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PHYLOGENETIC SCREENING FOR POSSIBLE NOVEL ANTIBIOTIC PRODUCING ACTINOMYCETES FROM RHIZOSPHERIC SOIL SAMPLES COLLECTED FROM NGAKA MODIRI MOLEMA DISTRICT IN NORTH WEST PROVINCE, SOUTH AFRICA

BY

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DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirely for the award of any degree.

Name: **Mobolaji Felicia Adegboye**

Signature:.....

Date: 20th July, 2014

DEDICATION

This work is dedicated to Almighty God for His faithfulness over my life and for making my helpers to be many.

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GENERAL ABSTRACT

Infectious disease is the number one cause of death in developing countries, accounting for approximately half of all fatalities. Emerging and re-emerging infections are thought to be driven largely by socio-economic, environmental and ecological factors. These negative health trends call for a renewed interest in infectious disease as well as effective strategies for treatment and prevention. This work is designed to search for antibiotic(s) from actinomycetes to address the treatment of infectious diseases.

In this study, 341 strains of actinomycetes were isolated from the rhizospheric soil samples collected from Ngaka Modiri Molema district in North West Province of South Africa. A combination of morphological, biochemical and physiological characteristics, sequencing of the 16S rDNA gene, and phylogenetic analysis of the nucleotide sequences determined from the 16S rDNA gene were carried out and showed that 253 (73.4%) of the isolates belong to the genus *Streptomyces* and 88 (26.6%) were rare actinomycetes. According to molecular taxonomical analysis, these actinomycete isolates was dominated by *Streptomyces* spp., followed by *Nocardia*, *Micromonospora*, *Rhodococcus*, *Streptosporangium*, *Nocardiopsis*, *Actinomadura*, *Pseudonocardia*, *Nonomuraea*, *Promicromonospora*, *Arthrobacter*, *Micrococcus*, *Rhodococcus*, and *Saccharothrix*. The isolates were screened for antibacterial activity against pathogenic bacteria. This revealed that 92 (27%) out of the 341 strains showed antagonistic activity against at least five of the eleven test organisms; 21.4% of the isolates were assigned as potent *Streptomyces* spp, and 5.5% as potent rare actinomycetes.

Polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced by microorganisms, especially actinomycetes. In order to assess

the occurrence of these biosynthetic systems in these actinomycete isolates, degenerative PCR primers targeted to specifically amplify PKS-I, PKS-II and NRPS genes from actinomycetes were used. Sixteen isolates (4.6%) were identified to have PKS-I gene positive strains, while the figures were 15.2% for PKS-II and 26% for NRPS gene. The amplification of the genes from some of the actinomycete isolates is an indication of their potential as antibiotic producers. Through the screening, it was found that *Streptomyces* have higher prevalence of PKS-I, PKS-II and NRPS genes compared to others genera. Phylogenetic analysis of the nucleotide sequences from the amplified biosynthetic genes confirmed that the isolates formed close phylogenetic relationship with known antibiotic producers.

Three isolates (NWU14, NWU49 and NWU91) were selected for fermentation optimization and partial characterization according to their phylogenetic diversity, antimicrobial activities and secondary-metabolite biosynthesis genes. For optimizing the bioactive secondary metabolite production from the 3 isolates, experiments with the supplementation of nutrients were conducted based on the method of one-factor-at-a-time. The chemical and physical parameters affecting the production of bioactive secondary metabolite were optimized. The result showed that the most effective carbon source was glucose and that the best sources of nitrogen were oatmeal and yeast extract. The results of this study showed that the temperature, pH, inoculum size, fermentation period and the culture medium directly influenced the production of bioactive secondary metabolites (antibiotics). Each isolate behaved differently, requiring specific conditions for the production of secondary metabolites. The results obtained allow an efficient production of bioactive secondary metabolites by the isolates of actinomycete used in this study.

To investigate the bioactive secondary metabolites of actinomycete isolates in order to find novel compounds or effective components, the resultant crude extracts after fermentation were partitioned using different organic solvents. The antimicrobial activities of partition

fractions collected were determined. The ethyl acetate, petroleum ether, benzene and n-hexane fractions of the crude extract from NWU91, ethyl acetate fractions of NWU49 and NWU14 were found to be active and were subjected to Gas Chromatography-Mass Spectrometry analysis (GC-MS) to establish the chemical components of these active fractions. The bioactive compounds identified include furosemide, phellopterin, 4,5-dihydroxyanthraquinone-2-carboxylic acid and milbemycin B. The compound 4,5-dihydroxyanthraquinone-2-carboxylic acid produced by NWU91 was not reported earlier from actinomycete.

This study demonstrates the significance of actinomycetes in the rhizosphere and their potential for producing biologically active compounds and novel material for genetic manipulation or combinatorial biosynthesis. These bioactive secondary metabolites have application in human medicine and agriculture.

CHAPTER 1

1.1 General Introduction

Infectious diseases are leading health problems with high morbidity and mortality in both developing and developed countries (Jones et al., 2008). The infectious diseases caused by pathogenic organisms range from tonsillitis to pneumonia, osteomyelitis, cough, diarrhea, measles, tuberculosis, septicemia, candidiasis, malaria, influenza, and many other diseases (Willey et al., 2010). Many people have lost their lives due to these infectious diseases which at times reach epidemic rates. Evolution of novel diseases, toxicity of currently used compounds and emergence of drugs resistant pathogens which can cause life threatening infections and risk undermining the viability of health care systems, especially in immunodeficiency patients, are on the increase at an alarming rate worldwide (Woolhouse, 2008). The development of resistance to multiple drugs is a major problem in the treatment of these infectious diseases caused by pathogenic microorganisms (Adegboye et al., 2012). This antimicrobial resistance is presently an urgent focus of research and continuous searching for new bioactive compounds is necessary to combat the menace of pathogens. With the serious problem of antibiotic resistance, there is a need for an intensive search for new antibiotics.

The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources (Harvey, 2008, Newman and Cragg, 2012). These natural products having novel skeletons have been found to possess important biological activities and produce a significant number of therapeutic agents in clinical use all around the world (Chin et al., 2006). They even serve as template for the synthesis of synthetic and semi-synthetic drugs. This involves the screening of microorganisms and plant extracts, using a variety of models (Gunatilaka, 2006, Adegboye et al., 2008). The

serendipitous discovery of penicillin by Alexander Fleming in 1929 from *Penicillium notatum* ushered in a new era in medicine known as the "Golden Age of Antibiotics". In 1940, Selman Waksman discovered that the soil bacteria he was studying made actinomycin, a discovery for which he received a Nobel Prize (Waksman and Wayne, 1963). This prompts the screening of soil microorganisms as sources for bioactive compounds. The importance of microorganisms' sources for the discovery of novel natural products with a pharmaceutical potential has been proved during the last few decades (Berdy, 2005). Since then, thousands of naturally occurring antibiotics have been discovered from microorganisms. Among all the known microbes, members of the actinomycetes have long been recognized as prolific producers of useful bioactive metabolites with a broad spectrum of activities. Among actinomycetes, the *Streptomyces* are the dominant while the non-*Streptomyces* are called rare actinomycetes comprising approximately 100 genera (Adegboye and Babalola, 2012).

Actinomycetes belong to the order actinomycetales comprising of 14 suborders, 49 families, and over 140 genera. This group is characterized as Gram-positive, heterogeneous with a high G+C content in their genetic makeup (Ventura et al., 2007). Initially they were classified based on the branching filamentous cellular morphology which occurs during the growth cycle. Thus, because of the presence of the filamentous forms (0.5-0.8 microns in diameter) which branch out, the organisms were for many years erroneously classified as fungi (Lechevalier et al., 1971). They produce branching mycelia which may be of two kinds namely substrate and aerial. They produce asexual spores and are not motile, but when motility is present it is confirmed to flagellated spores. Actinomycetes cell wall composition varies greatly among different groups and is of considerable taxonomic importance (Schleifer and Kandler, 1972). Four major cell wall types can be distinguished according to three features of peptidoglycan composition and structure, namely the amino acids in the tetrapeptide side chain position 3, the presence of glycine in the interpeptide bridges, and the

peptidoglycan sugar content. Cell extracts of actinomycetes with wall type II, III, IV also contain characteristic sugars that are useful in identification. Some other taxonomically valuable properties are the morphology and color of mycelia and sporangia, the surface features and arrangement of condiospores, the phospholipids composition of cell membranes, and spore heat resistance (Shirling and Gottlieb, 1966, Lechevalier et al., 1977).

Newer techniques are applied to actinomycetes taxonomy: comparisons of the 16S rDNA sequences have proven valuable (Zhi et al., 2009). Techniques for carrying out the comparisons include DNA-DNA hybridization and PCR based gene analysis (Heuer et al., 1997, Cole et al., 2003). The 16S rDNA gene sequences serves as a powerful tool for finding phylogenetic relationships among microorganisms. This gene is highly conserved among organisms and is therefore an excellent tool for studying evolutionary relationships. The 16S rRNA genes of many phylogenetic groups have characteristic nucleotide sequences called oligonucleotide signatures. Oligonucleotide signatures are short sequences which occur in most or all members of a particular phylogenetic group. This can be used in designing primers which are genus or species-specific for identification (Purohit et al., 2003). PCR and sequencing of the 16S rRNA gene give data that can be used to describe complete microbial community composition and can indicate possible nutritional requirements and physiological niches of many microbes based on information already available for known phylogenetic relatives. The use of molecular approaches for describing microbial diversity has greatly enhanced the knowledge of population structure in microbial communities (Babalola et al., 2009).

Nucleotide sequence similarity values now widely serve as one of the standard taxonomic criteria used at the species level along with estimates of DNA relatedness values (Stackebrandt and Ludwig, 1994). Phylogenetic relationships at higher taxonomic rank are mainly determined by constructing phylogenetic trees, that is, by what is known as

phylogenetic reconstruction. This procedure involves three sequential steps, namely choice of macromolecule, alignment and construction of phylogenetic trees.

Microbial diversity is a vast frontier and potential goldmine for the biotechnology industry since it offers numerous novel compounds, genes and biochemical pathways to probe for enzymes, and other useful molecules (Gurung et al., 2009). Actinomycetes are widely distributed in the environment and include some of the most common soil life, fresh water life, and marine life, but are primarily soil inhabitants (Trujillo, 2001). The number and types present in a particular soil would be greatly influenced by geographical location such as soil temperature, soil type, soil pH, organic matter content, cultivation, aeration and moisture content (Arifuzzaman et al., 2010). Actinomycetes can degrade an enormous number and variety of organic compounds and are extremely important in the mineralization of organic matter (Singh and Sharma, 2013). They play an active role in the decomposition of organic materials such as cellulose, xylan, lignin and chitin; thereby playing a vital part in organic matter turnover and carbon cycle. This replenishes the supply of nutrients in the soil and is an important part of humus formation. The production of extracellular hydrolytic enzymes such as amylases, xylanases, chitinases, cellulases, pectinases and proteases, makes it possible for actinomycetes to break down organic matter in their natural environment.

