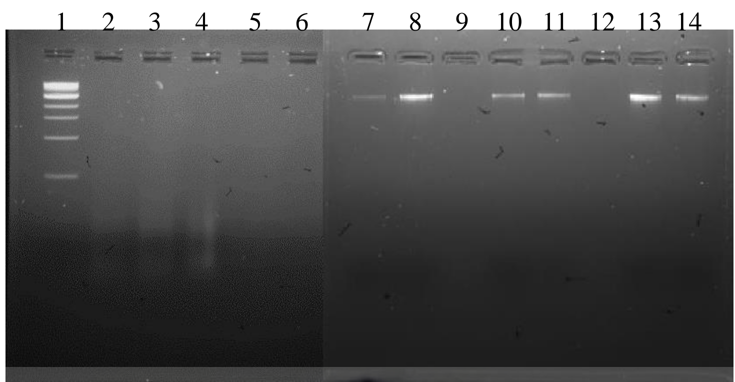

Figure 3.6: Gel image of plant and soil metagenomic DNA. Lane 1 includes the DNA marker. Lanes 2-4 show the plant DNA for samples BP1, TP1, and WP1. Lanes 7 and 8 are soil samples B1 and 2. Lanes 10 and 11 are soil samples T1 and 2. Lanes 13 and 14 are soil samples W1 and 2 respectively.

Microbial DNA from symbionts is notoriously difficult to isolate from plant material due to the presence of rigid plant cell walls. In addition, the cellulose, lipids, proteins, and polyphenols contained in the cell wall act as contaminants when extracting DNA (Heikrujam *et al.*, 2020). Figure 3.6 shows smears for the plant DNA in lanes 2-4 which could be a result of sample degradation, or a low concentration of sample extracted. DNA from soil samples was successfully extracted with clear bands being present on the gel image.

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Despite not seeing clear bands for the plant DNA samples, both the plant and soil DNA were used for 16S rRNA gene PCR. PCR for plant samples did not produce clear or significant bands and was therefore subjected to nested PCR. Unfortunately, this was also unsuccessful and as such, the plants samples were subsequently excluded from further investigation. The 16S rRNA gene of the soil samples was successfully amplified using Robust polymerase, chosen for its ability to amplify samples containing inhibitors especially prevalent in environmental samples. The resulting PCR products were electrophoresed and can be seen in Figure 3.7. While amplification was achieved it was relatively weak as observed by the faint bands, especially in lanes 6 and 7. The bands observed were subjected to gel clean-up using the using the Macherey-Nagel NucleoSpin gel and PCR Clean-up kit and the resulting product was used as a template for metagenomic cloning.

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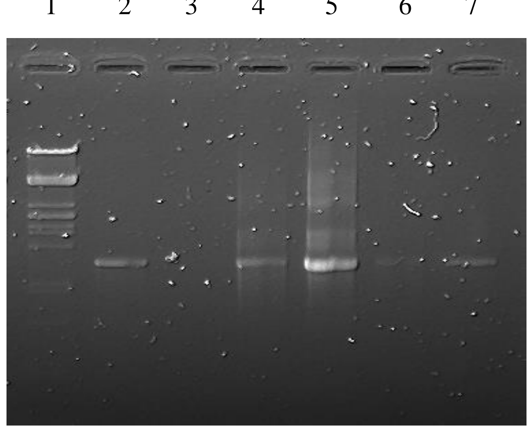

Figure 3.7: Gel image of 16S rRNA gene amplification of the metagenomic soil samples. Lane 1 includes λ Pst I DNA marker. Lane 2-3 includes the positive and negative controls respectively. Lane 4-7 include the soil samples B1,W1, T1.

3.5 Cloning of metagenomic DNA and sequencing

The purified PCR products of the soil samples were cloned using the pJet kit with electrocompetent and chemically competent dH5α *E. coli* cells. Due to the difficulty of the plant DNA extraction, the plant samples were not included in the cloning experiment. Cloning performed using electrocompetent cells and the standard procedure with Luria broth as the recovery media was unsuccessful. This was reattempted with fresh electrocompetent cells and produced the same results. Transformation was attempted again but this time using SOC media as the recovery medium, and this too did not yield the desired results. The procedure was repeated with a few modifications which included preheating the recovery media and further purification the ligation mix. Unfortunately, these changes were also unsuccessful. The fourth attempt involved the use of chemically competent cells along with the modifications to the procedure and this had limited successful as only a handful of transformants was obtained. Two colonies, labelled A and B, were selected from the W1 plate and the positive control plate for colony PCR. The W1 samples produced faint bands which resulted in samples being subjected to regular PCR as well as hot start PCR which can be seen in Figure 3.8.

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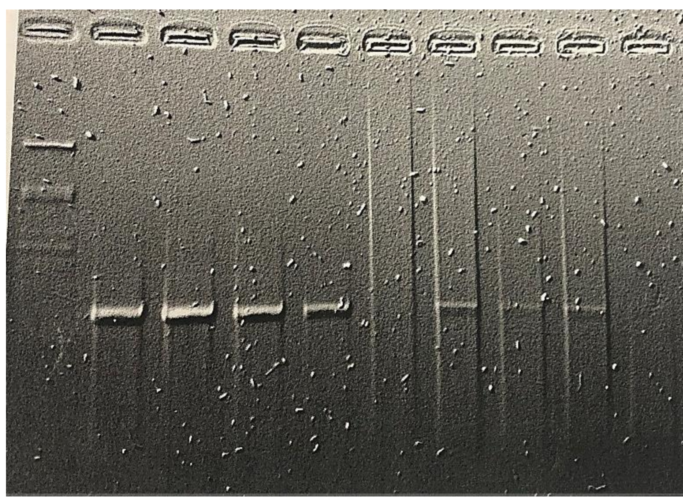

Figure 3.8: Gel electrophoresis image representing the amplified 16S rRNA gene of the metagenomic sample. Lane 1 includes the DNA marker. Lane 2-5 contains positive samples as colony A, A heated, B, and B heated. Lane 6 is a negative sample. Lane 7-10 contains the W1 samples as colony A, A heated, B, and B heated.

PCR clean up and sequencing was performed on the resulting amplicons obtained from the W1 plate. Good quality sequencing was obtained, and the clones were identified as *Pantoea eucrina* (86.96%) and *Pantoea dispersa* (84.05%) respectively. The genus *Pantoea* is made up of Gram-negative bacteria which have been isolated from a multitude of environments. Some species are able to produce antimicrobials and have bioremediation potential (Walterson and Stavrinides, 2015). While they have the potential to produce medically, agriculturally, and environmentally important products, they are not relevant to this study but could potentially be investigated in a separate study. These isolates were also identified at low similarities which could be an indication of novel strains of *Pantoea.*

3.6 Conclusion

As mentioned in Chapter 1 (section 2), there is a steady increase in the number of bacterial species becoming resistant to traditional antibiotics in clinical, as well as non-clinical settings. This has resulted to the need for novel antimicrobial compounds to effectively treat multi-drug resistant pathogens and combat rapidly emerging infections (Gohain *et al.*, 2019). Medicinal plants are seen as a unique source for the isolation of bioactive actinobacteria with the ability to provide these compounds (Abdalla and Mcgaw, 2018).

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One of the aims of this study was the isolation and characterisation of actinobacteria from *Aloe ferox*. Filamentous actinobacteria were successfully isolated and characterized using various established techniques. The 16S rRNA gene analysis identified the isolates as being similar to known antibiotic producing species. As expected for soil samples, most of the isolates were identified as *Streptomyces* species (Arifuzzaman *et al.*, 2010). Given the limitations of 16S rRNA gene analysis within the genus *Streptomyces,* further phylogenetic analysis is warranted, especially with isolates that have unique morphological features. To more accurately determine whether the isolates are known or novel species further ISP testing, and chemotaxonomy should be performed, as well as the analysis of additional housekeeping genes. Isolate 507 would be of particular interest as it was identified as being the most similar to *Amycolatopsis nivea* at 98.74% similarity, suggesting a likely novel strain. Full identification of this strain is warranted, however based on the current data we propose that Isolate 507 represents a novel *Amycolatopsis* species and the name *Amycolatopsis aloensis*^{T} is proposed. While only one of the isolates was identified as a novel strain, the results confirm that medicinal plants, specifically *A. ferox,* are a potential source of novel actinobacteria. Future studies should aim to optimise the isolation and maintenance of endophytes as well as the screening of other indigenous *Aloe* species which have been used medicinally.

When considering the limitations of culture-based screening, metagenomic DNA analysis was used in an attempt to identify unculturable isolates. However, despite numerous attempts to optimise all steps of the metagenomic DNA analysis, including modifying the DNA extraction protocol, use of a special DNA polymerase and many attempts to clone the resulting amplicons, it was unsuccessful. Only a low number of transformants were obtained and it was decided to rather focus on the analysis of culturable isolates. Metagenomic analysis should be reconsidered in future studies, using the protocols tested here as a starting point. In addition, new DNA extraction kits, specifically designed to extract DNA from environmental samples (including plant material) are now available which should be tested.

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Chapter 4: Results and discussion – Screening of actinobacterial isolates

4.1 Antimicrobial screening

The increasing global crisis of antibiotic resistance has led to the need to find new antimicrobial compounds, with medicinal plants seen as a good source of microbial (and thus bioactive) diversity. As previously mentioned in Chapter 1, actinobacteria are known to produce an array of antibiotics with streptomycetes producing ~80% of the currently available antimicrobials (Arifuzzaman *et al.,* 2010; Baskaran *et al.*, 2011).

In this study, antimicrobial screening was performed using perpendicular streaks as a primary screening method. This was followed by performing standard agar overlay assays on selected isolates against the six pathogenic test strains. The media used for antimicrobial screening was chosen based on previous screening results which indicated that activity was observed on SCN, oats, and YEME media (Isaacs, 2016). It should be noted that as the composition of each media is highly varied, providing alternate sources of carbon, nitrogen, and micronutrients which could affect the production of secondary metabolites by the isolates.

Based on the variety of media used for screening activity, the isolates performed better on specific media. Table 4.1 shows the highest average zone size (mm^2) for these isolates on their best performing media. These results lead to ten isolates being selected for further screening and identification.

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Table 4.1: Antimicrobial screening results against selected test strains.

Isolates	*Acinetobacter baumannii*	*Candida albicans*	*Enterococcus faecalis Van A*	*Escherichia coli*	*Mycobacterium aurum*	*Staphylococcus aureus*
1	-	115.45S	1161.6S	678.58Y	1021.02Y	668.37Y
9	181.43S	-	565.49S	565.49S	-	-
257	170.43Y	-	106.53O	2563.54S	-	319.66Y
507	3715.72Y	3760.1Y	247.4Y	1492.26S	-	804.25Y
560	202.63Y	-	1178.1S	-	181.43Y	-
563	270.98Y	-	1548.81S	-	-	-
579	812.89Y	395.84Y	574.13Y	467.71S	98.17Y	1272.34Y
590	-	1617.13Y	-	-	-	301.59O
611	1241.32Y	478.31Y	37.7O	-	93.46O	94.25S
789	-	-	-	1450.63S	-	2905.97S

*Zone sizes are given in mm^2. The S, O, and Y indicates the media (SCN, Oats, and YEME) on which these results were obtained.

The results in Table 4.1 represent the largest zone of activity for all isolates against the selected test strains. It was observed that the highest activity was detected on YEME and SCN media, indicating greater antibiotic production on these media. The ten selected isolates were active against multiple test organisms which could indicate that several different antimicrobial compounds are produced or that the compounds have a broad activity spectrum. Isolates were selected for further screening based on the high activity (zones larger than 1000 mm^2) they displayed against specific test strains.

The highest activity against *A. baumannii*, *C. albicans,* and *M. aurum* was seen on YEME media, while large zones of activity for the two enteric species, *E. faecalis* Van A and *E. coli,* were seen on SCN media. Isolate 507 produced the most significant activity against *A. baumannii* and *C. albicans* with zones of activity above 3500 mm^2. It also had moderate to high activity against *S. aureus* and *E. coli*. These results show the potential of the isolate to produce a broad-spectrum antibiotic(s) or the ability to produce several different antimicrobials. It should be noted that this isolate was most closely related to *Amycolatopsis nivea*, but no bioactivity has been reported for this species.

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Isolate 1 had activity against yeast and bacterial pathogens with the highest activity against Gram positive bacteria. When looking at its the closest relative, *Streptomyces afghaniensis*, Vijayakumar and colleagues (2012) reported activity against various Gram negative and positive bacteria as well as yeast, including *E. coli* and *C. albicans*. Isolate 9 had no activity against yeast, with low to moderate activity against bacteria. A study by Smaoui (2012) reported that antimicrobial compounds produced by its closest relative, *Streptomyces microflavus*, were active against Gram positive and negative, as well as fungi. Isolate 560 is also closely related to *S. microflavus* however, isolate 560 had no antifungal activity, with little to no activity against bacteria except the high activity against *E. faecalis*. This high level of activity might be indicative of a narrow spectrum antibiotic produced by the isolate which is worth further investigation.

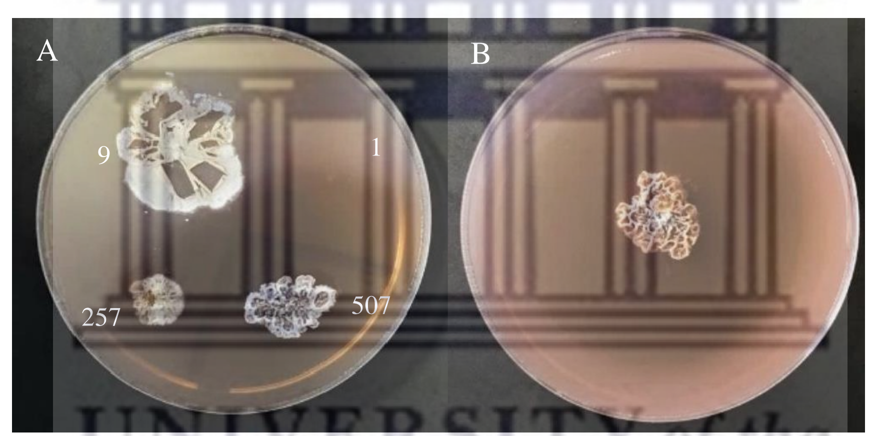

Figure 4.1: The activity of selected isolates against *Candida albicans* as tested on YEME media. A) Isolates 9, 1, 507, and 257 (arranged clockwise). No growth is seen for isolate 1 while the activity of isolate 507 is seen to overlap surrounding isolates. B) Isolate 507 grown individually in order to more accurately quantitate the zone of activity.

Isolates 579 and 590 were both identified as being most similar to *S. ambofaciens* which was shown to have strong activity against *C. albicans,* as well as both Gram negative and positive bacteria (Aouiche *et al.*, 2012). While isolate 579 had moderate activity against all test pathogens with high activity against *S. aureus,* 590 had no to low activity against bacteria with high activity against yeast. The high activity of isolate 590 against yeast might also indicate a narrow spectrum antibiotic but future screening against other pathogenic fungi is needed.

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Isolates 611 and 789 are both closely related to *S. pratensis* which, under metal stress, was found to produce novel antimicrobial compounds with activity against Gram positive and negative bacteria (Akhter *et al.*, 2018). Isolate 611 had low to moderate activity against Gram positive bacteria and yeast respectively, and high activity against Gram negative bacteria. On the other hand, isolate 789 displayed high activity against *E. coli* and *S. aureus* which implies that a possible broad range antibiotic or several compounds are produced by this strain. Future studies could focus on the isolation and identification of the active compounds. Isolate 789 produced interesting activity against *E. coli* as seen in Figure 4.2. It appears to have produced a "reverse halo" which could be due to the release of metabolites from the isolate which promoted the growth of the pathogen.

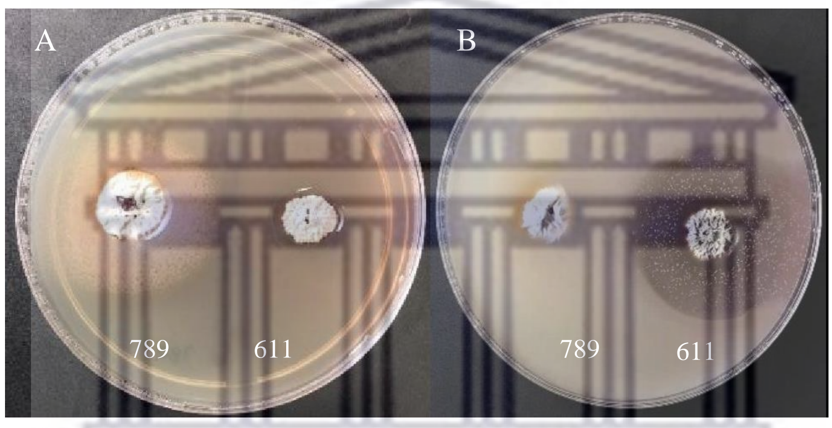

Figure 4.2: The activity of isolates 789 and 611 as seen on YEME media. A) Activity against *Escherichia coli* where a "reverse halo" can be seen around isolate 789. B) Activity of isolate 611 against *Acinetobacter baumannii*.

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4.2 Enzyme activity

Along with being prolific producers of antibiotic compounds, actinobacteria also produce a variety of industrially important enzymes. This includes enzymes such as xylanase, amylase, pectinase, and cellulases which were tested in this study. Figure 4.3 and 4.4 represents the zones obtained from enzyme screening as well as siderophore activity of the selected isolates.

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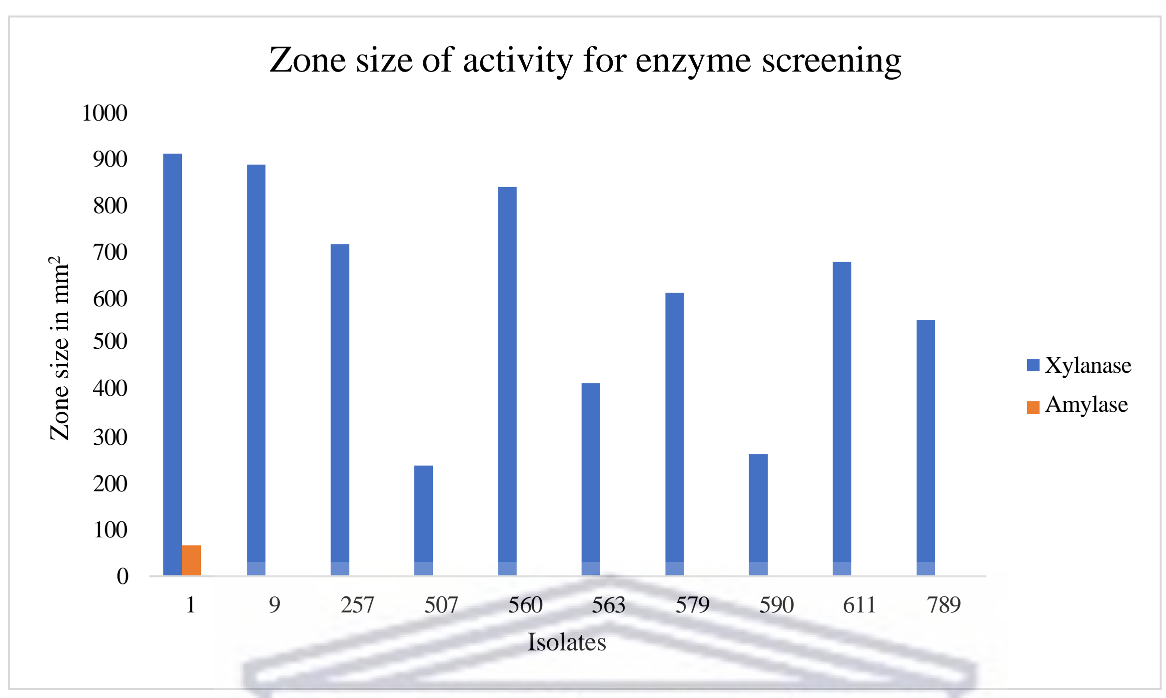

Figure 4.3: A bar graph representing the zones of activity obtained during amylase and xylanase screening (in mm^2) using soluble starch and beechwood xylan, respectively.

It was observed that none of the isolates were able to hydrolyse pectin or CM cellulose. Pectin and cellulose are abundant compounds within the cell wall of plants, and studies have shown that many plant-associated microbes can hydrolyse these substrates (Bhadrecha *et al.*,2020). The lack of activity could potentially suggest that the incorrect substrates were used/ the isolates may not be able to hydrolyse these specific substrates under the tested conditions (Lynd *et al.*, 2002). Future studies could therefore be done using different types of pectin (including pectin extracted from *Aloe ferox*) and cellulose, as well as testing this activity in a range of conditions (temperatures, pH etc) to determine if this may influence activity.

The results for amylase activity are unexpected when compared to the isolation results which showed that the isolates preferred SCN as a growth medium. However, only isolate 1 was able to hydrolyse starch (66.76 mm^2), despite both media containing soluble starch. This could indicate that the actinobacteria tested require specific components within the SCN media in order to hydrolyse the starch, which are not present in the Bennet's media which was used to test enzyme activity.

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The results for xylanase activity indicate the 70% of the isolates had zones of activity larger than 500 mm^2. Isolates 1, 9, 254, and 560 produced the largest zones with 911.85 mm^2, 888.29 mm^2, 716.28 mm^2, and 850 mm^2, respectively. It is interesting to note that when the strains were initially screened, they appeared to have significantly higher activity (isolates 1, 9, and 254 produced much larger zones with 1608.5 mm^2, 1143.54 mm^2, and 1470.26 mm^2, respectively). This could be an example of how the potency of secondary metabolites produced by isolates decreases with sequential subculturing/long term maintenance on agar.

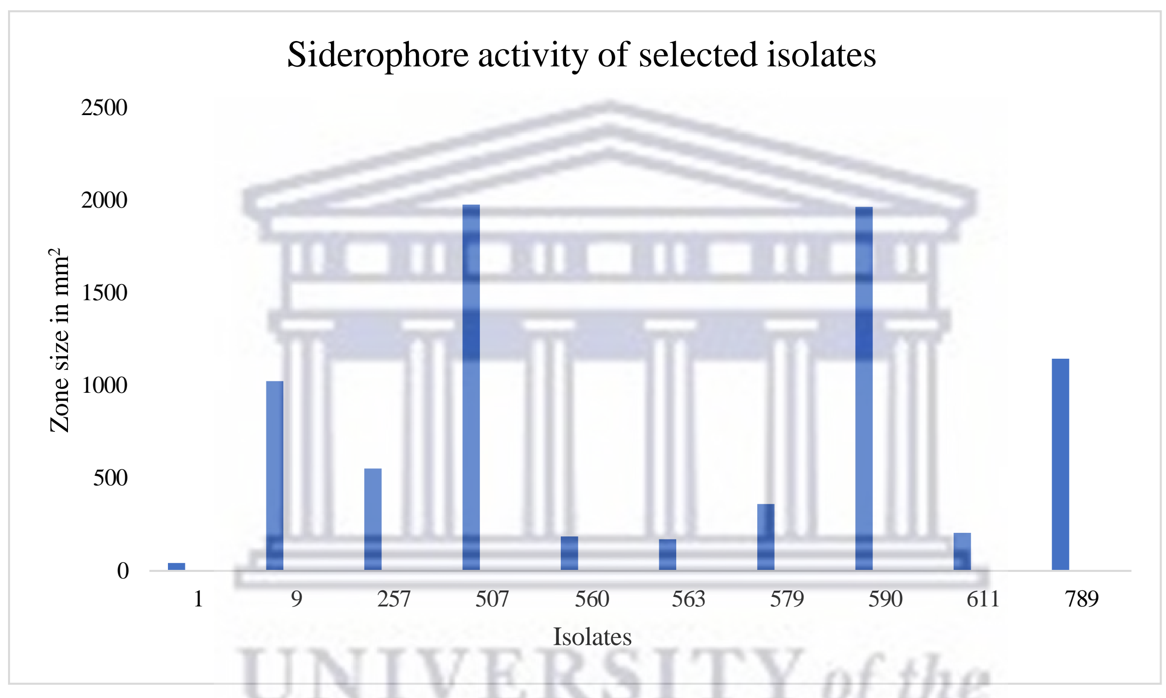

Figure 4.4: A bar graph representing the zones of activity obtained during siderophore screening.