Microbial secondary metabolites are compounds of low molecular weight (>3000 Da) biosynthesized during the stationary growth phase by a wider variety of synthetic pathways involving various enzymatic steps. Genes encoding for the enzymes responsible for the multistep process in the secondary metabolites biosynthesis are clustered together in the genome of the producing microorganisms. The gene clusters contain gene encoding enzymes such as polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS). These enzymes are referred to as signature genes/enzymes, since they give rise to the skeleton structures of most classes of secondary metabolites (Donadio et al., 2007). The clusters also

contain genes for tailoring enzymes, regulation of the cluster expression and resistance to the end products. Studying the biosynthetic gene cluster organization and regulatory pathways makes it possible to develop strategies to modulate the expression of the secondary metabolites (Thykaer and Nielsen, 2003).

Substantial advances made in the analysis of microbial genomes have enabled the identification of multitude gene clusters responsible for secondary metabolites regulation. This results in the discovery of novel secondary metabolites not detected in standard laboratory process, for example the discovery of a novel peptide, coelichelin from *Streptomyces coelicolor* (Lautru et al., 2005). Such genome mining also provided the tools for engineering the biosynthesis of novel "unnatural" natural compounds through gene shuffling, domain deletions and mutations. Novel aminocoumarins was derived from *Streptomyces* spp through genetic engineering (Li and Heide, 2005). Microbial biotechnology has opened up unexpected new horizons for finding promising microorganisms in order to exploit their potential resources. The microbial universe clearly presents a vast untapped resource for drug discovery.

Actinomycetes are the most economically and biotechnologically valuable prokaryotes due to their ability to produce a vast number of bioactive secondary metabolites. They are found to be responsible for the production of a wide range of secondary metabolites and more than 70% of the naturally derived antibiotics are currently in clinical use (Baltz, 2007). Actinomycetes have been the most attractive sources of antibiotics and other biologically active substances of high pharmacological and commercial value. They have produced all types of bioactive secondary metabolites that have important applications in human medicine as antibacterial, antifungal, antiprotozoal, antiviral, anticancer, anticholesterol, antihelminthic, immunosuppressant compounds and in agriculture as herbicides and insecticides (Ganesan, 2008). Because of the excellent track record of

actinomycetes in this regard, a significant amount of effort has been focused on the successful isolation of novel actinomycetes for drug screening programs in the past fifty years. Various types of bioactive metabolites from actinomycetes include β-lactams, aminoglycosides, lipirmycins, lipopeptides, glycopeptides, asamycins, anthracyclines, nucleosides, peptides, polyenes, polyethers, tetracyclines, macrolides, angucyclines, phenazine, piercidins, octaketides, heptadecaglycoside, and lactones

The diversity of terrestrial actinomycetes is of extraordinary significance in several areas of science and medicine, particularly in antibiotic production (Blodgett et al., 2008). Among the 140 described actinomycetes genera, only a few are responsible for the majority of over 20,000 microbial natural products identified so far. In particular, the genus *Streptomyces* accounts for about 80% of the actinomycete natural products reported to date (Newman and Cragg, 2012). A novel cyclic octaketide antibiotic named Fogacin was isolated from *Streptomyces sp* and exhibited a narrow spectrum of antibacterial activity (Radzom et al., 2006). Nomimicin, a new spirotetronate-class polyketide, was isolated from genus *Actinomadura* and showed antimicrobial activity against *M. luteus*, *C. albincans* and *Kluyveromyces fragilis* (Igarashi et al., 2012). A novel anticancer agent Chandrananimycins A-C was isolated from the culture broth of a marine *Actinomadura sp* (Maskey et al., 2003). Alchivemycin A, a novel polycyclic polyketide isolated from the culture extract of a plant-derived actinomycete *Streptomyces sp.* showed potent antimicrobial activity against *Micrococcus luteus* and inhibitory effects on tumour cell invasion (Igarashi et al., 2010). *Micromonospora* sp. Tü 6368 isolated from terrestrial habitant produced novel secondary metabolites retymicin, galtamycin B, saquayamycin Z and ribofuranosyllumichrome showed potent anticancer and antibiotic activities (Stroch et al., 2005). A new cytotoxic compound, pterocidin, isolated from the endophytic *Streptomyces hygroscopicus*, showed cytotoxicity against some human cancer cells (Igarashi et al., 2006). Yatakemycin, a novel antifungal

antibiotic produced by *Streptomyces sp.* TP-A0356, inhibited the growth of pathogenic fungi such as *Aspergillus fumigatus* and *Candida albicans* (Igarashi et al., 2003).

Thus, screening of terrestrial habitat for promising strains of actinomycetes with potential antibiotics is still a thrust area of research. The specific objectives of this study include:

1. Isolate, identify and characterize antibiotic producing actinomycete strains.
2. Analyze the phylogenetic and molecular evolutionary lineages of the antibiotic producing actinomycete using 16S rDNA.
3. Screen for specific antibiotic biosynthetic gene cluster in the antibiotic producing isolates.
4. Determine the antimicrobial activity of isolated strains against selected pathogens.
5. Determine the optimum conditions for maximum production of the antimicrobial compound(s).
6. Extract, purify and identify the antimicrobial compound(s).
7. Determine the minimum inhibitory concentrations (MIC) of the active extract(s).

LIST OF PUBLICATIONS

Chapter 2: Taxonomy and Ecology of Antibiotic Producing Actinomycetes. *Published in African Journal of Agricultural Research, 7:2255-2261*).

Authors: Mobolaji Felicia Adegboye and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, and wrote the first draft of the manuscript.

Chapter 3: Actinomycetes: A Yet Inexhaustive Source of Bioactive Secondary Metabolites. P*ublished as a book chapter In: Microbial pathogens and strategies for combating them: science, technology and education (A. Méndez-Vilas, Ed.)* (2013) Vol 2: pp 786-795, Formatex Research Center, Badajoz, Spain.

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Authors: Mobolaji Felicia Adegboye and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results and wrote the first draft of the manuscript.

Chapter 5: Isolation and Identification of Potential Antibiotic Producing Rare Actinomycetes from Rhizospheric Soils. *This chapter has been submitted in this format for publication in Biological Research.*

Authors: Mobolaji Felicia Adegboye and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results and wrote the first draft of the manuscript.

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Chapter 9: Isolation and Identification of Bioactive Compounds Produced by *Streptomyces* spp. *This chapter has been submitted in this format for publication in Microbiological Research*

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CHAPTER 2

Taxonomy and Ecology of Antibiotic Producing Actinomycetes

Abstract

The taxonomic and ecological positions of antibiotic producing actinomycetes are an integral part in antimicrobial agents' development program. Comprehensive understanding of the organisms gives useful insight on the secondary metabolites produced by them and other activities carried out by them in their habitat. Criteria for the identification of actinomycetes include morphological, physiological, ecological and molecular characterization. It is vital to identify the organism up to species level, since this will give an indication whether the antimicrobial agent being produced is novel or not. The suborder and habitat also act as pointers for possible secondary metabolites production and confer need for further exploration.

Keywords: Taxonomy, ecology, actinomycetes, antimicrobial agents, characterization

2.1 Introduction

Actinomycetes are prolific producers of novel antimicrobial agents (Atta et al., 2010). Vast numbers of these antimicrobial agents are discovered from actinomycetes by screening natural habitats such as soils and water bodies (Duraipandiyan et al., 2010, Gallagher et al., 2010, Zotchev, 2011). A wide taxonomic range of actinomycetes have the ability to produce secondary metabolites with biological activities such as antibiotic, antifungal, antiviral, anticancer, enzyme, immunosuppressant and other industrially useful compounds (Baltz, 2007, Newman and Cragg, 2007, Demain, 2009, Demain and Sanchez, 2009, Kekuda et al., 2010, Naine et al., 2011). Antibiotics have been isolated from almost all the suborders of

actinomycetes. Despite increase in antibiotic resistance to commonly used drugs, there is still a steady supply of novel antimicrobial agents from actinomycetes isolated from the natural environment (Baltz, 2006, Yang et al., 2011).

Taxonomy is the science of biological classification. The basic taxonomic group in microbial taxonomy is the species. Taxonomic characterization of antibiotic producing actinomycetes is a very important aspect in screening for novel antibiotics (Van Hop et al., 2011). This provides informative insight about the organism, possible kinds of secondary metabolite and whether the metabolite is new or not (Labeda, 1987). Major characteristics used in taxonomy for the classification and identification of microorganisms are morphological, physiological, ecological and molecular characteristics (Willey et al., 2010). Many novel actinomycetes species have been characterized and named, and secondary metabolites extracted from them using various techniques (Kim et al., 2011, Zotchev, 2011). Actinomycetes are phylogenetically grouped as Gram-positive bacteria with high Guanine + Cytosine in their DNA. Actinomycetes belong to the order actinomycetales comprising of 14 suborders, 49 families, and over 140 genera (Wikipedia, 2011).

Majority of actinomycetes are free living organisms that are widely distributed in nature. They are found in both aquatic and terrestrial habitats. These bacteria have high mechanisms of survival in adverse environments (Macagnan et al., 2006). The use of molecular techniques to study microbial diversity has brought a great advancement to microbial ecology, making it possible to determine the natural microbial population especially in the soil (Alam et al., 2010, Hirsch et al., 2010). One of the goals of ecology is to study the distribution and biodiversity of microbes in various climates and natural habitats.

Actinomycetes population have been identified as one of a prominent group of soil microbes which differ with soil type, soil pH, geographical location and climatic condition

(Arifuzzaman et al., 2010). The characterization of these microbes is as important as studying their existence in natural environments (Hirsch et al., 2010). Actinomycetes play a vital role in the soil such as mineralization of organic matter, immobilization of nutrients, antibiosis and production of plant promoters (Anderson et al., 2011, Sonia et al., 2011). This review focuses on the taxonomy and ecology of antibiotic producing actinomycetes. It is essential to understand the taxonomy and ecology of secondary metabolites producing actinomycetes to facilitate the exploration of the different strains for biotechnology.

2.2 Taxonomy of Antibiotic Producing Actinomycetes

Taxonomy is an integral aspect of science and is also important in the screening for novel organisms with the ability to produce secondary metabolites that can be of valuable use. A purposeful search for novel antibiotics will be worthwhile if there is good knowledge about the species that is producing them (Labeda, 1987). Actinomycetes taxonomy was previously based on morphology; this is inadequate to differentiate between different species of many genera. The use of phylogenetic and molecular evolutionary approaches has greatly helped the classification methods (Babalola et al., 2009, Hozzein and Goodfellow, 2011). Uncultivable or not easily cultivated actinomycetes can now be identified from environmental samples due to the advent of metagenomics (Mincer et al., 2005, Hirsch et al., 2010). Some organisms that were erroneously placed in inappropriate groups are now classified appropriately due to the advent of molecular techniques (Zhi et al., 2009). Phylogenies and species identification are now commonly derived from 16S rRNA and the use of polymerase chain reactions (PCR) for sequence analyses (Wood et al., 2007, Zhi et al., 2009).