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Siderophores aid in the uptake of iron by binding to Fe^{3+} present in the environment (Wang *et al.*, 2014). Figure 4.4 provides the siderophore results for all the strains. Looking at Figure 4.5 it is interesting to note the large activity zones sizes produced for some isolates despite the small size of colonies. Isolates 9, 507, 590, and 789 (Figure 4.4) could be of particular interest as they had the strongest activity, with zones above 1000 mm^2. Future studies focusing on plant growth promotion and the identification of the specific structure of the siderophore compound could be performed on these isolates.

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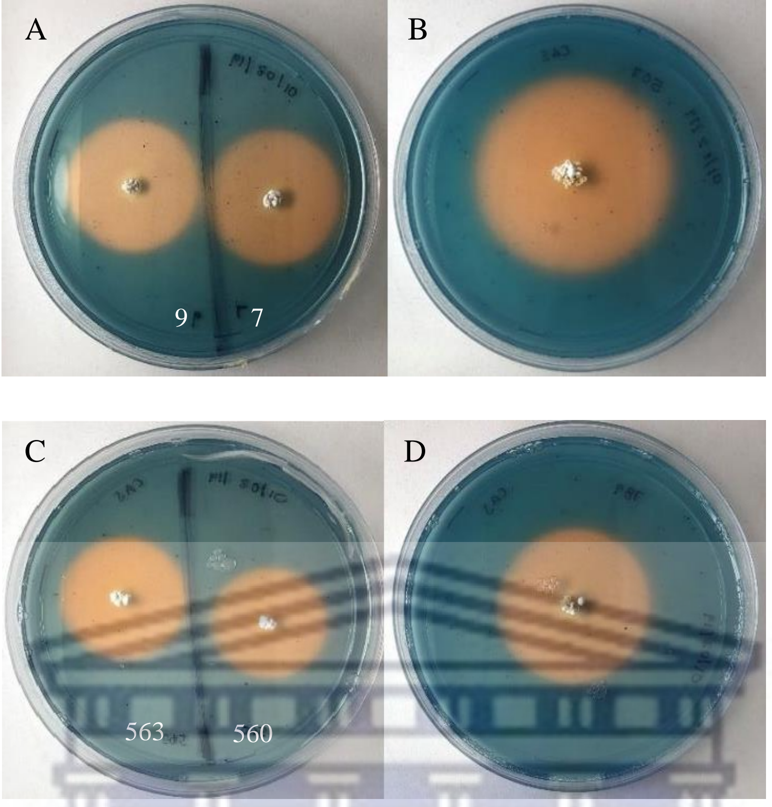

Figure 4.5: Zones of activity seen on CAS plates testing for siderophore activity. A) Isolates 9 and 7, B) isolate 507, C) isolates 563 and 560, and D) isolate 789.

4.3 Antimicrobial solvent extractions

Solvent extractions were performed for the top isolates and these extractions were tested using the Kirby-Bauer disc diffusion method (where 50 µl of the extract was spotted), and the well diffusion method (where 100 µl was added to each well). Unfortunately, the results obtained for the disc diffusion assay were inconclusive, with the only clear zone obtained for the positive controls and a small indistinct zone for isolate 579. It can be noted that the lack of results for the disc diffusion does not necessarily mean no compounds were extracted. It could indicate that low amounts of the compounds were isolated, that they were overly diluted, or that the compounds were bound to the disc and not available for diffusion. The latter explanation appears likely for some isolates when taken in conjunction with the well diffusion results.

The well diffusion method was used as an alternative screening method and proved to be more successful. The zones obtained for this method can be seen in Tables 4.2 and 4.3.

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Positive controls included vancomycin for *E. coli* and *C. albicans*, polymyxin B for *E. faecalis*, and ampicillin for *A. baumannii.* Negative control included disks made using extractions of uninoculated media.

Table 4.2: Zones of activity for well diffusion (mm^2) against *A. baumannii* and *C. albicans*.

Isolates	***Acinetobacter baumannii***			***Candida albicans***		
	Chloroform	**Ethyl acetate**	**Methanol**	**Chloroform**	**Ethyl acetate**	**Methanol**
579	42.67	103.41	-	78.8	42.02	32.23
611	85.06	130.12	-	109.43	146.08	-
Negatives	109.56	145.73	-	99.34	16.11	-

* Not included in the table are the results for the positive controls for each test strain which are 256.45 for *A. baumannii* and 285.88 for *C. albicans.*

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Table 4.3: Zones of activity for well diffusion (mm²) against E. faecalis and E. coli.

Isolates	*Enterococcus faecalis Van A*			*Escherichia coli*		
	Chloroform	**Ethyl acetate**	**Methanol**	**Chloroform**	**Ethyl acetate**	**Methanol**
1	50.27	156.56	67.55	-	-	-
9	37.31	58.51	65.97	-	-	-
257	-	-	-	73.3	39.8	24.87
560	50.79	48.96	60.52	-	-	-
563	50.27	43.46	120.17	-	-	-
579	-	-	-	151.58	172	40.1
789	-	-	-	54.98	75.02	32.98
Negatives	-	22	134.3	55.24	138.63	22.78

* Not included in the table are the results for the positive controls for each test strain which are 301.6 for *E. faecalis* and 18.85 for *E. coli.*

Based on the results in Tables 4.2 and 4.3, isolates that produced activity for *A. baumannii, C. albicans,* and *E. faecalis* had significantly lower activity when compared to the respective positive controls. Due to the fact that concentrated purified antibiotics were used for positive controls and the test compounds were crude extracted generated from small test cultures this is not unexpected. Conversely, activity produced against *E. coli* was slightly higher than the activity produced by the positive control. While this increase in activity might not appear very significant, it is significant given the scale of extraction. It could be worth looking at in the future to optimize growth and extraction methods on a larger scale as the anti-*E. coli* compound being produced appears to be highly potent.

As seen in Table 4.2, the chloroform and ethyl acetate extracts from isolates 579 and 611 both had activity against *A. baumannii* and *C. albicans*. However, only isolate 579 had activity against *C. albicans* when methanol was used for the cell mass extractions. In Table 4.3 it can be observed that isolates 1, 9, 560, and 563 were active against *E. faecalis,* while isolates 257, 579, and 789 were active against *E. coli*.

In addition, the ethyl acetate extract of isolate 1 produced the highest activity against *E. faecalis,* while the methanol and chloroform extracts had moderate activity. It was also

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determined that these extracts did not display activity against any of the other pathogens tested. When comparing these results to that of *S. afghaniensis*, it is clear that the ethyl acetate extracts have the highest activities for both strains. However, isolate 1 did not produce any activity against *C. albicans* unlike *S. afghaniensis* (Vijayakumar *et al.*, 2012). The solvent extractions for isolate 9 had moderate activity against *E. faecalis*, but no activity against any of the other pathogenic strains was observed. This is interesting as a study by Atta and Yassen (2015) isolated the antibiotic irumamycin from the ethyl acetate extraction of *S. microflavus* (isolate 9's closest relative). The antibiotic had activity against a wide range of bacterial pathogens as well as yeast strains – however, this compound was detected from large scale production (100 litres) in very rich production media (containing glycerol, glucose, and soybean meal) which could account for the lack of production detected in the current study.

As previously mentioned, (Section 4.1), Akhter and colleagues (2018) identified antimicrobial compounds active against various Gram positive and negative bacteria in the ethyl acetate extraction of *S. pratensis*. Isolates 560, 611, and 789 were all closely related to *S. pratensis.* Isolate 560 produced a compound with moderate activity against *E. faecalis,* while isolate 789 had moderate activity against *E. coli*. The chloroform and ethyl acetate extracts from isolate 611 was also found to have high activity against *A. baumannii* and *C. albicans*.

Similarly, the chloroform and ethyl acetate extracts of isolate 563 had moderate activity against *E. faecalis*, while the methanol extract had very strong activity. From the literature no activity has been reported for *S. fractus*, however, the ethyl acetate extract of *S. endophyticus* was shown to be active against Gram positive and negative bacteria (Charousová *et al.*, 2017).

Lastly, the extracts from isolate 579 had moderate activity against yeast and high activity against Gram negative bacteria. A study by Wei and associates (2015) isolated a novel telomycin-like antibiotic, ambobactin, from *S. ambofaciens*. This was achieved by the elution of the filtrate using methanol and running the concentrate on silica gel column. This was repeated for the active compound with a mixture of methanol and ethyl acetate and finally a mixture of methanol and acetic acid. Future work could apply this production and purification protocol for isolate 579 to determine whether it is producing the same/similar compounds.

When comparing the results of the well diffusion assays to that of the overlays in Table 4.1, we can see a significant decrease in activity. It can also be observed that for some isolates the

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activity detected in the stab inoculations was not detected in the solvent extracts. This could be a result of a generational decrease in potency of the secondary metabolite activity or a low amount of compounds being extracted. It has previously been noted that liquid broth provides more accessible nutrients for isolates compared to solid media due to aeration and agitation of the media. It would therefore be easy to assume higher levels of activity should be seen for isolates grown in liquid media. However, while low availability of nutrients might hinder the growth of isolates, the same can be said for an excess amount of nutrients (Bonnet *et al.*, 2020). The growth and extraction conditions should therefore be further optimized to potentially increase the extraction of compounds and subsequently increase activity. The results obtained for the negative controls could be due to spillage of the solvents when plating.

4.4 Thin layer chromatography (TLC) and bioautography

To further study the compounds presents in the solvent extractions, the extracts of isolates 257, 579, and 789 were subjected to thin layer chromatography and subsequent bioautography analysis against *E. coli*. Figure 4.6 shows the TLC plate results of the bioautography after staining with MTT (0.25%).

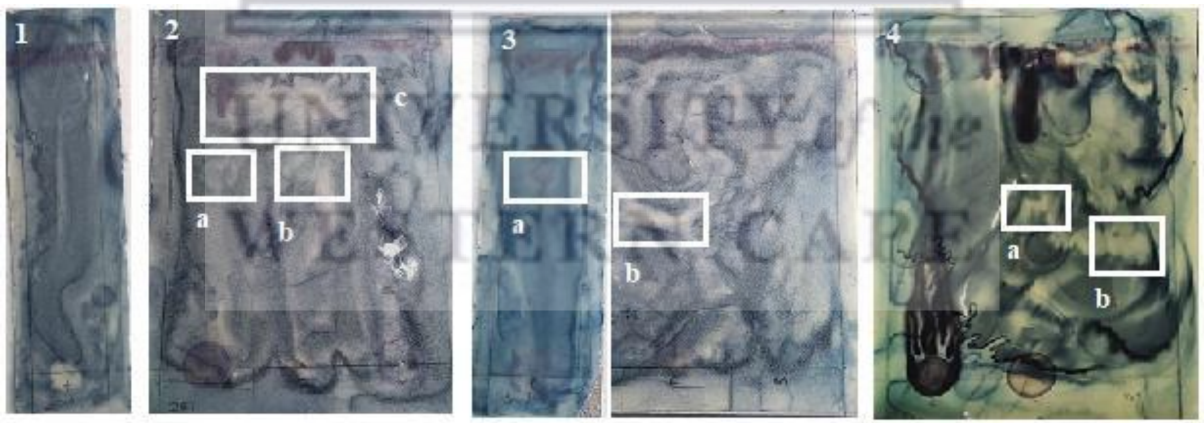

Figure 4.6: Bioautography plates of isolates 257, 579, and 789. 1) Positive control, vancomycin. 2) Isolate 257 with the R_f value 0.6 for chloroform (2a) and 0.61 for ethyl acetate (2b). The large "zone" (2c) that appears to be between the chloroform and ethyl acetate lanes has a $R_{f\ value}$ of 0.85. 3) Isolate 579 with the R_f value 0.65 for chloroform (3a) and 0.46 for ethyl acetate (3b). 4) Isolate 789 with R_f value 0.52 for ethyl acetate (4a) and 0.43 for methanol (4b).

Isolates 257, 579, and 789 all produced two clear individual bands when tested against *E. coli.* Isolates 257 and 579 had bands for the chloroform and ethyl acetate extract but unfortunately

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not the methanol extract which had activity for the well diffusions assay. Isolate 257 also produced a band that appears between the chloroform and ethyl acetate lanes but most likely came from the second lane (ethyl acetate). This band could be due to running error where the extract ran into neighbouring lanes. Isolate 789 produced bands for the ethyl acetate and methanol extracts, but not the chloroform extract which displayed activity for the well diffusion assays. It can be noted that the "flames" seen on isolate 789 could be caused by overloading the sample. Further studies could include the purification and identification of the isolated compounds using LC-MS and/or HPLC, with results from this initial TLC analysis being used to show how many compounds are present, which solvent system to use and when the compounds are likely to be eluted based on their apparent polarity

4.5 Conclusion

The secondary metabolites produced by actinobacteria have a multitude of bioactive functions in nature and are also important in a wide range of industries. One of the aims of this study was to screen for bioactivity of actinobacterial isolates. This includes antimicrobial as well as enzymatic activity. The production of these secondary metabolites is, however, dependent on the environmental/cultural conditions in which the actinobacteria are grown (Barka *et al.*, 2015; Wohlleben *et al*., 2017). To account for this factor, antimicrobial screening was conducted on various media that differed in their composition of carbon and nitrogen sources.

The varying compositions of the media were used to test the effect of different food sources had on the activity of isolates. It is interesting to note that most activity was seen on YEME media. This could indicate that the composition of YEME media aided in the production of secondary metabolites. Some interesting antimicrobial results were discovered, and future studies should focus on these. Of particular interest is the compounds produce by isolates 257 and 789 which appear to have potent, narrow-activity spectrums such as *E. coli* and *S. aureus*, respectively. In addition, isolates 507 and 579 appear to be producing either broad-spectrum or several potent antimicrobials which should be investigated.

Surprisingly these isolates appeared to have relatively weak enzyme production, with no cellulase or pectinase activity being detected. Some isolates displayed significant xylanase and siderophore activity which should be considered for further study to identify the active

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compounds. The siderophore activity is of particular interest from an agricultural-biotechnology standpoint as there is growing interest in siderophore-producing native strains (with GRAS status) for inclusion in biofertilizers (A. Burger, personal communication). It should also be noted that isolates 507 and 590 were included in a subsequent MSc study which focused on plant-growth promoting actinobacteria and the strains performed well in plant growth promoting assays (M. Jupin, personal communication).

While there is a high rate of re-isolation of *Streptomyces* species, it is easy to see why they remain a source of interest for bioactive compounds. For example, isolate 579 is most closely related to *S. ambofaceins.* In a previous study a novel telomycin-like antibiotic, ambobactin, was isolated from *S. ambofaciens* (Wei *et al.,* 2015). Given that the methanol extracts from isolate 579 has similar activity profiles as to what was reported for ambobactin future studies should be performed to optimise the growth conditions/purification conditions to determine whether this strain is producing an ambobactin-like compound or a novel compound.

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Chapter 5: General Conclusion

The discovery of antibiotics has been one of the major advances in medical history. They have positively affected life expectancy by not only influencing the outcome of infectious diseases, but also facilitating advances in medical practices. However, the misuse of available antibiotics over the years has led to the increase of multidrug resistant pathogens. This has subsequently narrowed the availability of effective treatments for diseases, increasing the need to find novel bioactive compounds (Rossolini *et al.*, 2014). As stated in Chapter 1 (section 1), natural products, including secondary metabolites produced by microorganisms, remain the most successful sources of these compounds (Gould and Bal, 2013; Spellberg and Gilbert, 2014). Actinobacteria are known producers of potent secondary metabolites and thus remain a source of interest. In order to avoid the re-isolation of known species, novel sources of isolation are required. Medicinal plants, particularly those from unique environments have been proposed as an ideal source to screen (Abdalla and Mcgaw, 2018).

This study aimed to isolate and characterize bioactive actinobacterial organisms from a medicinally important plant, namely *Aloe ferox*. This was achieved through the use of well-established isolation and screening techniques. The results revealed the successful isolation of 879 bacteria, most of which were filamentous actinobacteria. A large number of isolates (822) were obtained from soil samples, while 57 were isolated from leaf samples. It was also noted that a high number of isolates were obtained from the soil samples of the TP2 and WP2 in comparison to the TP1 and WP1 samples that were roughly around the same location. Sixty-one strains were selected for antimicrobial testing based on their morphological characteristics. They were shown to have significant antimicrobial activity against various human pathogens. The isolates were also shown to have enzymatic activity including xylanase and siderophore activity.

Using 16S rRNA gene analysis, 80% of the selected isolates were identified as being most closely related to known *Streptomyces* species, as expected for soil samples. (Arifuzzaman, Khatun and Rahman, 2010). Interestingly, half of the identified isolates (isolates 1, 9, 579, and 590) were found to be closely related to known antibiotic producers. The remaining isolates were either not related to antibiotic producers or no literature is currently available on their activity. Among the identified isolates, isolate 507 was proposed as a novel

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Amycolatopsis species due to its low identity percentage (98.74%). The name *Amycolatopsis aloensis* is proposed for this isolate. Future work will include the full polyphasic characterisation of the isolate and publication of the species description.

The selected isolates were screened for antimicrobial activity against a variety of human pathogens. This included *Acinetobacter baumannii, Candida albicans, Enterococcus faecalis Van A, Escherichia coli, Mycobacterium aurum,* and *Staphylococcus aureus*. The results in Table 4.1 (Chapter 4, section 1) only show the largest zones of activity for the isolates against these pathogens. It was noted that several of the isolates (1, 560, 563, 579, 590, and 611) displayed high activity (above 1000 mm^2) against either Gram positive, Gram negative, or yeast pathogens only. This could suggest that these strains produce antimicrobial compound(s) with a narrow activity spectrum. Conversely, isolates 507 and 789 had high activity against multiple strains, which led to the conclusion that they potentially produced several antimicrobial compounds or that their compounds have broad spectrum activity.

The enzymatic potential of the isolated actinobacteria was tested by screening the hydrolysis of compounds abundant in plants, including pectin, cellulose, starch, and xylan. It was observed that none of the isolates had pectinase or cellulase activity, while only isolate 1 had amylase activity. In comparison, all isolates displayed xylanase activity with 70% of the isolates producing significant zones of activity larger than 500 mm^2. Isolates also displayed great potential for siderophore activity, especially isolates 9, 507, 590, and 789 with zone sizes above 1000 mm^2.

Despite making use of established techniques throughout this study, there were limitations, and these should be combated in future studies. This includes the limitations of 16S rRNA gene analysis for species identification, especially within the genus *Streptomyces*. To more accurately distinguish between known and novel strains more extensive phylogenetic analysis, including the analysis of other housekeeping genes, should be considered in the future. Complete phenotypic screening as well as chemotaxonomy should also be performed. Lastly, to combat the limitation of culture-based screening, sequence based whole genome analysis should be considered for future studies. It is noted that attempts to perform metagenomic analysis in this study were unsuccessful, despite numerous attempts. This can be attributed to the fact that DNA extraction methods were not optimised for extraction from plants (especially those with high phenolics). As metagenomics is a rapidly advancing field, since we attempted this analysis several new kits, including those which are specific for

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extraction from plants have been developed. As such in future studies new methods (including protocols mentioned in Chapter 3, sections 4 and 5) and/or extraction kits should be considered.

While only one potentially novel strain was isolated in this study, this supports the idea that medicinal plant associated environments should be explored for the isolation of rare and novel actinobacteria. Based on the results obtained throughout this study, it is apparent why actinobacteria remain a source of interest when screening for bioactive compounds. Although known strains are constantly being reisolated from soil samples, they still have the potential to produce significant antimicrobial compounds. However, the novelty and potency of these compounds remains unknown until they are screened and characterized.

Future studies should focus on further characterizing and screening the activity of active isolates, with special emphasis on isolate 507 as it was proposed as a novel species. The analysis of additional housekeeping genes should be performed for a more accurate identification of isolates identified as *Streptomyces* species. Finally, focus should be put on the isolation and identification of active compounds produced by the actinobacterial strain, and screening should be extended to include a wider range of pathogenic organisms, including resistant strains.

The Fynbos biome is home to an estimated 8000 plant species. From our findings it is apparent that this represents an exciting, untapped source of novel bacteria. More widespread screening of these plants, especially medicinal species, is warranted.

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References

Abdalla, M.A. and Mcgaw, L.J. (2018) 'Bioprospecting of South African Plants as a Unique Resource for Bioactive Endophytic Microbes.', *Front. Pharmacol*, 9, p. 456. Available at: https://doi.org/10.3389/fphar.2018.00456.

Ahmad Khan, M.S. and Ahmad, I. (2018) 'Herbal Medicine: Current Trends and Future Prospects.', in *New Look to Phytomedicine: Advancements in Herbal Products as Novel Drug Leads*. Elsevier, pp. 3–13. Available at: https://doi.org/10.1016/B978-0-12-814619-4.00001-X.

Ait Barka, E., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H., Clément, C., Ouhdouch, Y. and Van Wezel, G.P. (2015) 'Taxonomy, Physiology, and Natural Products of Actinobacteria.' Available at: https://doi.org/10.1128/MMBR.00019-15.

Akhter, N., Liu, Y., Auckloo, B.N., Shi, Y., Wang, K., Chen, J., Wu, X., Wu, B. (2018) 'Stress-Driven Discovery of New Angucycline-Type Antibiotics from a Marine *Streptomyces pratensis* NA-ZhouS1.', *Marine Drugs 2018, Vol. 16,* 16(9), p. 331. Available at: https://doi.org/10.3390/MD16090331.

Allkin, B. (2017) Useful Plants – Medicines: At Least 28,187 Plant Species are Currently Recorded as Being of Medicinal Use, *State of the World's Plants 2017*.

Alvan, G., Edlund, C. and Heddini, A. (2011) 'The global need for effective antibiotics - A summary of plenary presentations.', in *Drug Resistance Updates*. Churchill Livingstone, pp. 70–76. Available at: https://doi.org/10.1016/j.drup.2011.01.007.

Alvarez, A. Saez, J.M., Davila Costa, J.S., Colin, V.L., Fuentes, M.S., Cuozzo, S.A., Benimeli, C.S., Polti, M.A. and Amoroso, M.J. (2017) 'Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals.', *Chemosphere*, 166, pp. 41–62. Available at: https://doi.org/10.1016/J.CHEMOSPHERE.2016.09.070.

Amir, H.M., Grace, O.M., Wabuyele, E. and Manoko, M.L.K. (2019) 'Ethnobotany of *Aloe L.* (*Asphodelaceae*) in Tanzania.', *South African Journal of Botany*, 122, pp. 330–335. Available at: https://doi.org/10.1016/j.sajb.2019.01.038.

UNIVERSITY of the
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http://etd.uwc.ac.za/

Aouiche, A., Sabaou, N., Meklat, A., Zitouni, A., Mathieu, F. and Lebrihi, A. (2012) 'Antimicrobial activity of a Saharan *Streptomyces spp*. PAL111 strain against various clinical and toxinogenic microorganisms resistant to antibiotics.', *Journal de Mycologie Medicale*, 22(1), pp. 42–51. Available at: https://doi.org/10.1016/J.MYCMED.2011.12.077.

de Araújo, J.M.D., Silva, A.C.D. and Azevedo, J.L. (2000) 'Isolation of endophytic actinomycetes from roots and leaves of maize (*Zea mays L.*).', *Brazilian Archives of Biology and Technology*, 43(4), pp. 447–451.

Arifuzzaman, M., Khatun, M.R. and Rahman, H. (2010) 'Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity.', *African Journal of Biotechnology*, 9(29), pp. 4615–4619. Available at: http://www.academicjournals.org/AJB.

Arseneault, T. and Filion, M. (2017) 'Biocontrol through antibiosis: exploring the role played by subinhibitory concentrations of antibiotics in soil and their impact on plant pathogens.', *Canadian Journal of Plant Pathology,* 39(3), pp. 267–274. Available at: https://doi.org/10.1080/07060661.2017.1354335.

Atlas, R.M. (2010) *Handbook of microbiological media*. 4th New ed. Boca Raton: Taylor & Francis Inc.

Atta, H.M. and Yassen, A.M. (2015) 'Antimicrobial Activities of a *Streptomyces microflavus* Isolated From KSA: Taxonomy, Fermentation, Extraction and Biological activities.', *International Journal of Life Sciences*, 4(4), pp. 260–269. Available at: www.crdeep.com (Accessed: 28 November 2022).