Taxonomic characterization of antibiotic producing actinomycetes is a tremendously significant step in screening for novel antibiotics (Labeda, 1987). Actinomycetes exhibits

considerable physiological and biochemical diversity, the order is diverse in term of morphology, phylogeny and chemotaxonomy (Kekuda et al., 2010). This group was initially classified based on their branching filamentous morphology which occurs during the growth cycle (Willey et al., 2010). Thus, due to the presence of the filamentous forms which branches out, these organisms were wrongly classified as fungi for many years before they were rightly placed in the bacteria kingdom (Madigan et al., 2009). Certain criteria are used for the classification and identification up to the species level; these include growth on different media, mycelial pigment, cell wall composition, utilization of carbon and nitrogen sources, production of spores, and mol % of G+C of DNA (Willey et al., 2010). Recent, phylogenetic and molecular techniques including 16S rRNA analysis and DNA-DNA hybridization are commonly used (Ventura et al., 2007, Hirsch et al., 2010).

Actinomycetes are morphologically diverse ranging from rod to coccoid, fragmenting hyphal forms to those with a highly differentiated branched mycelium (Trujillo, 2001). Many of these bacteria also produce external spores. The cell wall composition of actinomycetes is of significant taxonomic value which differs among the different suborders. There are four types of cell wall distinguished based on the characteristics of peptidoglycan composition and structure (Willey et al., 2010). These characteristics are: the type of amino acids in tetrapeptide side chain position 3, the presence of glycine in interpeptide bridges, and peptidoglycan sugar content (Willey et al., 2010). Cell extracts of actinomycetes with wall type II, III, and IV also contain characteristic sugars that are useful in identification. Some other taxonomically important features are the cellular morphology, color of mycelia and sporangia, the surface features and arrangement of conidiospores, the presence of high G+C content in DNA, the phospholipids composition of cell membranes and heat resistant spores (Willey et al., 2010). Modern techniques are applied to actinomycetes taxonomy: comparisons of the 16S rRNA sequences have proven valuable (Zhi et al., 2009). The pattern

of 16S rRNA signatures consists of nucleotides at positions 688:699 (G–C), 701 (C), 823:877 (G–C) and 1060:1197 (U–A) (Zhi et al., 2009). Based on the molecular and chemical composition data, the order actinomycetales is grouped into 14 suborders (Wikipedia, 2011). These include the following suborders Actinomycineae, Actinopolysporineae, Catenulisporineae, Corynebacterineae, Frankineae, Glycomycineae, Jiangellineae, Kineosporineae, Micrococineae, Micromonosporineae, Propionibacterineae, Pseudonocardineae, Streptomycineae and Streptosporangineae (Euzéby, 1997).

2.2.1 Suborder Actinomycineae

The genera *Actinobaculum*, *Actinomyces*, *Arcanobacterium*, *Falcivibrio*, *Mobiluncus*, *Trueperella* and *Varibaculum* are located in this suborder (Euzéby, 1997). Most genera in this suborder have irregular shaped, fragmenting filaments without aerial hyphae and spores. They are Gram-positive rods with aerobic anaerobic or facultative metabolism (Willey et al., 2010). Their rods may be straight or slightly curved and usually have swelling, club shapes or other deviations from normal rod-shaped morphology (Trujillo, 2001). The cell wall compositions contain lysine but not diaminopimelic acid or glycine (Sumbali and Mehrotra, 2009). The 16S rRNA nucleotide signature is at position 127:234 (R–U), 598:640 (Y–G), 828 (R), 829:857 (G–C), 832:854 (G–Y), 952:1229 (C–G) and 986:1219 (A–U) (Zhi et al., 2009). They play an important ecological role by producing enzymes that help degrade organic matter in the soil such as lignin and chitin. They also help in the formation of compost (Zhi et al., 2011).

2.2.2 Suborder Streptomycineae

The suborder *Streptomycineae* has only one family, *Streptomycetaceae*, and ten genera, which are *Actinopycnidium*, *Actinosporangium*, *Chainia*, *Elytrosporangium*, *Kitasatoa*, *Kitasatospora*, *Microellobosporia*, *Streptacidiphilus*, *Streptomyces* and *Streptoverticillium* (Euzéby, 1997). The most important of these genera is *Streptomyces*. Bacteria in these genera

have aerial hyphae that divide in a single plane to form chains of 3-50 or non–motile conidiospores with surface texture ranging from smooth to spiny and warty (Willey et al., 2010). All have a type 1 cell wall and G+C content of around 69 to 78 mol % (Farris et al., 2011). The pattern of 16S rRNA signatures consists of nucleotides at positions 127:234 (G–C), 449 (A), 672:734 (C–G), 950:1231 (U–G), 952:1229 (U–A), 955:1225 (C–G), 965 (C), 986:1219 (A–U) and 1362 (C) (Zhi et al., 2009).

This suborder is very important both ecologically and medically (Farris et al., 2011). The natural habitat of most genera in this suborder is the soil, where they constitute from 1-20% of the culturable population (Trujillo, 2001). The odour of moist earth is largely as a result of *Streptomyces* production of volatile substances such as geosmin (Jiang et al., 2007). They play a major role in mineralization and immobilization of soil nutrients (Sonia et al., 2011). They are flexible nutritionally and can aerobically degrade resistant substances such as pectin, lignin, chitin, keratin, latex and aromatic compounds (Shi et al., 2011). *Streptomyces* are best known for their synthesis of a vast array of antibiotics, some of which are useful in medicine and agriculture (Watve et al., 2001).

2.2.3 Suborder Corynebacterineae

This suborder contains 7 families with several genera including *Amycolicicoccus*, *Bacterionema*, *Caseobacter*, *Corynebacterium*, *Dietzia*, *Gordonia*, *Micropolyspora*, *Millisia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Segniliparus*, *Skermania*, *Smaragdicoccus*, *Tomitella*, *Tsukamurella*, *Turi* and *Williamsia* (Euzéby, 1997). The most important ones are *Corynebacterium*, *Mycobacterium* and *Nocardia.* This suborder is composed of aerobic and facultative, catalase positive, straight to slightly curved rods or filamentous rods. Corynebacterineae are characterized by an unusual cell wall structure, having peptidoglycan with meso-diaminopimelic acids and no peptide interbridge in the cell wall (Van Hop et al., 2011). The wall usually contains carbohydrate composed of arabinose, galactose lipid and

mycolic acids, which are usually present in different compositions depending on the genera. They are characterized by the presence of mycolic acids. Although some species are non-pathogenic, many are plant and animal pathogens (Pelczar et al., 2006). They have about 64 to 74 mol % G+C content in their DNA. The pattern of 16S rRNA signatures consists of nucleotides at positions 127:234 (G–Y), 564 (C), 672:734 (U–G), 833:853 (U–G), 952:1229 (U–A) and 986:1219 (U–A) (Zhi et al., 2009).

Members of this suborder are mostly soil inhabitants and are good survivors in harsh environments. Because of this attribute they are found in a wide range of environmental niche. They play an important role in bioremediation of heavily oil contaminated environment due to their ability to utilize diverse substrates (Shen et al., 2008). Some genera like *Rhodococcus*, *Gordonia* and *Mycobacterium* also play an active role in the mineralization of polycyclic aromatic hydrocarbons. The antibiotic griseusin was isolated from this family (Gandhimathi et al., 2008).

2.2.4 Suborder Micrococcineae

The suborder *Micrococcineae* has 17 families and a wide variety of genera. The suborder contains the families, Beutenbergiaceae, Bogoriellaceae, Brevibacteriaceae, Cellulomonadaceae, Demequinaceae, Dermabacteraceae, Dermacoccaceae, Dermatophilaceae, Intrasporangiaceae, Jonesiaceae, Microbacteriaceae, Micrococcaceae, Promicromonosporaceae, Rarobacteraceae, Sanguibacteraceae and Yaniellaceae (Euzéby, 1997). The two best-known genera are *Micrococcus* and *Arthrobacter*. This suborder contains aerobic, catalase positive, cocci or rods and usually non-motile. The peptidoglycan layer of the cell wall contains lysine. They have about 59 to 70 mol % G+C content (Willey et al., 2010). The pattern of 16S rRNA signatures consists of nucleotides at positions 127:234 (A–U), 598:640 (U–G), 657:749 (U–A), 953:1228 (G–C), 986:1219 (A–U), 987:1218 (A–U) and 1362 (A) (Zhi et al., 2009). They are widespread in soil, water and on mammalian skin.

This suborder is unusually flexible nutritionally and can even degrade some herbicides and pesticides; it is probably important in the mineralization and assimilation of organic molecules (Viamajala et al., 2007, Matsui et al., 2009, Nawel et al., 2011).

2.2.5 Suborder Micromonosporineae

This suborder contains only one family *Micromonosporaceae* with up to thirty genera. The suborder contains genera like *Actinoplanes, Catenuloplanes*, *Couchioplanes*, *Dactylosporangium*, *Micromonospora*, *Pilimelia, Salinispora* and. *Verrucosispora* (Euzéby, 1997). They are aerobic like most actinomycetes and their peptidoglycan contain meso-diaminopimelic acid (DAP), glycine, Arabinose and xylose. They have an extensive substrate mycelium and are wall type IID. Often the hyphae are highly colored and diffusible pigments may be produced. Conidiospores are usually formed within a sporangium raised above the surface of the substratum at the end of special hyphae called a sporangiosphore (Willey et al., 2010). The spores can be either motile or non-motile. The G+C content is about 72 to 73 mol %. The pattern of 16S rRNA signatures consists of nucleotides at positions 127:234 (A–U), 209 (G), 534 (G), 831:855 (U–G), 832:854 (G–Y), 833:853 (U–G), 840:846 (Y–G), 845 (G), 955:1225 (A–U), 986:1219 (U–A) and 987:1218 (G–C) (Zhi et al., 2009). They are widely distributed in nature; growing in almost all the soil habitats ranging from forest litter to beach sand. Due to their ability to produce hydrolytic enzymes like xylanases, chitinases, they are able to degrade a wide range of organic matter in their natural habitat. Members of this suborder also help in nitrogen fixation (Hirsch and Valdés, 2010).

2.2.6 Suborder Propionibacteriaceae

This suborder contains two families and 25 genera including *Aestuariimicrobium*, *Actinopolymorpha*, *Auraticoccus*, *Luteococcus*, *Nocardioides*, *Propionibacterium* and *Thermasporomyces*. The most important genus is *Propionibacterium*, which are usually catalase positive, contains pleomorphic, non-motile, non-sporing rods that are often club-

shaped with one end tapered and the other end rounded (Willey et al., 2010). Members of the family have cell wall peptidoglycans including LL-diaminopimelic acid (DAP), meso-DAP or lysine as the diagnostic diamino acid depending on genus (Jung et al., 2007). The genus is facultatively anaerobic or aerotolerant; lactate and sugars are fermented to produce large qualities of propionic and acetic acids, and carbon (IV) oxide (Willey et al., 2010). The G+C content varies from 53 to 67 mol %. The pattern of 16S rRNA signatures consists of nucleotides at positions 127:234 (A–U), 598:640 (U–A), 657:749 (G–C), 828 (U), 829:851 (A–C), 832:854 (U–C), 833:853 (G–U), 952:1229 (C–G) and 986:1219 (U–A) (Zhi et al., 2009). The genus is found growing on the skin and the digestive tract of animals and in dairy products such as cheese (Sumbali and Mehrotra, 2009).