Baltz, R. (2006) 'Marcel Faber Roundtable: Is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration?', *Journal of Industrial Microbiology and Biotechnology*, 33(7), pp. 507–513.

Bankar, A. and Nagaraja, G. (2018) 'Recent Trends in Biosorption of Heavy Metals by Actinobacteria', in *New and Future Developments in Microbial Biotechnology and Bioengineering: Actinobacteria: Diversity and Biotechnological Applications*. Elsevier, pp. 257–275. Available at: https://doi.org/10.1016/B978-0-444-63994-3.00018-7.

Barcroft, A. and Myskja, A. (2003) *Aloe Vera: Nature's Silent Healer*. London: BAAM.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Basilio, A., González, I., Vicente, M.F., Gorrochategui, J., Cabello, A., González, A. and Genilloud, O. (2003) 'Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity', *Journal of Applied Microbiology*, 95(4), pp. 814–823. Available at: https://doi.org/10.1046/j.1365-2672.2003.02049.x.

Baskaran, R. and Mohan, P.M. (2011) 'Enrichment Method for the Isolation of Bioactive Actinomycetes from Mangrove Sediments of Andaman Islands, India Precipitation isotopes and hydro-meteorological processes View project'. Available at: https://doi.org/10.21161/mjm.24410.

Bbosa, G.S., Mwebaza, N., Odda, J., Kyegombe, D.B. and Ntale, M. (2014) 'Antibiotics/antibacterial drug use, their marketing and promotion during the post-antibiotic golden age and their role in emergence of bacterial resistance.', *Health*, 06(05), pp. 410–425. Available at: https://doi.org/10.4236/health.2014.65059.

Benson, D.R. and Silvester, W.B. (1993) 'Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants.', *Microbiological Reviews*, 57(2), pp. 293–319. Available at: https://doi.org/10.1128/mr.57.2.293-319.1993.

Bérdy, J. (2005) 'Bioactive microbial metabolites: A personal view', *Journal of Antibiotics*. Japan Antibiotics Research Association, pp. 1–26. Available at: https://doi.org/10.1038/ja.2005.1.

Bhadrecha, P., Bala, M., Khasa, Y.P., Arshi, A., Singh, J. and Kumar, M. (2020) '*Hippophae rhamnoides* L. rhizobacteria exhibit diversified cellulase and pectinase activities', *Physiology and Molecular Biology of Plants*, 26(5), pp. 1075-1085. Available at: https://doi.org/10.1007/s12298-020-00778-2.

Bisen, P.S., Debnath, M. and Prasad, G.B. (2012) *Microbes: concepts and applications.* New Jersey: John Wiley & Sons.

Biswas, K., Choudhury, J.D., Mahansaria, R., Saha, M. and Mukherjee, J. (2017) '*Streptomyces euryhalinus* sp. nov., a new actinomycete isolated from a mangrove forest.', *Journal of Antibiotics*, 70(6), pp. 747–753. Available at: https://doi.org/10.1038/ja.2017.3.

http://etd.uwc.ac.za/

Blackall, L.L., Parlett, J.H., Hayward, A.C., Minnikin, D.E., Greenfied, P.F. and Harbers, A.E. (1989) '*Nocardia pinensis* sp. nov., an Actinomycete Found in Activated Sludge Foams in Australia.', *Microbiology*, 135(6), pp. 1547–1558. Available at: https://doi.org/10.1099/00221287-135-6-1547.

Bodiba, D., Szuman, K.M. and Lall, N. (2018) 'The Role of Medicinal Plants in Oral Care.', in *Medicinal Plants for Holistic Health and Well-Being*. Academic Press, pp. 183–212. Available at: https://doi.org/10.1016/B978-0-12-812475-8.00006-8.

Bonnet, M., Lagier, J.C., Raoult, D. and Khelaifia, S. (2020) 'Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology.', *New Microbes and New Infections*, 34, p. 100622. Available at: https://doi.org/10.1016/J.NMNI.2019.100622.

Cao, L., Qiu, Z., You, J., Tan, H. and Zhou, S. (2005) 'Isolation and characterisation of endophytic streptomycete antagonists of fusarium wilt pathogen from surface-sterilized banana roots.', *FEMS Microbiology Letters*, 247(2), pp. 147–152. Available at: https://doi.org/10.1016/j.femsle.2005.05.006.

Carvalho, J.C.T. (2020) 'Market Analysis on Traditional Medicine-2020', in *7th Asian Herbal and Traditional Medicines Summit*. Tokyo.

Ceylan, O., Okmen, G. and Ugur, A. (2008) 'Isolation of soil *Streptomyces* as source antibiotics active against antibiotic-resistant bacteria.', *Eurasian Journal of Biosciences*, 2(1), pp. 73–82.

Charousová, I. Medo, J., Halenárová, E. and Javoreková, S. (2017) 'Antimicrobial and enzymatic activity of actinomycetes isolated from soils of coastal islands.', *Journal of Advanced Pharmaceutical Technology and Research*. Available at: https://doi.org/10.4103/japtr.JAPTR_161_16.

Cheah, Y.K., Lee, L.H., Chieng, C.Y.C. and Wong, V.L.C.M. (2015) 'Isolation, identification and screening of actinobacteria in volcanic soil of deception island (the Antarctic) for antimicrobial metabolites.', *Polish Polar Research*. Available at: https://doi.org/10.1515/popore-2015-0001.

Chen, W., Van Wyk, B.E., Vermaak, I. and Viljoen, A.M. (2012) 'Cape aloes—A review of the phytochemistry, pharmacology and commercialisation of *Aloe ferox.*', *Phytochemistry Letters*, 5(1), pp. 1–12. Available at: https://doi.org/10.1016/J.PHYTOL.2011.09.001.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Cleenwerck, I. and De Vos, P. (2008) 'Polyphasic taxonomy of acetic acid bacteria: An overview of the currently applied methodology.', *International Journal of Food Microbiology*, 125(1), pp. 2–14. Available at: https://doi.org/10.1016/J.IJFOODMICRO.2007.04.017.

Cock, I.E. (2015) 'Novel Natural Products: Therapeutic Effects in Pain, Arthritis and Gastro-intestinal Diseases.', *Progress in Drug Research*, 70, pp. 179–235. Available at: https://doi.org/10.1007/978-3-0348-0927-6_6.

Cook, A.E. and Meyers, P.R. (2003) 'Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns.', *International Journal of Systematic and Evolutionary Microbiology*, 53(6), pp. 1907–1915. Available at: https://doi.org/10.1099/ijs.0.02680-0.

Cross, T. (1981) 'Aquatic *Actinomycetes*: A Critical Survey of the Occurrence, Growth and Role of *Actinomycetes* in Aquatic Habitats.', *Journal of Applied Bacteriology*, 50(3), pp. 397–423. Available at: https://doi.org/10.1111/j.1365-2672.1981.tb04245.x.

Dagne, E., Bisrat, D., Viljoen, A. and Van Wyk, B.E. (2005) 'Chemistry of Aloe Species.', *Current Organic Chemistry*, 4(10), pp. 1055–1078. Available at: https://doi.org/10.2174/1385272003375932.

Dold, A.P. and Cocks, M.L. (2002) 'The trade in medicinal plants in the Eastern Cape Province, South Africa.' *South African Journal of Science*, 98(11), pp. 589-897.

Doumbou, C.L., Akimov, V. and Beaulieu, C. (1998) 'Selection and Characterisation of Microorganisms Utilizing Thaxtomin A, a Phytotoxin Produced by *Streptomyces scabies.*', *Applied and Environmental Microbiology*, 64(11), pp. 4313–4316. Available at: https://doi.org/10.1128/AEM.64.11.4313-4316.1998.

Duraipandiyan, V., Sasi, A.H., Islam, V.I.H., Valanarasu, M. and Ignacimuthu, S. (2010) 'Antimicrobial properties of actinomycetes from the soil of Himalaya.', *Journal de Mycologie Medicale*, 20(1), pp. 15–20. Available at: https://doi.org/10.1016/j.mycmed.2009.11.002.

Edwards, C. (1993) 'Isolation properties and potential applications of thermophilic actinomycetes', *Applied Biochemistry and Biotechnology*, 42(2–3), pp. 161–179. Available at: https://doi.org/10.1007/BF02788050.

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

ElSohly, M.A., Gul, W. and Murphy, T.P. (2004) ‘Analysis of the anthraquinones aloe-emodin and aloin by gas chromatography/mass spectrometry.’, *International immunopharmacology*, 4(14), pp. 1739–1744.

Farrelly, V., Rainey, F.A. and Stackebrandt, E. (1995) ‘Effect of Genome Size and rrn Gene Copy Number on PCR Amplification of 16S rRNA Genes from a Mixture of Bacterial Species.’ *Applied and Environmental Microbiology*, 61(7), pp. 2798-2801.

Fenical, W., Baden, D., Burg, M., De Goyet, C.V., Grimes, J.D. and Katz, M. (1999) ‘Marine-derived pharmaceuticals and related bioactive compounds.’, in *Monsoons to Microbes: Understanding the Ocean’s Role in Human Health*. Washington, DC: National Academies Press, pp. 71–86.

Flärdh, K. and Buttner, M.J. (2009) ‘*Streptomyces* morphogenetics: Dissecting differentiation in a filamentous bacterium.’, *Nature Reviews Microbiology*. Nature Publishing Group, pp. 36–49. Available at: https://doi.org/10.1038/nrmicro1968.

Fong, I.W., Shlaes, D. and Drlica, K. (2018) *Antimicrobial Resistance in the 21st Century*. 2nd edn. Edited by I.W. Fong, D. Shlaes, and K. Drlica. Springer International Publishing. Available at: https://doi.org/10.1007/978-3-319-78538-7.

Gallagher, K.A., Fenical, W. and Jensen, P.R. (2010) ‘Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes.’, *Current Opinion in Biotechnology*, 21(6), pp. 794–800. Available at: https://doi.org/10.1016/J.COPBIO.2010.09.010.

Germishuizen, G. and Meyer, N.L. (2003) Plants of southern Africa: an annotated checklist. *Strelitzia* 14, pp. 133-136. National Botanical Institute, Pretoria.

Getha, K., Vikineswary, S., Wong, W.H., Seki, T., Ward, A. and Goodfellow, M. (2005) ‘Evaluation of *Streptomyces sp*. strain g10 for suppression of Fusarium wilt and rhizosphere colonization in pot-grown banana plantlets.’, *Journal of Industrial Microbiology and Biotechnology*, 32(1), pp. 24–32. Available at: https://doi.org/10.1007/s10295-004-0199-5.

Glick, B.R. (2012) ‘Plant Growth-Promoting Bacteria: Mechanisms and Applications.’, *Scientifica*, pp. 1–15. Available at: https://doi.org/10.6064/2012/963401.

Gohain, A., Sarma, R.K., Debnath, R., Saikia, J., Singh, B.P., Sarmah, R. and Saikia, R. (2019) ‘Phylogenetic affiliation and antimicrobial effects of endophytic actinobacteria

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

associated with medicinal plants: prevalence of polyketide synthase type II in antimicrobial strains.', *Folia Microbiologica*, 64(4), pp. 481–496. Available at: https://doi.org/10.1007/s12223-018-00673-0.

Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H. and Rai, M. (2015) 'Endophytic actinobacteria of medicinal plants: Diversity and bioactivity.', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. Kluwer Academic Publishers, pp. 267–289. Available at: https://doi.org/10.1007/s10482-015-0502-7.

Gould, I.M. and Bal, A.M. (2013) 'New antibiotic agents in the pipeline and how they can help overcome microbial resistance.', *Virulence*, 4(2), pp. 185–191. Available at: https://doi.org/10.4161/viru.22507.

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

Hayakawa, M. (2008) 'Studies on the Isolation and Distribution of Rare Actinomycetes in Soil', *Actinomycetologica*, 22(1), pp. 12–19. Available at: https://doi.org/10.3209/saj.SAJ220103.

Heikrujam, J., Kishor, R. and Mazumder, P.B. (2020) 'The Chemistry Behind Plant DNA Isolation Protocols.', in *Biochemical Analysis Tools: Methods for Bio-Molecules Studies*. London: IntechOpen. Available at: https://doi.org/10.5772/intechopen.82530.

Hogenhout, S.A. and Loria, R. (2008) 'Virulence mechanisms of Gram-positive plant pathogenic bacteria.', *Current Opinion in Plant Biology*, 11(4), pp. 449–456. Available at: https://doi.org/10.1016/J.PBI.2008.05.007.

Huang, Y., Wang, X., Yang, J., Lu, S., Lai, X.H., Jin, D., Pu, J., Huang, Y., Ren, Z., Zhu, W., Meng, J., Dong, K., Ge, Y., Zhou, J., Zhang, G., Zhang, S., Lei, W., Liang, H. and Xu, J. (2019) '*Nocardioides yefusunii sp*. Nov., isolated from *Equus kiang* (tibetan wild ass) faeces.', *International Journal of Systematic and Evolutionary Microbiology*, 69(11), pp. 3629–3635. Available at: https://doi.org/10.1099/ijsem.0.003674.

Huguet-Tapia, J.C., Badger, J.H., Loria, R. and Pettis, G.S. (2011) '*Streptomyces turgidiscabies* Car8 contains a modular pathogenicity island that shares virulence genes with other actinobacterial plant pathogens.', *Plasmid*, 65(2), pp. 118–124. Available at: https://doi.org/10.1016/j.plasmid.2010.11.002.

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

Hutchings, M., Truman, A. and Wilkinson, B. (2019) 'Antibiotics: past, present and future.', *Current Opinion in Microbiology*. Elsevier Ltd, pp. 72–80. Available at: https://doi.org/10.1016/j.mib.2019.10.008.

Isaacs, N. (2016) 'Determining the Biodiversity and Bioactivity of Actinobacteria Associated with the Indigenous South African Plant, Aloe ferox.' University of the Western Cape.

Izadpanah, F., Homaei, A., Fernandes, P. and Javadpour, S. (2018) 'Marine microbial L-asparaginase: Biochemistry, molecular approaches and applications in tumor therapy and in food industry.', *Microbiological Research*. Elsevier GmbH, pp. 99–112. Available at: https://doi.org/10.1016/j.micres.2018.01.011.

Janda, J.M. and Abbott, S.L. (2007) '16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls.', *Journal of Clinical Microbiology*, 45(9), pp. 2761–2764. Available at: https://doi.org/10.1128/JCM.01228-07.

Jia, Y., Zhao, G. and Jia, J. (2008) 'Preliminary evaluation: The effects of *Aloe ferox Miller* and Aloe arborescens Miller on wound healing.', *Journal of Ethnopharmacology*, 120(2), pp. 181–189. Available at: https://doi.org/10.1016/j.jep.2008.08.008.

Kaewkla, O. and Franco, C.M.M. (2013) 'Rational Approaches to Improving the Isolation of Endophytic Actinobacteria from Australian Native Trees.', *Microbial Ecology*, 65(2), pp. 384–393. Available at: https://doi.org/10.1007/s00248-012-0113-z.

Katz, L. and Baltz, R.H. (2016) 'Natural product discovery: past, present, and future.', *Journal of Industrial Microbiology and Biotechnology*. Springer Verlag, pp. 155–176. Available at: https://doi.org/10.1007/s10295-015-1723-5.

Khan, M.R. and Williams, S.T. (1975) 'Studies on the ecology of actinomycetes in soil-VIII. Distribution and characteristics of acidophilic actinomycetes.', *Soil Biology and Biochemistry*, 7(6), pp. 345–348. Available at: https://doi.org/10.1016/0038-0717(75)90047-4.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000) *Practical Streptomyces Genetics*. The John Innes Foundation.

Kitouni, M., Boudemagh, A., Oulmi, L., Reghioua, S., Boughachiche, F., Zerizer, H., Hamdiken, H., Couble, A., Mouniee, D., Boulahrouf, A. and Boiron, P. (2005) 'Isolation of actinomycetes producing bioactive substances from water, soil and tree bark samples of the

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

north-east of Algeria.', *Journal de Mycologie Medicale*, 15(1), pp. 45–51. Available at: https://doi.org/10.1016/j.mycmed.2004.12.005.

Goethals, K., Vereecke, D., Jaziri, M., Van Montagu, M. and Holsters, M. (2001) 'Leafy gall formation by *Rhodococcus fascians.*', *The Annual Review of Phytopathology*, 39, pp. 27–52. Available at: https://doi.org/10.1146/ANNUREV.PHYTO.39.1.27.

Kollef, M.H. and Fraser, V.J. (2001) 'Antibiotic resistance in the intensive care unit.', *Annals of Internal Medicine*. American College of Physicians, pp. 298–314. Available at: https://doi.org/10.7326/0003-4819-134-4-200102200-00014.

Kuete, V. (2013) *Medicinal Plant Research in Africa: Pharmacology and Chemistry*. 1st edn. Elsevier.

Kumarasamy, K.K. *et al.* (2010) 'Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: A molecular, biological, and epidemiological study.', *The Lancet Infectious Diseases*, 10(9), pp. 597–602. Available at: https://doi.org/10.1016/S1473-3099(10)70143-2.

Laskaris, P., Tolba, S., Calvo-Bado, L. and Wellington, L. (2010) 'Coevolution of antibiotic production and counter-resistance in soil bacteria.', *Environmental Microbiology*, 12(3), pp. 783–796. Available at: https://doi.org/10.1111/j.1462-2920.2009.02125.x.

Lazzarini, A., Cavaletti, L., Toppo, G. and Marinelli, F. (2000) 'Rare genera of actinomycetes as potential producers of new antibiotics.', *Antonie van Leeuwenhoek*, 78(3/4), pp. 399–405. Available at: https://doi.org/10.1023/A:1010287600557.

LeBlanc, J.C., Gonçalves, E.R. and Mohn, W.W. (2008) 'Global response to desiccation stress in the soil actinomycete *Rhodococcus jostii* RHA1.', *Applied and Environmental Microbiology*, 74(9), pp. 2627–2636. Available at: https://doi.org/10.1128/AEM.02711-07.

Lee, L.H., Cheah, Y.K., Sidik, S.M., Mutalib, N.S.A., Tang, Y.L., Lin, H.P. and Hong, K. (2012) 'Molecular characterisation of Antarctic actinobacteria and screening for antimicrobial metabolite production.', *World Journal of Microbiology and Biotechnology*, 28(5), pp. 2125–2137. Available at: https://doi.org/10.1007/s11274-012-1018-1.

Li, J., Zhao, G., Zhu, W., Huang, H., Xu, L., Zhang, S. and Li, W. (2013) '*Streptomyces endophyticus sp. nov*., an endophytic actinomycete isolated from *Artemisia annua L.*', *International Journal of Systematic and Evolutionary Microbiology*, 63(1).

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

Li, J.T., Yang, J., Chen, D.C., Zhang, X.L. and Tang, Z.S. (2007) 'An optimized mini-preparation method to obtain high-quality genomic DNA from mature leaves of sunflower.', *Genetics and Molecular Research*, 6(4), pp. 1064–1071.

Li, Q., Chen, X., Jiang, Y. and Jiang, C. (2016) 'Morphological Identification of Actinobacteria', in D. Dhanasekaran and Y. Jiang (eds) *Actinobacteria - Basics and Biotechnological Applications*. InTech. Available at: https://doi.org/10.5772/61461.

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R.B., Taghavi, S., Mezgeay, M. and Van Der Lelie, D. (2002) 'Endophytic Bacteria and Their Potential Applications', *Critical Reviews in Plant Sciences*, 21(6), pp. 583–606. Available at: https://doi.org/10.1080/0735-260291044377.

Loots, D.T., van der Westhuizen, F.H. and Botes, L. (2007) '*Aloe ferox* Leaf Gel Phytochemical Content, Antioxidant Capacity, and Possible Health Benefits.' *Journal of Agricultural and Food Chemistry*, 55, pp. 6891-6896. Available at: https://doi.org/10.1021/JF071110T.

Lourens, A.C.U., Reddy, D., Başer, K.H.C., Viljoen, A.M. and Van Vuuren, S.F. (2004) 'In vitro biological activity and essential oil composition of four indigenous South African *Helichrysum* species.', *Journal of Ethnopharmacology*, 95(2–3), pp. 253–258. Available at: https://doi.org/10.1016/j.jep.2004.07.027.

Lynd, L.R., Weimer, P.J., van Zyl, W.H. and Pretorius, I.S. (2002) 'Microbial Cellulose Utilization: Fundamentals and Biotechnology.', *Microbiology and Molecular Biology Reviews,* 66(3), pp. 506-577. Available at: https://doi.org/10.1128/MMBR.66.3.506-577.2002.

Macagnan, D., Romeiro, R.D.S., De Souza, J.T. and Pomella, A.W.V. (2006) 'Isolation of actinomycetes and endospore-forming bacteria from the cacao pod surface and their antagonistic activity against the witches' broom and black pod pathogens.', *Phytoparasitica*, 34(2), pp. 122–132. Available at: https://doi.org/10.1007/BF02981312.

Mathabe, M.C., Hussein, A.A., Nikolova, R.V., Basson, A.E., Meyer, J.J.M. and Lall, N. (2008) 'Antibacterial activities and cytotoxicity of terpenoids isolated from *Spirostachys Africana.'*, *Journal of Ethnopharmacology*, 116(1), pp. 194–197. Available at: https://doi.org/10.1016/J.JEP.2007.11.017.

http://etd.uwc.ac.za/

Matsuo, Y., Kanoh, K., Jang, J.H., Adachi, K., Matsuda, S., Miki, O., Kato, T. and Shizuri, Y. (2011) 'Streptobactin, a tricatechol-type siderophore from marine-derived *Streptomyces sp*. YM5-799.', *Journal of Natural Products*, 74(11), pp. 2371–2376. Available at: https://doi.org/10.1021/np200290j.

McKay, D.L. and Blumberg, J.B. (2007) 'A review of the bioactivity of South African herbal teas: Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*).', *Phytotherapy Research*, 21(1), pp. 1–16. Available at: https://doi.org/10.1002/PTR.1992.

Meyers, P.R., Goodwin, C.M., Bennett, J.A., Aken, B.L., Price, C.E. and Van Rooyen, J.M. (2004) '*Streptomyces africanus sp. nov*., a novel streptomycete with blue aerial mycelium.', *International Journal of Systematic and Evolutionary Microbiology*, 54(5), pp. 1531–1535. Available at: https://doi.org/10.1099/IJS.0.02738-0/CITE/REFWORKS.

Miyajima, K., Tanaka, F., Takeuchi, T. and Kuninaga, S. (1998) '*Streptomyces turgidiscabies* sp. nov.', *International Journal of Systematic Bacteriology*, 48(2), pp. 495–502. Available at: https://doi.org/10.1099/00207713-48-2-495.

Ndhlala, A.R., Amoo, S.O., Stafford, G.I., Finnie, J.F. and Van Staden, J. (2009) 'Antimicrobial, anti-inflammatory and mutagenic investigation of the South African tree aloe (*Aloe barberae*).', *Journal of Ethnopharmacology*, 124(3), pp. 404–408. Available at: https://doi.org/10.1016/J.JEP.2009.05.037.

Ngoma, L., Babalola, O.O. and Ahmad, F. (2012) 'Ecophysiology of plant growth promoting bacteria.', *Scientific Research and Essays*, 7(47), pp. 4003–4013. Available at: https://doi.org/10.5897/SRE12.646.

Nimnoi, P., Pongsilp, N. and Lumyong, S. (2010) 'Endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lec and screening of plant growth promoters production.', *World Journal of Microbiology and Biotechnology.*, 26(2), pp. 193–203.

Nithya, K., Muthukumar, C., Duraipandiyan, V., Dharumadurai, D. and Thajuddin, N. (2015) 'Diversity and antimicrobial potential of culturable actinobacteria from desert soils of Saudi Arabia.', *Journal of Pharmaceutical Sciences and Research.,* 7(3), pp. 117-122

Niu, M.M., Ming, H., Cheng, L.J., Zhao, Z.L., Ji, W.L., Li, M., Yi, B.F., Xia, T.T. and Nie, G.X. (2020) '*Amycolatopsis nivea* sp. Nov., isolated from a yellow river sample.', *International Journal of Systematic and Evolutionary Microbiology*, 70(5), pp. 3084–3090. Available at: https://doi.org/10.1099/IJSEM.0.004134/CITE/REFWORKS.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Nouioui, I., Carro, L., García-López, M., Meier-Kolthoff, J.P., Woyke, T., Kyrpides, N.C., Pukall, R., Klenk, H.P., Goodfellow, M. and Göker, M. (2018) 'Genome-based taxonomic classification of the phylum *Actinobacteria.'*, *Frontiers in Microbiology*, 9, pp. 1-119 Available at: https://doi.org/10.3389/fmicb.2018.02007.