2.2.7 Suborder Streptosporangineae

The suborder *Streptosporangineae* contains three families and 23 genera including *Actinoallomurus*, *Actinomadura*, *Microbispora*, *Microtetraspora*, *Nonomuraea*, *Nocardiopsis*, *Planobispora*, *Planomonospora*, *Planotetraspora*, *Sphaerisporangium*, *Spirillospora*, *Streptomonospora*, *Streptosporangium*, *Thermomonospora* and *Thermopolyspora*. They have type III cell walls containing meso-diaminopimelic acid and whole cell hydrolysate containing madurose and galactose. Their G+C content is about 64 to 74 mol %. Aerial mycelia bear pairs of short chains of spores, and the substrate mycelium is branched. Some genera form sporangia; spores are not heat resistant (Willey et al., 2010). This suborder contains some thermophiles that have been isolated from high temperature habitants such as compost piles and hay. It can grow at 40 to 48°C (Willey et al., 2010). The pattern of 16S rRNA signatures consists of nucleotides at positions 127:234 (A–U), 829:857 (G–C), 830:856 (G–C), 953:1228 (U–A), 950:1231 (U–A), 955:1225 (C–G), 986:1219 (A–U) and 987:1218 (A–U) (Zhi et al., 2009).

2.2.8 Suborder Frankineae

The suborder contains six families and twelve genera including *Acidothermus*, *Cryptosporangium*, *Fodinicola*, *Frankia*, *Blastococcus*, *Geodermatophilus*, *Modestobacter*, *Humicoccus*, *Nakamurella*, *Saxeibacter* and *Sporichthya* (Euzéby, 1997). They are aerobic, catalase positive with type III cell walls containing sugars like glucose, xylose and galactose, although the cell extract sugar patterns differ among the different genera (Lee et al., 2004). They have motile sporangiospores in a sporogenous body. The G+C content varies from 57 to 75 mol %. The pattern of 16S rRNA signatures consists of nucleotides at positions 184:193 (A–C), 195 (A), 196 (U), 582:758 (U–A), 601:637 (G–U), 602:636 (C–G), 841 (C), 952:1229 (U–A), 986:1219 (A–U), 1059:1198 (C–G) and 1308:1329 (C–G) (Zhi et al., 2009). Members of this suborder have been isolated from various habitats such as rhizosphere, hot springs, activated sludge and geographically diverse soils (Carlsohn et al., 2008). They are involved in nitrogen fixation in association with non-leguminous plants.

2.3 Ecology of Antibiotic Producing Actinomycetes

Microbial diversity is a substantial leading edge and prospective goldmine for the biotechnology industry because it offers countless secondary metabolites to probe for enzymes, antibiotics, antioxidants, cytotoxics and many other useful substances (Singh and Pelaez, 2008, Gurung et al., 2009, Williams, 2009). The actinomycetes occur in a vast diversity of habitats, either natural or artificial, growing on different kinds of substrates. The diversity of actinomycetes is of exceptional impact in several areas of pharmaceutics, medicine and agriculture, particularly in antibiotic production (Blodgett et al., 2008). Actinomycetes are ubiquitous and have been isolated from various locations, in the soil, fresh water, marine, hot spring, mining sites, and also in extreme environments.

2.3.1 Actinomycetes as Soil Inhabitant

Soil is a unity entity that inhabits varieties of microorganisms and the microbial community is an integral part of the soil. Actinomycetes are primarily soil inhabitants and are also very widely distributed in nature (Babalola et al., 2009; Gurung et al., 2009). They are also well known as soil saprophytes and are responsible for the distinctive earthy odour of freshly ploughed soil due to the production of geosmin. The most dominant actinomycetes in soil is the genus *Streptomyces* although others like *Nocardia*, *Microbispora*, *Micromonospora*, *Actinomyces*, *Actinoplanes* and *Streptosporangium* have also been isolated from the soil. The number and variety of actinomycetes present in any soil sample would be significantly influenced by geographical location, soil temperature, soil type, soil pH, organic matter content, agricultural activities, aeration, nutrient availability, moisture content and soil vegetation (Arifuzzaman et al., 2010). Actinomycetes have been isolated from diverse soil types and locations such as arid, tropical forest, mining, cave, swamp, desert and savannah. They are particularly abundant in slightly alkaline soils rich in organic matters and produce several structurally diverse secondary metabolites of pharmaceutical and agricultural importance.

Actinomycetes play an important ecological role by recycling and mineralization of nutrients in the soil. They help to recycle nutrients by degrading vast numbers of organic matter in the soil and are found most commonly in compost. They act as plant growth promoters by helping in nitrogen fixation, solubilization of nutrients, immobilization of nutrients, siderophores production, biological control and soil structure maintenance (Macagnan et al., 2008, Rascio et al., 2008, Vargas Gil et al., 2009, Kekuda et al., 2010). Actinomycetes are of great practical importance in nature and seem to be ultimately involved in soil ecology (Van Hop et al., 2011).

2.3.2 Actinomycetes as Rhizobacteria

Rhizosphere is defined as the soil ecological zone surrounding the roots of growing plants. It is a unique biological niche within the soil environment having high nutrients content and housing numerous and diverse soil microbes. The high nutrient content in the rhizosphere is as a result of sloughed off plant cell (rhizodeposition) and exudates from the roots such as proteins and sugars. The high nutrient content in the rhizosphere makes the microbial load higher than that of the surrounding bulk soil. The bacteria which colonize plant root are called rhizobacteria. They specifically multiply and inhabit the growing plant root system and continue throughout the life span of the plant. A rhizobacterium may form a symbiotic relationship with a plant, for example when the bacterium produces antibiotic that inhibit plant pathogens in exchange for nutrients. Such microbes are also referred to as plant growth promoting rhizobacteria (Compant et al., 2010).

Actinomycetes are one of the prominent soil microbes and they grow in close association with the plant organs. They form thread-like filaments in the soil which give them an advantage in colonizing the rhizosphere effectively. As a rhizobacteria, they influence plant growth, antagonize plant pathogens and makes nutrients available for the plants (Maheshwari and Shimizu, 2011). Actinomycetes are known to be versatile degraders of complex organic matters such as cellulose, lignin, xylan, chitin and other complex polysaccharides (Macagnan et al., 2008). The production of hydrolytic enzymes makes it possible for actinomycetes to break down organic matter in their natural environment (Marsh and Wellington, 2007). Several reports have shown that actinomycetes are one of the important groups of root-colonizing microorganisms (Franco-Correa et al., 2010, Nimnoi et al., 2010).

Table 2.1: Examples of some rhizospheric actinomycetes and their functions to plants

Rhizospheric Actinomycetes	Function	Plant species	Reference
Micromonospora endolithica	Phosphate solubilization to promote plant growth	Bean (*Phaseolus vulgaris* L.)	(El-Tarabily et al., 2008)
Streptomyces griseus	Protection against damping off disease caused by *Pythium ultimum*	Wheat (*Triticum spp.*)	(Hamdali et al., 2008)
Frankia species	Biological fixation of nitrogen	Actinorhizal plant (*Casuarina equisetifolia*)	(Rascio et al., 2008)
Norcardia levis	Biological control of *Fusarium oxysporum* wilt disease	Sorghum (*Sorghum bicolor)*	(Kavitha et al., 2010)
Streptomyces species	Act as biocontrol against *Rhizoctonia solani*	Tomato (*Solanum lycopersicum*)	(Patil et al., 2011)
Streptomyces species	Bioremediation of contaminated soil	Maize (*Zea mays*)	(Benimeli et al., 2008)

2.4 Conclusion

Actinomycetes play a significant role in the production of antimicrobial agents and other industrially important substances like enzymes. It is essential to have a good knowledge about their taxonomy and ecology, for maximum exploration, since they are of great use for economic and industrial development. In soil ecology, they are also active in bioremediation, biofertilizer, biocontrol and as plant growth promoters, making them indispensable in agricultural practice. Although a great deal of work has been carried out on actinomycetes, more comprehensive studies are needed in the area of taxonomy and ecology. This will help to predict the productivity of members of this order and their possible exploitation.

CHAPTER 3

Actinomycetes: A Yet Inexhaustive Source of Bioactive Secondary Metabolites

Abstract

The rapid emergence of antimicrobial resistance among pathogens has led to a renewed interest in searching for novel antimicrobial agents. The history of new drug discovery processes shows that novel skeletons have, in the majority of cases, come from natural sources. This involves screening of microorganisms or plant extracts. They have been the source of, or inspiration for the development of chemical entities introduced as pharmaceuticals. Among microorganisms, actinomycetes are an enthralling resource due to their ability to produce novel bioactive secondary metabolites with antimicrobial activities. They have proven to be an inexhaustive mine of antimicrobial agents, especially those potent against pathogenic organisms. Microbial secondary metabolites, especially those from actinomycetes have been a phenomenal success for the discovery of novel drugs. They produced a wide range of secondary metabolites and more than 70% of the naturally derived antibiotics currently in clinical use. They remain a fundamental source of new chemical diversity and an important part of drug discovery. Their ingenuity and immense industrial value is extremely noteworthy. The discovery of streptomycin from actinomycetes has been imperative to the exploration of this group as a source of novel bioactive compounds. This group of organisms have produce antibiotics in different classes such as aminoglycosides, ansamycins, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines. Existence of actinomycetes has been reported in both terrestrial and marine habitats. This chapter highlights the bioactive metabolites produces by actinomycetes and their mode of action.

Keywords: actinomycetes; antimicrobial agents; secondary metabolites, mechanism of action

3.1 Introduction

History has shown that the discovery of novel antimicrobial agents has often come from natural sources (Chin et al., 2006, Ganesan, 2008). These natural products having novel skeletons have been found to possess important biological activities and produce a significant number of therapeutic agents in clinical use all around the world. They even serve as a template for the synthesis of synthetic and semi-synthetic drugs. These discoveries involve the screening of microorganisms and plants from nature, using various techniques (Newman and Cragg, 2012). Microorganisms over the years have proved to be fascinating sources of natural products for industries especially, pharmaceutical industries (Berdy, 2005). The importance of microorganisms' sources for the discovery of novel natural products with a pharmaceutical potential has been proved fruitful during the last decade and was highlighted in various excellent review articles. Microbial natural products are noteworthy for their high therapeutic index and desirable pharmacological activities.

Most of the microbial bioactive compounds discovered so far originated from actinomycetes, accounting for about two-third of antibiotics, including those in clinical uses. Actinomycetes are the most economically and biotechnologically worthwhile microorganisms (Baltz, 2007, Naine et al., 2011, Raja and Prabakarana, 2011). They have produced a wide range of secondary metabolites of various medical importances such as antibiotics, antifungal, antiprotozoal, antiviral, anticholesterol, antihelminthic, anticancer, and immunosuppressant. Among the 140 described actinomycetes genera, only a few are responsible for the over 10,000 bioactive compounds in clinical use.

Actinomycetes are Gram-positive bacteria of the order Actinomycetales; they are characterized by filamentous morphology, DNA with a high in G+C content presence of LL-Diaminopimelic acid (LL-DAP) and the presence or absence of characteristic sugars in the cell wall. Actinomycetes are ubiquitous and form a stable and persistent population in various

ecosystems (Dietz et al., 1996, Adegboye and Babalola, 2012). The discovery of new actinomycete taxa from diverse habitats with unique metabolic activity often led to the discovery of novel antimicrobial agents. Various antimicrobial agents have been isolated and characterized from actinomycetes including aminoglycosides, anthracyclines, glycopeptides, macrolides, β-lactams, polyenes, phenazine, and tetracyclines. In this chapter, we point out biological activities of secondary metabolites produced by actinomycetes in an effort to combat infectious diseases.