O'Brien, C., Van Wyk, B.-E. and Van Heerden, F.R. (2011) 'Physical and chemical characteristics of *Aloe ferox* leaf gel.', *South African Journal of Botany*, 77(4), pp. 988–995. Available at: https://doi.org/10.1016/J.SAJB.2011.08.004.

Oskay, M., Tamer, A.Ü. and Azeri, C. (2004) 'Antibacterial activity of some actinomycetes isolated from farming soils of Turkey.', *African Journal of Biotechnology*, 3(9), pp. 441–446.

Palaniyandi, S.A., Yang, S.H., Zhang, L. and Suh, J.W. (2013) 'Effects of actinobacteria on plant disease suppression and growth promotion.', *Applied Microbiology and Biotechnology*. Springer, pp. 9621–9636. Available at: https://doi.org/10.1007/s00253-013-5206-1.

Praveen, V., Tripathi, C.K.M. and Bihari, V. (2008) 'Studies on optimum fermentation conditions for actinomycin-D production by two new strains of *Streptomyces* spp.', *Medicinal Chemistry Research*, 17(2–7), pp. 114–122. Available at: https://doi.org/10.1007/S00044-007-9042-7/FIGURES/2.

Pridham, T.G., Hesseltine, C.W. and Benedict, R.G. (1958) 'A guide for the classification of streptomycetes according to selected groups; placement of strains in morphological sections.', *Applied microbiology*, 6(1), pp. 52–79. Available at: https://doi.org/10.1128/aem.6.1.52-79.1958.

Qin, S., Li, J., Zhao, G.Z., Chen, H.H., Xu, L.H. and Li, W.J. (2008) '*Saccharopolyspora endophytica* sp. nov., an endophytic actinomycete isolated from the root of *Maytenus austroyunnanensis*.', *Systematic and Applied Microbiology.*, 31(5), pp. 352–357.

Qin, S., Li, J., Chen, H.H., Zhao, G.Z., Zhu, W.Y., Jiang, C.L. and Li, W.J. (2009) 'Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna China.', *Applied and Environmental Microbiology*, 75(19), pp. 6176–6186. Available at: https://doi.org/10.1128/AEM.01034-09.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Rai, M., Agarkar, G. and Rathod, D. (2014) 'Multiple Applications of Endophytic Colletotrichum Species Occurring in Medicinal Plants', in *Novel Plant Bioresources*. Chichester, UK: John Wiley & Sons, Ltd, pp. 227–236. Available at: https://doi.org/10.1002/9781118460566.ch18.

Ranjani, A., Dhanasekaran, D. and Gopinath, P.M. (2016) 'An Introduction to Actinobacteria', in *Actinobacteria - Basics and Biotechnological Applications*. InTech. Available at: https://doi.org/10.5772/62329.

Rautengarten, C., Birdseye, D., Pattathil, S., McFarlane, H.E., Saez-Aguayo, S., Orellana, A., Persson, S., Hahn, M.G., Scheller, H.V. and Ebert, B. (2017) 'The elaborate route for UDP-Arabinose delivery into the Golgi of plants.', *Proceedings of the National Academy of Sciences of the United States of America*, 114(16), pp. 4261–4266. Available at: https://doi.org/10.1073/PNAS.1701894114/SUPPL_FILE/PNAS.1701894114.ST06.DOCX.

Reinten, E. and van Wyk, B.E. (2018) 'Floriculture industry benefits from southern African floral biodiversity.', *Acta Horticulturae*, 1201(1201), pp. 659–664. Available at: https://doi.org/10.17660/ActaHortic.2018.1201.89.

Reysenbach, A.L., Pace, N.R., Robb, F.T. and Place, A.R. (1995) 'Archaea: a laboratory manual-thermophiles.', *CSHLP*, Protocol (16), pp. 101–107.

Rohland, J. and Meyers, P.R. (2015) '*Streptomyces fractus* sp. nov., a novel streptomycete isolated from the gut of a South African termite.', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 107(5), pp. 1127–1134. Available at: https://doi.org/10.1007/S10482-015-0404-8/FIGURES/2.

Rong, X., Doroghazi, J.R., Cheng, K., Zhang, L., Buckley, D.H. and Huang, Y. (2013) 'Classification of Streptomyces phylogroup pratensis (Doroghazi and Buckley, 2010) based on genetic and phenotypic evidence, and proposal of *Streptomyces pratensis* sp. nov.', *Systematic and Applied Microbiology*, 36(6), pp. 401–407. Available at: https://doi.org/10.1016/J.SYAPM.2013.03.010.

Rossolini, G.M., Arena, F., Pecile, P. and Pollini, S. (2014) 'Update on the antibiotic resistance crisis.', *Current Opinion in Pharmacology*. Available at: https://doi.org/10.1016/j.coph.2014.09.006.

UNIVERSITY of the WESTERN CAPE
http://etd.uwc.ac.za/

Sánchez-Castro, I., Ruiz-Fresneda, M.A., Bakkali, M., Kämpfer, P., Glaeser, S.P., Busse, H.J., López-Fernández, M., Martínez-Rodríguez, P. and Merroun, M.L. (2017) '*Stenotrophomonas bentonitica* sp. nov., isolated from bentonite formations.', *International Journal of Systematic and Evolutionary Microbiology*, 67(8), p. 2779. Available at: https://doi.org/10.1099/IJSEM.0.002016.

Sathya, A., Vijayabharathi, R. and Gopalakrishnan, S. (2017) 'Plant growth-promoting actinobacteria: a new strategy for enhancing sustainable production and protection of grain legumes.', *3 Biotech*, 7(102). Available at: https://doi.org/10.1007/s13205-017-0736-3.

Schmid, R.D. and Verger, R. (1998) 'Lipases: Interfacial enzymes with attractive applications.', *Angewandte Chemie - International Edition*. Wiley-VCH Verlag, pp. 1608–1633. Available at: https://doi.org/10.1002/(sici)1521-3773(19980703)37:12<1608:aid-anie1608>3.0.co;2-v.

Schneider, K., Rose, I., Vikineswary, S., Jones, A.L., Goodfellow, M., Nicholson, G., Beil, W., Süssmuth, R.D. and Fiedler, H.P. (2007) 'Nocardichelins A and B, siderophores from N*oca*rdia strain Acta 3026#.', *Journal of Natural Products*, 70(6), pp. 932–935. Available at: https://doi.org/10.1021/np060612i.

Schrempf, H. (2008) 'S*treptomycetaceae*: Lifestyle, Genome, Metabolism and Habitats.', in *Encyclopedia of Life Sciences*. Chichester, UK: John Wiley & Sons, Ltd. Available at: https://doi.org/10.1002/9780470015902.a0020393.

Schwyn, B. and Neilands, J.B. (1987) 'Universal chemical assay for the detection and determination of siderophores.', *Analytical Biochemistry*, 160(1), pp. 47–56.

Sengupta, S., Chattopadhyay, M.K. and Grossart, H.P. (2013) 'The multifaceted roles of antibiotics and antibiotic resistance in nature.', *Frontiers in Microbiology*. Frontiers Research Foundation. Available at: https://doi.org/10.3389/fmicb.2013.00047.

Seong, C.N., Park, J.H. and Baik, K.S. (2001) 'An Improved Selective Isolation of Rare Actinomycetes from Forest Soil.', *Journal of Microbiology*, 39(1), pp. 17–23.

Shirling, E.B. and Gottlieb, D. (1966) 'Methods for characterisation of Streptomyces species.', *International Journal of Systematic Bacteriology*, 16(3), pp. 313–340. Available at: https://doi.org/10.1099/00207713-16-3-313.

Singh, V., Haque, S., Singh, H., Verma, J., Vibha, K., Singh, R., Jawed, A. and Tripathi, C.K.M. (2016) 'Isolation, screening, and identification of novel isolates of *Actinomycetes* from India for antimicrobial applications.', *Frontiers in Microbiology*. Available at: https://doi.org/10.3389/fmicb.2016.01921.

Smaoui, S., Mathieu, F., Elleuch, L., Coppel, Y., Merlina, G., Karray-Rebai, I. and Mellouli, L. (2012) 'Taxonomy, purification and chemical characterisation of four bioactive compounds from new *Streptomyces* sp. TN256 strain.', *World Journal of Microbiology and Biotechnology*, 28(3), pp. 793–804. Available at: https://doi.org/10.1007/S11274-011-0872-6/TABLES/4.

Solans, M., Scervino, J.M., Messuti, M.I., Vobis, G. and Wall, L.G. (2016) 'Potential biocontrol actinobacteria: Rhizospheric isolates from the Argentine Pampas lowlands legumes.', *Journal of Basic Microbiology*, 56(11), pp. 1289–1298. Available at: https://doi.org/10.1002/jobm.201600323.

Spellberg, B. and Gilbert, D.N. (2014) 'The future of antibiotics and resistance: A tribute to a career of leadership by John Bartlett.', *Clinical Infectious Diseases*, 59(Suppl 2), pp. S71–S75. Available at: https://doi.org/10.1093/cid/ciu392.

Stach, J.E.M., Maldonado, L.A., Ward, A.C., Goodfellow, M. and Bull, A.T. (2003) 'New primers for the class Actinobacteria: Application to marine and terrestrial environments.', *Environmental Microbiology*, 5(10), pp. 828–841. Available at: https://doi.org/10.1046/j.1462-2920.2003.00483.x.

Staniek, A., Woerdenbag, H.J. and Kayser, O. (2008) 'Endophytes: exploiting biodiversity for the improvement of natural product-based drug discovery.', *Journal of Plant Interactions*, 3(2), pp. 75–93. Available at: https://doi.org/10.1080/17429140801886293.

Stevenson, A. and Hallsworth, J.E. (2014) 'Water and temperature relations of soil Actinobacteria.', *Environmental Microbiology Reports*, 6(6), pp. 744–755. Available at: https://doi.org/10.1111/1758-2229.12199.

Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R. and Hess, W.M. (1996) 'Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*.', 142, pp. 43–48.

http://etd.uwc.ac.za/

Sultanpuram, V.R., Mothe, T. and Mohammed, F. (2015) '*Nocardioides solisilvae* sp. nov., isolated from a forest soil.', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 107(6), pp. 1599–1606. Available at: https://doi.org/10.1007/s10482-015-0455-x.

Taddei, A., Rodríguez, M.J., Márquez-Vilchez, E. and Castelli, C. (2006) 'Isolation and identification of *Streptomyces* spp. from Venezuelan soils: Morphological and biochemical studies.', *Microbiological Research*. Available at: https://doi.org/10.1016/j.micres.2005.08.004.

Taechowisan, T., Chuaychot, N., Chanaphat, S., Wanbanjob, A. and Shen, Y. (2008) 'Biological activity of chemical constituents isolated from *Streptomyces* sp. Tc052, an endophyte in Alpinia galanga.', *International Journal of pharmacology.*, 4(2), pp. 95–101.

Takahashi, Y. and Omura, S. (2003) 'Isolation of new actinomycete strains for the screening of new bioactive compounds.', *The Journal of General and Applied Microbiology*, 49(3), pp. 141–154. Available at: https://doi.org/10.2323/JGAM.49.141.

Tanvir, R., Sajid, I., Hasnain, S., Kulik, A. and Grond, S. (2016) 'Rare actinomycetes *Nocardia caishijiensis* and *Pseudonocardia carboxydivorans* as endophytes, their bioactivity and metabolites evaluation.', *Microbiological Research*, 185, pp. 22–35. Available at: https://doi.org/10.1016/j.micres.2016.01.003.

Thakur, D., Yadav, A., Gogoi, B.K. and Bora, T.C. (2007) 'Isolation and screening of *Streptomyces* in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites.', *Journal de Mycologie Medicale*, 17(4), pp. 242–249. Available at: https://doi.org/10.1016/j.mycmed.2007.08.001.