3.2. Actinomycetes as producers of secondary metabolites

Microbial secondary metabolites have been in the frontier in the discovery of novel antimicrobial agents for the pharmaceutical industry, and today all evidence suggests that novel compounds with potential therapeutic applications are still waiting to be discovered from secondary metabolites especially those produced by actinomycetes. Actinomycetes are prolific producers of secondary metabolites with biological activities (Marinelli and Marcone, 2011). Secondary metabolites are metabolic products that are not essential for vegetative growth of the producing organisms but they are considered differentiation compounds conferring adaptive roles, for example, by functioning as defense compounds or signalling molecules in ecological interactions. They are produced at the end of the exponential growth phase and their syntheses greatly depend on the growth conditions. Production is usually when growth is limited by the exhaustion of one key nutrient such as carbon or nitrogen (Sanchez and Demain, 2002, Barrios-González and Mejýa, 2008). They are structurally diverse and most of them are endowed with biological activities, such as antimicrobial agents, toxins, pesticides, ionophores, bioregulators, and quorum signalling. These bioactive metabolites are profoundly used as antimicrobial agents for the treatment of diverse ailments (Vaishnav and Demain, 2010).

Secondary metabolites usually comprise various chemical moieties, such as polyketide backbones, amino acid derivatives and sugars. Biosynthesis of secondary metabolite is catalyzed by a number of enzymes, usually encoded by genes. These genes occur adjacent to one another in clusters. The gene cluster contains all the necessary genes for the synthesis of a particular secondary metabolite. These include: the genes that encode the biosynthetic enzymes, regulatory proteins, genes for resistance to the toxic action of secondary metabolites and genes for secretion of the metabolites. Enzymes such as polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) are involved in the synthesis of secondary metabolites (Donadio et al., 2007). Other enzymes responsible for the synthesis of other constitutive compounds, such as sugars, are often encoded by genes adjacent to the gene cluster. Through processes such as elongation, synthesis, glycosylation, alkylation and oxidation, structurally diverse and complex metabolites are produced. The whole process of production and transportation of secondary metabolites is strictly regulated by transcriptional regulators and transporters (Ichikawa et al., 2013). The genes encoding for tailoring enzymes, transcriptional regulators and transporters are often located adjacent to PKS and NRPS genes. The size of the gene cluster responsible for the synthesis of each secondary metabolite is usually between 10-100 kb.

Previous studies showed that the gene cluster responsible for the production of secondary metabolites is not found in all bacteria and even in those present it is not uniformly distributed among them. For example *Streptomyces coelicolor* possesses more than 20 gene clusters while *S. avermitilis* possesses 30 gene clusters for the synthesis of secondary metabolites (Arias et al., 2011). Genome mining for new candidate secondary metabolic pathways based on clustering and co-expression has proved to be a highly successful approach in microbes (Osbourn, 2010). This helps to predict the types of antibiotic one might expect to find after extraction and purification. With the growing number of genome

nucleotide sequence information in the GenBank and the advent of next generation sequencing it will be possible to search for candidate secondary metabolite gene clusters in a wide range of actinomycetes species. The evolution of microbial natural product collections and development of high-throughput screening methods have attracted researchers to the use of natural product libraries in drug discoveries. Ecopia Biosciences Inc used high-throughput genome scanning to detect secondary metabolite gene clusters, then used the signature sequences to predict the structures (Baltz, 2008). Actinomycetes continue to be a productive and successful focus for natural products research, with many novel compounds being of eminent pharmacological value.

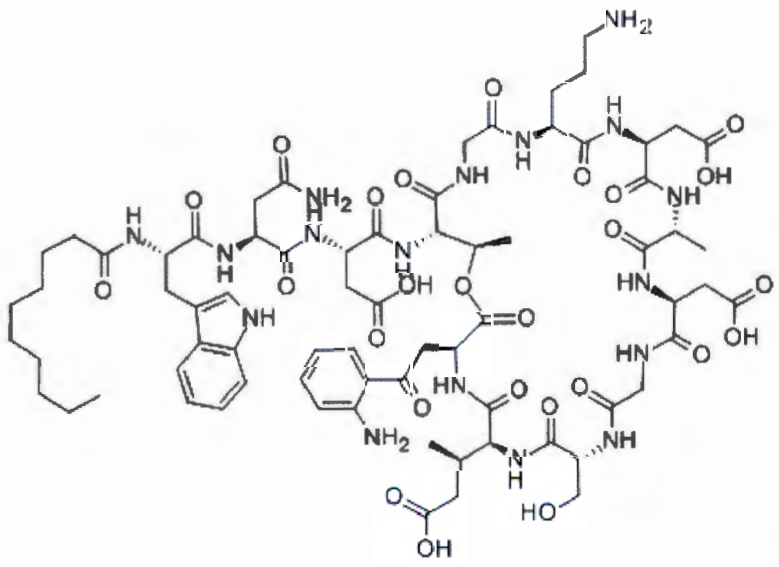
3.3. Antimicrobial activities of actinomycete secondary metabolites

Secondary metabolites produced by actinomycetes exhibit a great number of diverse and versatile biological effects, first of all antimicrobial activities. The members of the order Actinomycetales are renowned producers of bioactive metabolites with a track record of over 10,000 antimicrobial agents in clinical use (Demain, 2009). The secondary metabolites produced by actinomycetes reveal multifarious biological activities such as antibacterial, antifungal, antiviral, anticancer, antiprotozoal, anticholesterol, antiageing, antihelminthic and immunosuppressant. This group of compounds forms a heterogeneous assemblage of biologically potent molecules with diverse structures and mechanisms of action. The discovery of antimicrobial agents from actinomycetes led to a breakthrough in the world of medicine, due to their tremendous contribution in saving human beings from infectious diseases. Most bacterial infections with no cure in the 19^{th} century can easily be cured now with a short course of antibiotics, for example tuberculosis (McDermott et al., 1947). About 75% of the antibacterials are produced by actinomycetes. Many of these antibacterials exhibit a broad spectrum of activities. The diversity in structure of these antibacterials is responsible

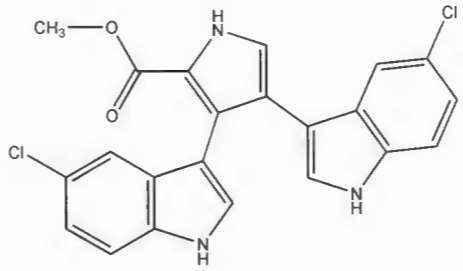
for their broad spectrum antimicrobial activities and diverse mechanisms of action. They show high potency against a large number of Gram-positive and Gram-negative organisms. Historically, actinomycetes have been the origin of the largest number of new antibiotic drug candidates and lead molecules with applications in many other therapeutic areas. This order alone produced 45% of the presently known bioactive microbial metabolites; over 10,000 compounds were still isolated from various actinomycetes species, 34% from *Streptomyces* and 11% from the rare actinomycetes. The most frequent producers, the *Streptomyces* species, produce 7600 compounds (74% of all actinomycetes), while the rare actinomycetes represent 26%, altogether 2500 compounds. Members of this group include: *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora* and *Streptosporangium*.

Daptomycin a cyclic lipopeptide antibiotic produced by *S. roseosporus* through NRPS mechanism is clinically used in the treatment of antibiotic resistant pathogens such as methicillin resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. It exhibit bacteriocidal mode of action by causing rapid depolarization on the bacterial cell membrane, inhibition of biosynthesis of protein, cell wall and lipoteichoic acid (Baltz, 2009). Lynamicins A-E are chlorinated bisindole pyrrole antibiotics produced by *Marinospora* sp. These alkaloids demonstrated strong broad spectrum antimicrobial activities against drug-resistant pathogens (McArthur et al., 2008). Marinopyrroles A-F are alkaloids composed of two salicyloyl substituents on a 1, 3′-bipyrrole core. They are the first naturally occurring 1, 3′-bipyrrole reported and produced by *Streptomyces* spp isolated from marine sediment. These compounds show cytotoxicity against human cancer and antibacterial activities against resistant pathogens (Hughes et al., 2008). Echinomycin is a quinoxaline compound isolated from *S. echinatus*. This compound demonstrated antibacterial, antiviral and antitumor activities (Foster et al., 1985, Kong et al., 2005).

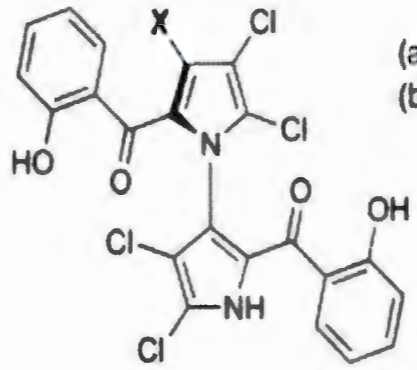
Platensimycin is a benzoic acid moiety antibiotic produced by *S. platensis*. Platensimycin exerts its action by selectively inhibiting β-ketoacyl-acyl carrier protein (ACP) synthase II (FabF) in the synthetic pathway of fatty acids (Wang et al., 2006). This compound is acknowledged as an effective broad-spectrum antibiotic against drug-resistant microorganism strains for example MRSA (Singh et al., 2006). Diazepinomicin is a dibenzodiazepine alkaloid isolated from *Micromonospora* sp (McAlpine et al., 2008). The compound shows significant antitumor, antiparasitic, antioxidant and antiprotease activities (Abdelmohsen et al., 2012). The streptogramins are cyclic hexa or hepta depsipeptides antibiotics produced by the *Streptomyces* spp through NRPS mechanism. They are used in the treatment of infection caused by multiple drug-resistant pathogens. The mechanism of action is by inhibiting protein synthesis by preventing polypeptide elongation (Bonfiglio and Furneri, 2001). Spinosyns are glycosylated polyketide insecticides produce by *Saccharopolyspora species* through PKS mechanism. These compounds are used as biocontrol for insect pests. They have a novel mechanism of action that involves disrupting the binding sites on nicotinic acetylcholine receptors of the insect nervous system (Kirst, 2010).



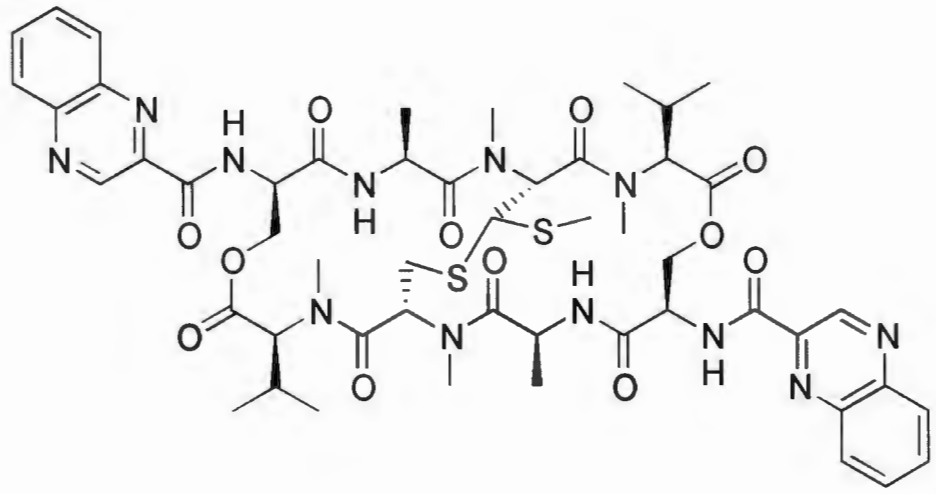
Daptomycin



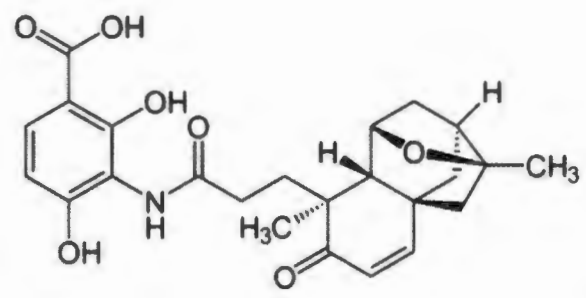
Lynamicin A



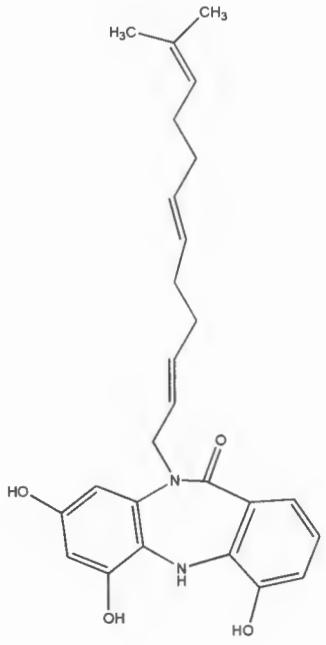
(a) (-)-marinopyrrole A X = H
(b) (-)-marinopyrrole B X = Br



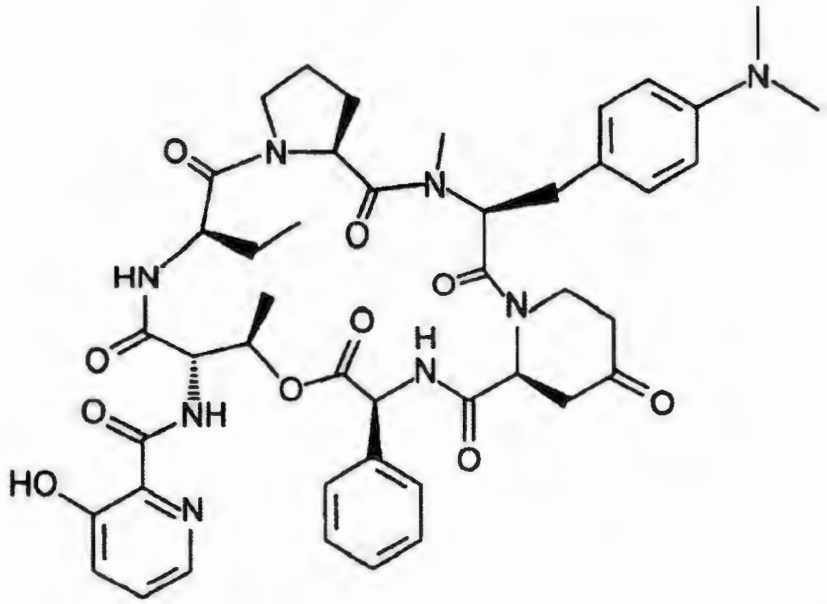
Echinomycin



Platensimycin



Diazepinomicin



Streptogramins

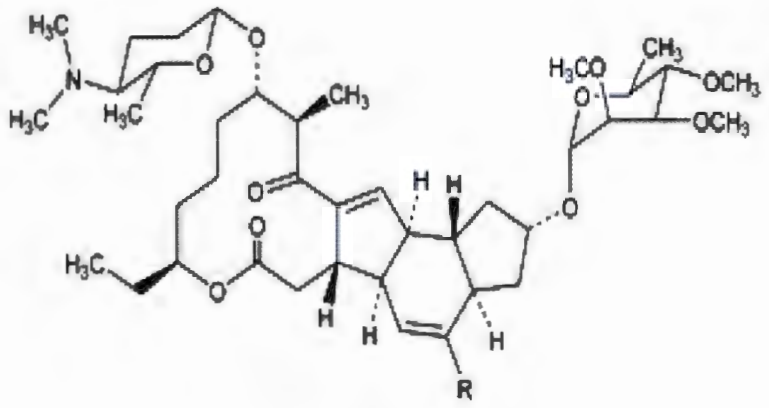


Figure 3.1: Structures of various compounds produced by members of the order *Actinomycetales*

3.4. Important classes of antibiotics produced by actinomycetes

Streptomyces, Micromonospora, Actinomadura, Amycolatopsis, Streptoverticillium, Actinoplanes, Nocardia, Saccharopolyspora Streptosporangium, Streptoalloteichus, Dactylosporangium, Frankia and *Streptosporangium* spp. are increasingly playing a significant role in the production of a wide range of antimicrobial metabolites of enormous importance to the pharmaceutical industries. Important classes of antibiotics produced by actinomycetes include: β-lactams, aminoglycosides, lipopeptides, glycopeptides, asamycins, anthracyclines, nucleosides, peptides, polyenes, polyethers, tetracyclines and macrolides.

3.4.1 β-Lactams

β-lactams are one of the most important classes of antibiotics in clinical use. This class include penicillins, cephalosporins, cephamycins, carbapenems, monobactams and clavulanic acid (Elander, 2003). They are bacteriocidal in nature and exhibit a broader spectrum of antibiotic activities, which are active against both the Gram-positive and Gram-negative bacteria. The main producers of these antibiotics in the order Actinomycetales are *Nocardia lactamdurans*, *Streptomyces clavuligerus, S. chartreusis, S. panayaensis, S. viridochromogenes, S. wadayamensis, S. jumonjinenesis* and *S. limanii* (Thykaer and Nielsen, 2003).

The biosynthesis of β-lactams contains specific enzymatic steps: the first three steps are common for penicillin, cephalosporin and cephamycins (Martín et al., 1999). The first step is the condensation of the three amino acids L-α-aminoadipate (L-α-AAA), L-cysteine and L-valine to form the tripeptide α-aminoadipyl-cysteinyl-valine (ACV) catalyzed by the enzyme ACV synthetase, which also performs an epimerization of L-valine to D-valine. The second step is the cyclization of LLD-ACV to form isopenicillin N (IPN), catalysed by isopenicillin N synthase and in this reaction the characteristic penam ring structure is formed. The third step is the conversion of the L-configuration of the α-AAA lateral chain in the isopenicillin N

to the D-isomer of penicillin N by IPN epimerase. For penicillin, the next step is the exchange of hydrophilic L-α-AAA side chain of IPN with CoA-thioester activated form of hydrophobic acyl group such as phenylacetic acid or phenoxyacetic acid leading to the formation of penicillin G or penicillin V respectively (Martín et al., 2010). This side chain exchange is catalyzed by the enzyme acyl-CoA:IPN acyltranferase. For cephalosporin, the next step is the expansion of the 5-membered thiazolidine ring in penicillin N to a 6-dihydrothiazine ring in deacetoxycephalosporin (DAOC) by the DAOC synthase. Then, the hydroxylation at the C-3 carbon of DAOC to form deacetylcephalosporin (DAC) by DAC synthase. The final step is the addition of acetyl group to the hydroxyl group of DAC catalyzed by acetyl-CoA:DAC acetyltransferase to form cephalosporin. For cephamycins, the next step is the replacement of the C-3 acetoxy group of DAC by carbamyl group catalyzed by the enzyme DAC-carbaoyl transferase. The final step is the hydroxylation and methylation of the C-7 to form cephamycins. The hydroxylation is catalyzed by 3'-carbamoyl-DAC 7-hydroxylase and methylation by 7- hydroxyl-3'-carbamoyl-DAC methyltransferase (Thykaer and Nielsen, 2003).

The mechanism of action of β-lactams is by inhibiting the synthesis of the peptidoglycan layer in the bacterial cell wall, especially the Gram-positive bacteria, by blocking the action of transpeptidases. Transpeptidases, also called penicillin-binding proteins (PBPs), are involved in the assembly of the bacterial cell wall. It is during this stage that linear strands of peptidoglycan are cross-linked into a fishnet-like polymer. D-alanyl-D-alanine, is the terminal amino acid residue on the precursor N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG)-peptide subunit of the nascent peptidoglycan layer. The structural similarity between β-lactam antibiotics and d-alanyl-d-alanine facilitates their binding to the active site of transpeptidases. The carboxylate or sulfonate group of the β-lactam reacts with the serine residue of the transpeptidase to give an acyl enzyme, an acylated enzyme is

inactive (Van Bambeke et al., 2010). This irreversible inhibition prevents the enzyme from carrying out transpeptidation of the nascent peptidoglycan layer. Inhibition of the transpeptidation by β-lactam causes a build-up of peptidoglycan precursors; this triggers the digestion of existing peptidoglycan by autolytic hydrolases without the formation of new peptidoglycan (Anderson et al., 2012). As a result, the action of the β-lactam antibiotics causes inhibition of cell wall synthesis. N-thiolated β-lactams have been found to possess antibacterial, anticancer and antifungal activities. The mechanism of action of this new member of β-lactams involves the inhibition of fatty acid biosynthesis especially in pathogens expressing high levels of coenzyme A (O'Driscoll et al., 2008).

3.4.2 Aminoglycosides

Aminoglycosides are one of the most important broad spectrum antibiotics in clinical use. These antibiotics are structurally similar with a core aminocyclitol moiety and various unusual sugars including aminosugars and deoxysugars (Busscher et al., 2005). They are classified into two groups depending on the structure of the aglycone. One group containing streptamine originated from *myo*-inositol pathway and another group 2-deoxystretamine originated from 2-deoxy-*scyllo*-inosose pathway (Tsai et al., 2013). Aminoglycosides include important antibiotics such as streptomycin, neomycin, amikacin, paromomycin, tobramycin, kanamycin, ribostamycin, nebramycin, apramycin, gentamicin, netilmicin, istamycin, sisomicin, lividomycin, spectinomycin, hygromycin, verdamicin and astromicin (Vakulenko and Mobashery, 2003). These antibiotics have been produced by genus *Streptomyces, Streptosporangium, Saccharopolyspora, Streptoalloteichus, Micromonospora, Dactylosporangium* and *Frankia*.

Biosynthesis of aminoglycosidases is catalyzed by different enzymatic steps. The first step is the biosynthesis of paromamine is the conversion of D-glucose-6-phosphate to 2-deoxy-scyllo-inosose (2DOI) catalyzed by 2-deoxy-scyllo-inosose (2DOI) synthase (Kudo and

Eguchi, 2009). The 2DOI formed is converted into 2-deoxy-scyllo-inosamine (2DOIA) by Gln: 2DOI aminotransferase. At C-1 of 2DOIA is hydrogenated to give 3-amino-2, 3-dideoxy-scyllo-inosone (amino-DOI) catalyzes by the enzyme NAD-dependent dehydrogenase. Amino-DOI is then converted to 2DOS by dual functional Gln: 2DOI aminotransferase to complete the 2DOS biosynthesis. Then, 2DOS is glycosylated using glycosyltransferase with UDPN- acetyl-D-glucosamine (UDP-GlcNAc) as a glycosyl donor to give N-acetylparomamine, whose N-acetyl group is removed using a deacetylase to give paromamine (Llewellyn and Spencer, 2006). Paromamine is believed to be a branching intermediate to neomycin-related aminoglycosides, kanamycin and gentamicin.