Thamchaipenet, A., Indananda, C., Bunyoo, C., Duangmal, K., Matsumoto, A. and Takahashi, Y. (2010) '*Actinoallomurus acaciae* sp. nov., an endophytic actinomycete isolated from *Acacia auriculiformis A*. Cunn. ex Benth.', *International Journal of Systematic and Evolutionary Microbiology.*, 60(3), pp. 554–559.

Thibessard, A., Haas, D., Gerbaud, C., Aigle, B., Lautru, S., Pernodet, J.L. and Leblond, P. (2015) 'Complete genome sequence of *Streptomyces ambofaciens* ATCC 23877, the spiramycin producer', *Journal of Biotechnology*, 214, pp. 117–118. Available at: https://doi.org/10.1016/J.JBIOTEC.2015.09.020.

http://etd.uwc.ac.za/

Tork, S.E., Aly, M.M. and Elsemin, O. (2018) 'A new l-glutaminase from *Streptomyces pratensis* NRC 10: Gene identification, enzyme purification, and characterisation.', *International Journal of Biological Macromolecules*, 113, pp. 550–557. Available at: https://doi.org/10.1016/J.IJBIOMAC.2018.02.080.

Trabelsi, I., Oves, D., Manteca, A., Genilloud, O., Altalhi, A. and Nour, M. (2016) 'Antimicrobial Activities of Some Actinomycetes Isolated from Different Rhizospheric Soils in Tunisia.', *Current Microbiology*. Available at: https://doi.org/10.1007/s00284-016-1053-5.

Traxler, M.F., Watrous, J.D, Alexandrov, T., Dorrestein, P.C. and Kolter, R. (2013) 'Interspecies interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome.', *mBio*, 4(4). Available at: https://doi.org/10.1128/mBio.00459-13.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. and Van Sinderen, D. (2007) 'Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum.', *Microbiology and Molecular Biology Reviews*, 71(3), pp. 495–548. Available at: https://doi.org/10.1128/mmbr.00005-07.

Verma, M., Lal, D., Haur, J., Saxena, A., Kaur, J., Anand, S. and Lal, R. (2013) 'Phylogenetic analyses of phylum *Actinobacteria* based on whole genome sequences.', *Research in Microbiology*, 164(7), pp. 718–728. Available at: https://doi.org/10.1016/j.resmic.2013.04.002.

Verma, V.C., Gond, S.K., Kumar, A., Mishra, A., Kharwar, R.N. and Gange, A.C. (2009) 'Endophytic actinomycetes from *Azadirachta indica* A. Juss.: Isolation, diversity, and antimicrobial activity.', *Microbial Ecology*, 57(4), pp. 749–756. Available at: https://doi.org/10.1007/s00248-008-9450-3.

Vijayakumar, R., Panneerselvam, K., Muthukumar, C., Thajuddin, N., Panneerselvam, A. and Saravanamuthu, R. (2012) 'Optimization of Antimicrobial Production by a Marine Actinomycete *Streptomyces afghaniensis* VPTS3-1 Isolated from Palk Strait, East Coast of India.', *Indian Journal of Microbiology*, 52(2), pp. 230–239. Available at: https://doi.org/10.1007/S12088-011-0138-X/TABLES/4.

http://etd.uwc.ac.za/

Vollmar, D., Thorn, A., Schuberth, I. and Grond, S. (2009) ‘A comprehensive view on 4-methyl-2-quinazolinamine, a new microbial alkaloid from *Streptomyces* of TCM plant origin.’, *The Journal of Antibiotics 2009 62:8*, 62(8), pp. 439–444. Available at: https://doi.org/10.1038/ja.2009.68.

van Vuuren, S.F., Viljoen, A.M., Van Zyl, R.L., Van Heerden, F.R. and Başer, K.H.C. (2006) ‘The antimicrobial, antimalarial and toxicity profiles of helihumulone, leaf essential oil and extracts of *Helichrysum cymosum* (L.) D. Don subsp. *Cymosum*.’, *South African Journal of Botany*, 72(2), pp. 287–290. Available at: https://doi.org/10.1016/J.SAJB.2005.07.007.

van Vuuren, S.F. (2008) ‘Antimicrobial activity of South African medicinal plants.’, *Journal of Ethnopharmacology*, 119(3), pp. 462–472. Available at: https://doi.org/10.1016/J.JEP.2008.05.038.

Walterson, A.M. and Stavrinides, J. (2015) ‘*Pantoea*: insights into a highly versatile and diverse genus within the *Enterobacteriaceae*.’, *FEMS Microbiology Reviews*, 39(6), pp. 968–984. Available at: https://doi.org/10.1093/FEMSRE/FUV027.

Wang, W., Qui, Z., Tan, H. and Cao, L. (2014) ‘Siderophore production by actinobacteria.’, *BioMetals*. Kluwer Academic Publishers, pp. 623–631. Available at: https://doi.org/10.1007/s10534-014-9739-2.

Wang, X. *et al.* (2018) ‘*Nocardioides houyundeii* sp. nov., isolated from Tibetan antelope faeces.’, *International Journal of Systematic and Evolutionary Microbiology*, 68(12), pp. 3874–3880. Available at: https://doi.org/10.1099/ijsem.0.003076.

Wang, Y., Zhang, Z. and Ruan, J. (1996) ‘A Proposal to Transfer *Microbispora bispora* (Lechevalier 1965) to a New Genus, *Thermobispora* gen. nov., as *Thermobispora bispora comb.* nov.’, *International Journal of Systematic Bacteriology*, 46(4), pp. 933–938. Available at: https://doi.org/10.1099/00207713-46-4-933.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P. and McPhail, A.T. (1971) ‘Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia.’*, *Journal of the American Chemical Society*, 93(9), pp. 2325–2327. Available at: https://doi.org/10.1021/ja00738a045.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Wei, S., Zhang, W. and Ji, Z. (2015) 'Structure and Antibacterial Activity of Ambobactin, a New Telomycin-Like Cyclic Depsipeptide Antibiotic Produced by *Streptomyces ambofaciens* F3.', *Molecules 2015, Vol. 20, Pages 16278-16289*, 20(9), pp. 16278–16289. Available at: https://doi.org/10.3390/MOLECULES200916278.

Williams, S.T. *et al.* (1993) 'Detection and identification of novel actinomycetes.', *Research in Microbiology*, 144(8), pp. 653–656. Available at: https://doi.org/10.1016/0923-2508(93)90069-E.

Wohlleben, W., Bera, A., Mast, Y. and Stegmann, E. (2017) 'Regulation of Secondary Metabolites of Actinobacteria.', *Biology and Biotechnology of Actinobacteria*, pp. 181–232. Available at: https://doi.org/10.1007/978-3-319-60339-1_8.

World Health Organization (2014) '*Antimicrobial resistance: Global Report on Surveillance*, *World Health Organization*.' World Health Organization.

Wright, G.D. (2014) 'Something old, something new: revisiting natural products in antibiotic drug discovery.', *Canadian Journal of Microbiology*, 60(3), pp. 147–154. Available at: https://doi.org/10.1139/cjm-2014-0063.

van Wyk, B.E. (2008) 'A broad review of commercially important southern African medicinal plants.', *Journal of Ethnopharmacology*, 119(3), pp. 342–355. Available at: https://doi.org/10.1016/J.JEP.2008.05.029.

van Wyk, B.E. (2011) 'The potential of South African plants in the development of new medicinal products.', *South African Journal of Botany*, pp. 812–829. Available at: https://doi.org/10.1016/j.sajb.2011.08.011.

van Wyk, B.E., van Oudtshoorn, B. and Gericke, N. (2009) *Medicinal Plants of South Africa*. 2nd edn. Briza Publications.

van Wyk, B.E., van Rheede van Oudtshoorn, M.C. and Smith, G.F. (1995) 'Geographical Variation in the Major Compounds of *Aloe ferox* Leaf Exudate', *Planta Medica*, 61(03), pp. 250–253. Available at: https://doi.org/10.1055/s-2006-958066.

van Wyk, B.E. and van Staden, J. (2002) 'A review of ethnobotanical research in southern Africa', *South African Journal of Botany*, 68(1), pp. 1–13. Available at: https://doi.org/10.1016/S0254-6299(16)30447-1.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Young, M. (1979) *A Modified Spread Plate Technique*, *Methodology for Biomass Determinations and Microbial Activities in Sediments: A Symposium*.

Zhang, J. (2011) 'Improvement of an Isolation Medium for *Actinomycetes.*', *Modern Applied Science*, 5(2), pp. 124–127. Available at: https://doi.org/10.5539/mas.v5n2p124.

Zhao, K. *et al.* (2011) 'The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau, China.', *Current Microbiology*, 62(1), pp. 182–190. Available at: https://doi.org/10.1007/s00284-010-9685-3.

Zhi, X.Y., Li, W.J. and Stackebrandt, E. (2009) 'An update of the structure and 16S rRNA gene sequence-based definition of higher ranks of the class Actinobacteria, with the proposal of two new suborders and four new families and emended descriptions of the existing higher taxa', *International Journal of Systematic and Evolutionary Microbiology*, 59(3), pp. 589–608. Available at: https://doi.org/10.1099/ijs.0.65780-0.

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Appendix: Antimicrobial screening results

Table 1: Overlay results (in mm^2) of the initial 61 actinobacterial isolate as tested against *A. baumannii, C. albicans,* and *E. faecalis.*

	Acinetobacter baumannii			***Candida albicans***			***Enterococcus faecalis* Van A**		
Isolates	**SCN**	**OATS**	**YEME**	**SCN**	**OATS**	**YEME**	**SCN**	**OATS**	**YEME**
1				115,45			1161,6		483,02
3									
4									
5				104,46					
6									
7				138,23					
8									
9	181,43						565,49		
20								219,13	
21									
23									
25									
256							150,8	37,7	296,1
257			170,43				58,9	100,53	
258									
262				204,99		188,5			
269									
283									
289									
290									
300									
301									
431									
432				84,82					125,66
433							138,23		
501									
502				263,89					
507			3715,72			3760,1			247,4
512									
528									
530	62,83								

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531									
536									
539									
544									
548									
560			202,63				1178,1		
563	74,61		270,98				1548,81		
578								104,46	
579	239,16		812,89			395,84			574,13
582							233,26	65,97	
583							215,98	82,47	
590					643,24	1617,13			
601							87,96		
602	93,46						162,58		
603	125,66			148,44			254,04	106,03	
610						226,19		285,88	
611	82,47		1241,32	82,47		478,31		37,7	
616			691,15						56,55
619					100,53				
634									
635									
636									
637									
657							285,1		
658									
741									
789									
794									
863									
864	148,05			193,99					

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Table 2: Overlay results (in mm^2) of the initial 61 actinobacterial isolate as tested against *E. coli, M. aurum,* and *S. aureus.*

	Escherichia coli			***Mycobacterium aurum***			***Staphylococcus aureus***		
Isolates	**SCN**	**OATS**	**YEME**	**SCN**	**OATS**	**YEME**	**SCN**	**OATS**	**YEME**
1			678,58	412,33		1021,02			668,37
3	452,39	301,59			365,21				
4			170,43						
5									
6				188,5					
7				69,12					
8									
9	565,49								
20									
21			282,74			94,25			
23	122,52								
25							134,3		126,45
256		157,08	435,9	138,23		90,32	150,8		219,91
257	2563,54								319,66
258									
262									
269									
283									
289									
290									
300									
301									
431									
432	49,48		251,33			176,71			138,23
433	204,99				58,9				51,05
501									
502	126,45						204,2		
507	1492,26						296,1	18,85	804,25
512							193,99		104,46
528						148,44		452,39	593,76
530	440,61						687,22		126,45
531					267,04	88			506,58
536									
539									
544									
548									
560				126,45	65,97	181,43			
563									
578									

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579	467,71		242,15			98,17			1272,34
582					176,71				
583	66,76	43,98		103,67	169,65				
590							169,65	301,59	
601			75,4	58,9		93,46			
602	58,9		275,67		214,41		157,08	75,4	157,08
603	336,94	263,89		94,25	125,66		534,07		537,07
610							477,5		459,46
611					93,46	59,69	94,25		
616					65,97	77,75	94,25		
619							148,44		
634							115,45		
635									
636									
637	125,66						75,4		
657				113,88					
658									
741									
789	1450,63		957,4				2905,97		
794									
863							106,03		
864						251,33	125,66		

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