The mechanism of action of aminoglycosides is by binding to the aminoacyl site of 16S rRNA within the 30S ribosomal subunit, leading to misreading of the genetic code and inhibiting translocation of the tRNA-mRNA complex (Yang et al., 2006, Hermann, 2007). The initial steps required for peptide synthesis are uninterrupted, such as binding of mRNA and the association of the 50S ribosomal subunit, but elongation fails to occur due to disruption of the mechanisms for ensuring translational accuracy by the antibiotic (Finch et al., 2010). The irreversible binding of the antibiotic to the 30S bacterial ribosome inhibits the polypeptide chain elongation phase resulting in protein synthesis inhibition. It has also been found that some aminoglycosides prevent the transfer of the peptidyl-tRNA from the A-site to the P-site, thus preventing the elongation of the polypeptide chain. Depending on concentration their effect can be bacteriostatic or bacteriocidal (Vakulenko and Mobashery, 2003).

3.4.3 Glycopeptides

Glycopeptides are a class of antibiotics composed of glycosylated cyclic or polycyclic non ribosomal peptides (Jeya et al., 2011). They are comprised of a heptapeptide core consisting of both common and unusual amino acids. They are characterized by the presence of a unique

long aliphatic chain attached to a sugar moiety. This class of antibiotics includes vancomycin, teicoplanin, telavancin, bleomycin, ramoplanin, balhimycin, chloroeremomycin decaplanin, avoparcin and actinoidin. They are produced by *S. orientalis*, *S. candidus, S. toyocaensis, N. actinoides, Actinoplanes teichomyceticus, Amycolatopsis orientalis* and *Amy. balhimycina* (Donadio et al., 2009). The glycopeptide antibiotics are used as drugs of last resort to combat resistant Gram-positive pathogens, especially methicillin-resistant *Staphylococcus aureus* (Wohlleben et al., 2009).

Biosynthesis of the glycopeptide aglycones includes firstly the biosynthesis of unusual amino acids and carbohydrates, secondly the assembly of amino acids into peptides by non-ribosomal peptide synthetases and lastly the attachment of methyl groups at the N-terminus and the addition of carbohydrates. The biosynthesis of glycopeptide begins with the conversion of to prephenate to *p*-hydroxyphenylpyruvate by prephenate dehydrogenase (Pdh); *p*-hydroxyphenylpyruvate is decarboxylated and hydroxylated by hydroxymandelate synthase (HmaS) to yield *p*hydroxymandelate, which is oxidized to *p*-hydroxybenzoylformate by hydroxymandelate oxidase (Hmo) followed by transamination by p-hydroxyphenylglycine transaminase (HpgT), using Tyr as the amino donor, to yield HPG (Hubbard et al., 2000). Four molecules of malonyl-CoA are converted to 3,5-dihydroxyphenylacetyl-CoA by DpgA with the assistance of DpgB and DpgD. The oxidase DpgC converts 3,5-dihydroxyphenylacetyl-CoA to 3,5-dihydroxyphenylglyoxylate, which generates DHPG on transamination by HpgT (Chen et al., 2001). Tyrosine is activated and bound as a thioester to the enzyme BpsD and OxyD adds an oxygen atom at position 6. β-hydroxylation occurs on a thioester-bound tyrosine to form β-hydroxytyrosine (β-HT). The putative hydrolase Bhp cleaves β-hydroxytyrosine from BpsD in order to deliver non-ribosomal peptide synthetases NRPS with β-hydroxytyrosine (Puk et al., 2004). The non-proteinogenic amino acids: 4-hydroxyphenylglycine (HPG), 3,5-dihydroxyphenylglycine (DPG), and β-hydroxytyrosine

(β-HT) are incorporated. These aromatic acid side chains are linked to each other to form two diaryl ether rings and one biaryl ring, and the aglycone thus formed is modified by sugar substituents (Pootoolal et al., 2002, Süssmuth and Wohlleben, 2004).

The mechanism of action of glycopeptides is by inhibiting the maturation of the peptidoglycan layer in bacterial cell wall biosynthesis at the transglycosylation and transpeptidation steps (Donadio et al., 2009). The antibiotic binds to the amino acids within the cell wall preventing the addition of new units to the peptidoglycan layer. In particular, they bind to acyl-D-alanyl-D-alanine (D-Ala-D-Ala) terminus of lipid II. This interaction occurs through formation of five hydrogen bonds between the glycopeptide and the amide and carbonyl groups of the dipeptide moiety (Donadio and Sosio, 2008). The antibiotic forms a tight complex with the lipid II intermediate; by shielding the D-Ala-D-Ala terminus, this prevents the subsequent action of the transpeptidase(s) (TP) and/or transglycosylase(s) (TG). The failure to form crosslinks between lipid II and the nascent PG chain lowers the rigidity of the cell wall and renders the bacterial cell susceptible to osmotic lysis (Donadio and Sosio, 2008).

3.4.4 Anthracyclines

Anthracyclines are among the most effective anticancer drugs in clinical use with the widest spectrum of activity (Minotti et al., 2004). Their diversity is based on structural differences in the aglycone and on a wide array of attached sugar residues. This class of drugs include daunorubicin, doxorubicin, epirubicin, pirarubicin, idarubicin, valrubicin, nogalamycin, aclacinomycin, amrubicin rabelomycin and rhodomycin. They are produced by *Streptomyces* spp (*S. peuceticus, S. galilaeus, S. nogalater* and *S. purpurascens*) and *Micromonospora lupine.* These compounds are used in clinical treatment of a wide variety of cancers such as acute myeloid leukaemia, lymphomas, and a diversity of solid tumours including breast, small cell lung, cervical, head, and neck cancer (Abdelfattah, 2008).

Biosynthesis of anthracycline skeleton is produced by a Type II polyketide synthase (PKS). First, a 21-carbon decaketide chain is synthesized from propionyl-CoA and malonyl-CoA. Each malonyl-CoA unit contributes a 2-carbon ketide unit to the growing polyketide chain. Each addition is catalyzed by the "minimal PKS" consisting of an acyl carrier protein (ACP), a ketosynthase (KS)/chain length factor (CLF) heterodimer and a malonyl-CoA: ACP acyltransferase (MAT). After the 21-carbon decaketide chain is completed, successive modifications are made to eventually produce a tetracyclic anthracycline aglycone. The daunosamine amino sugar activated by addition of thiamine diphosphate (TDP) is produced. It is joined to the anthracycline aglycone and further modifications are done to produce first daunorubicin then doxorubicin.

Anthracycline has three mechanisms of action: (1) it inhibits DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand. In order for a cell to divide, the DNA in the cell's nucleus must be unravelled and then duplicated (transcription). Anthracyclines bind to portions of the unwound strand of nuclear DNA, halting the transcription process, thus preventing the replication of rapidly-growing cancer cells (Nepomuceno, 2011, Kizek et al., 2012). (2) it inhibits topoisomerase II enzyme, an enzyme that unzips the DNA molecule for replication. Anthracyclines interfere with topoisomerase II by preventing the relaxing of supercoiled DNA, thus blocking DNA transcription and replication (Lynch et al., 2003). (3) it creates iron-mediated free oxygen radicals that damage the DNA, proteins and cell membranes (Muindi et al., 1984).

3.4.5 Macrolides

Macrolides are a class of antibiotics composed of a large macrocyclic lactone ring to which one or more deoxy sugars are attached. This class of antibiotics includes erythromycin, clarithromycin, azithromycin, dirithromycin, roxithromycin, flurithromycin, josamycin, midecamycin, rokitamycin, miocamycin, spiramycin, rapamycin, telithromycin and

pikromycin (Abu-Gharbieh et al., 2004). They are produced by *Saccharopolyspora erythraea*, *S. hydroscopicus* and *S. venezuelae.* This class of compounds includes a variety of bioactive agents such as antibiotics, antifungal, prokinetics, and immunosuppressants (Kanoh and Rubin, 2010). The antimicrobial spectrum of macrolides includes activities against streptococci, enterococci, staphylococci and some pathogenic *Haemophilus influenzae*, *Neisseria* species, *Bordetella*, *Corynebacterium*, *Chlamydia*, *Mycoplasma*, *Rickettsia* and *Legionella* species (Zuckerman, 2004).

Biosynthesis of macrolides consists of three basic stages: the formation of an aglycone, the biosynthesis of sugar moieties and the terminal reactions that include sugar attachment and final modifications of the macrolide intermediate. The polyketide synthase (PKS) catalyzes sequential condensation of one unit of propionyl-CoA and six units of methylmalonyl-CoA to give 6-deoxyerythronolide B (6dEB) (Corcoran and Vygantas, 1982). The conversion of glucose-1-phosphate to TDP-L-mycarose is catalyzed by 6dEB mycarosyl ransferase and 6dEB 6-hydroxylase. TDP-4-keto-deoxyglucose is converted to TDP-D-desosamine catalyzed by desosamine transferase, 6dEB 12-hydroxylase, and rRNA methyltransferase (Peirú et al., 2005). The conversion 6dEB to erythronolide-B is catalysed by EryF. The hydroxylation of to erythronolide-B to erythromycin is catalyzed by EryK (Stassi et al., 1993).

The mechanism of action of macrolides is inhibition of bacterial protein biosynthesis. They reversibly bind to the 23S rRNA in the 50S subunit of the bacterial ribosome, and inhibit mRNA-directed protein synthesis. This action is carried out by preventing peptidyltransferase from adding the peptidyl attached to tRNA to the next amino acid, this also inhibits ribosomal translocation (Hansen et al., 2003, Griffin and Pace, 2007). Another mechanism is premature dissociation of the peptidyl-tRNA from the ribosome. Macrolide antibiotics bind reversibly to

the P site on the subunit 50S of the bacterial ribosome (Gaynor and Mankin, 2005). Generally considered to be bacteriostatic, they may be bactericidal at higher doses.

3.4.6 Tetracyclines

Tetracyclines are antibiotics derivatives of polycyclic naphthacene carboxamide with octahydrotetracene-2-carboxamide skeleton. Examples of this class of antibiotics include tetracycline, demeclocycline, methacycline, minocycline, oxytetracycline rolitetracycline, lymecycline and chlortetracycline (Nelson and Levy, 2011). They are produced by members of the genus *Streptomyces*: *S. aureofaciens, S. rimosus* and *S. viridofaciens*. They exhibit activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae and protozoan parasites.

Biosynthesis of tetracycline starts with the oxidative decarboxylation of pyruvic acid to acetyl-CoA as the basic building unit of oligoketides; this reaction is catalyzed by pyruvate dehdrogenase. The carboxylation of acetyl-CoA by acetyl-CoA carboxylase produces malonyl-CoA. L-asparagine catalysed by asparagine-oxo-acid transaminase to give 2-oxosuccinamate. The condensation of malonyl-CoA and 2-oxosuccinamate yield malonamoyl-CoA. Aldol condensation of malonamoyl-CoA by OxyK and OxyN leads to the production of pretetramid (tetracyclic intermediate) (Zhang et al., 2006). The C-methyltransferase OxyF methylates pretetramid, producing 6-methylpretetramide. Keto-enol functionality is installed on the hydrophilic side of 6-methylpretetramide by oxygenases. OxyL catalyzes the formation of 4-hydroxy-6-methylpretetramide, using 6-methylpretetramide as substrate and OxyG is involved in the quinone formation of ring A in the compound. OxyE, a flavin adenine dinucleotide-dependent monooxygenase, catalyses the C-5 oxidation of 4-hydroxy-6-methylpretetramide to yield 4-dedimethylamino-4-oxo-hydrotetracycline (Zakeri and Wright, 2008).

Tetracyclines' mechanism of action is by preventing the attachment of charged aminoacyl-tRNA to the ribosomal acceptor (A) site on the ribosome (Chopra and Roberts, 2001). Thus, they prevent introduction of new amino acids to the nascent peptide chain. They also simultaneously inhibit other steps of the protein biosynthesis. Tetracyclines can also alter the cytoplasmic membrane and this in turn causes leakage of nucleotides and other compounds out of the cell (Pato, 1977). Their action is bacteriostatic in nature.

3.4.7 Polyenes

Polyenes are antifungal agents characterized by a hydroxylated macrocycle containing one (amino) sugar but their distinct characteristic is the presence of a chromophore formed by a system of three to seven conjugated double bonds in the macrolactone ring. This class of antibiotics includes amphotericin B, nystatin, natamycin, rimolidin, filipin, candicin, etruscomycin, hamycin and perimycin (Lee et al., 2006). They are produced by the members of the genus *Streptomyces*: *S. nodosus, S. noursei* and *S. natalensis.* Polyenes have a broad spectrum of action and are useful in treating fungal infections such as candidosis, cryptococcosis, histoplasmosis, blastomycosis, paracoccidioidomycosis, coccidioidomycosis, aspergillosis, extracutaneous sporotrichosis, mucormycosis, hyalohyphomycosis and phaeohyphomycosis (Ellis, 2002).

Biosynthesis of polyene (nystatin) starts with loading of the acetyl-CoA onto the NysA protein, and proceeds through condensation of three methylmalonyl-CoA and 15 malonyl-CoA extender units by NysB, NysC, NysI, NysJ and NysK PKS. After cleavage of the mature polyketide chain from the PKS complex by the TE domain of NysK, the chain is cyclized to form the nystatin aglycone (Fjaervik and Zotchev, 2005, Brautaset et al., 2011). The next two steps in the nystatin biosynthesis are accomplished by the NysL and NysN monooxygenases, which perform hydroxylation and oxidation of the macrolactone ring at C-10 and C-16, respectively. Formation of mycosamine starts with the L-fructose-6-phosphate, which is

converted to GDP-D-mannose through the action of a phosphomannoisomerase, phosphomannomutase, and a GDP–mannose pyrophosphorylase. GDP-D-mannose serves as a substrate for the NysDIII protein, which converts it to the GDP-4-keto-6-deoxy-D-mannose. A GDP-3-keto-6-deoxy-D-mannose isomerase performed the next step in mycosamine biosynthesis, followed by the NysDII-mediated amidation leading to formation of GDP-mycosamine. The NysDI protein, a putative glycosyltransferase, presumably completes the pathway by attaching the mycosamine moiety to the modified nystatin aglycone (Brautaset et al., 2011).

The mechanism of action of polyenes is by binding to ergosterol, the primary sterol in the fungal cell membrane. The consequence of this binding includes disruption of the osmotic integrity of the membrane, with leakage of intracellular potassium and magnesium, and also the disruption of oxidative enzymes in target cells (Ellis, 2002, Paquet and Carreira, 2006). Polyenes act by inserting into the fungal membrane in close association with ergosterol. The subsequent formation of porin channels leads to loss of transmembrane potential and impaired cellular function. Bioactive secondary metabolites from actinomycetes have extensive application in clinical treatment with different mechanisms of actions.

These bioactive compounds from actinomycetes are inexhaustible and only a fraction of these compounds have been identified so far. Discovering novel antimicrobial agents of clinical importance from actinomycetes is still a thrust area of research.

CHAPTER 4

Isolation, Characterization and Antibacterial Activity of Streptomycetes from Rhizosphere Soils in North West Province, South Africa

Abstract

Due to the issue of infection and development of resistance to clinical drugs, this work was carried out as a part of a screening process for antibiotic producing actinomycetes from the rhizosphere. In this study, 341 strains of actinomycetes were isolated from the rhizosphere soil samples collected from Ngaka Modiri Molema district in North West Province of South Africa. The actinomycetes isolates were screened for antibacterial potential against pathogenic organisms; and the potent ones were found to exhibit activity against at least 3 of the test organisms. A combination of morphological, biochemical and physiological characteristics, sequencing of the 16S rDNA gene, and phylogenetic analysis of the nucleotide sequences determined from the 16S rDNA gene showed that 73 (21.4%) of the potent bacterial isolates belong to the genus *Streptomyces*. The isolation, characterization and identification of the potent *Streptomyces* can be useful in the discovery of novel bioactive compounds that can be harnessed as biocontrol agents against pathogenic organisms in agriculture or medical industries.

Keywords: *Streptomyces*; antibiotic; rhizosphere; pathogens; characterization; 16S rDNA; molecular identification

4.1 Introduction

Nature has continued to be in the frontline as a source and inspiration of substances that can be used in combating infectious diseases which are a great menace to mankind (Newman and Cragg, 2007). More often than not a novel drug originates from nature either

as the new drug or blueprint for the development of a new bioactive entity (Berdy, 2005; Chin et al., 2006). This has been accomplished either by screening the plants or microorganisms from various habitats (Adegboye et al., 2008, Arifuzzaman et al., 2010). Microorganisms are inexhaustible producers of important bioactive compounds of great significance in agriculture and medicine. The importance of microorganisms as sources for the discovery of novel natural products with a pharmaceutical potential has been proved over time and was highlighted in various excellent review articles (Marinelli and Marcone, 2011, Raja and Prabakarana, 2011).

The soil is an integral part of the ecosystem; it is inhabited by a wide range of organisms including bacteria, fungi, protozoa, algae, nematodes, viruses and insects. Actinomycetes are one of the major groups of bacteria found in the soil, most importantly the rhizosphere. The rhizosphere serves as a natural reservoir for the exploration of secondary metabolites due to the biological activities of the wide array of soil organisms that dwell there. Filamentous soil bacteria belonging to the order Actinomycetales are of enormous importance both economically and biotechnologically (Baltz, 2007). Actinomycetes are prolific producers of a wide range of bioactive secondary metabolites (Liu et al., 2011). Such bioactive compounds have applications in medicine as antibacterial, antifungal, antiviral, antitumor and immunosuppressant agents and enzymes, and in agriculture as pesticides, herbicides, plant growth promoters and antiparasitic compounds (Li and Heide, 2005, Lam, 2006, Li et al., 2010, Kumar et al., 2012)

Most of the microbial bioactive compounds discovered so far originated from order Actinomycetales, mainly from the taxon *Streptomyces* (Demain and Sanchez, 2009). The genus *Streptomyces* accounts for about 80% of the actinomycetes natural products reported to date. The genus *Streptomyces* is composed of aerobic, Gram-positive bacteria with high G+C content in their genetic makeup. All have a type 1 cell wall with the presence of LL-

Diaminopimelic acid (LL-DAP) and absence of characteristic sugars. They are filamentous with extensively branched substrate and aerial mycelia (Trujillo, 2001). They are mainly soil dwelling bacteria and are widely distributed in nature. They constitute from 1-20% of the culturable population of microorganisms from the soil (Marsh and Wellington, 2007). *Streptomyces* are best known for their synthesis of a vast array of antibiotics, antitumor, enzymes and agroactive compounds, some of which are useful in medicine and agriculture (Watve et al., 2001, Kekuda et al., 2010). It is amazing that the majority of novel bioactive compounds still come from this group of organisms.

Since pathogenic organisms are gaining resistance to existing antibiotics (Alanis, 2005), there is a continuous need to screen nature for antimicrobial compounds. This study is an effort to isolate and identify rhizosphere *Streptomyces* having antibacterial activity using both conventional and molecular methods. These isolates can be explored for possible novel antibiotic(s) which can be used to combat the menace of pathogens. *Streptomyces* strains with novel antibiotics may still exist in nature due to their track record as antibiotic producers.

4.2 Methods and Materials

4.2.1 Sampling area

The study area covered the Ngaka Modiri Molema District in North West Province of South Africa (Adegboye and Babalola, 2013). The latitude and longitude of the district is 25°55'N and 25°50'E respectively. It covers a total of 28,206 km^2 area. Temperatures range from 17°C to 31°C (62° to 88°F) in the summer and from 3° to 21°C (37° to 70°F) in the winter. The average rainfall is 360 mm.

4.2.2 Sample collection

A total of seventeen soil samples were collected from plant crops in Ratlou, Tswaing, Mahikeng, Ditsobotla and Ramotshere all in Ngaka Modiri Molema district in North West

Province, South Africa (Table 1). Soil samples were collected from different locations between May-June 2011. From each crop about 100 g of sample was collected at 5 to 10 cm depth from each of the surfaces using a hand trowel. Soil samples were placed in sterile plastic bags to avoid contamination and taken to the laboratory. The soil samples were air-dried at room temperature for 5 to 7 days and stored at 4°C in plastic containers until further use.

4.2.3 Measurement of pH

The pH of all the samples was determined by pH meter (Crison Basic 20+, Spain). Ten grams of each sample was suspended in 20 ml of distilled water and allowed to stand for 20 min. with occasional stirring to reach equilibrium. After being left to settle, the pH value was measured in triplicate and the average value computed.

4.2.4 Isolation of actinomycetes

Isolation and enumeration of actinomycetes present in the soil sample was performed by serial dilution plate technique using 6 different media: Actinomycetes Isolation Agar (Sigma Aldrich), Luria Bertani agar (Merck), Starch Casein Agar (SCA), Inorganic Salt Starch Agar (ISSA), Czapek Agar (CA), Humic Acid Vitamin Agar (HAVA). All the media were supplemented with 0.2 µm pore size filtered cycloheximide (100 µg/ml) and nalidixic acid (25 µg/ml) to inhibit the growth of fungi and bacteria respectively (Aouiche et al., 2012). A 10 fold dilution series was prepared of the soil samples in sterile distilled water and aliquots (0.5 ml) from selected dilutions were spread plated in triplicate onto the surface of the isolation media. The isolation plates were air-dried in a biological safety cabinet for 20 min. The inoculated plates were incubated at 30°C for 2 weeks, following which the colonies were enumerated on the basis of the morphological characteristics of the previously described actinomycete taxa (Hong et al., 2009).

4.2.5 Biochemical and physiological characteristics

Various biochemical tests were performed for the characterization of the actinomycete isolates using standard methods. These tests include growth on MacConkey agar, indole test, methyl red test, Voges-Proskauer test, citrate utilization, gas production from glucose, casein hydrolysis, nitrate reduction, hydrogen sulphide production, oxidase test, catalase test, gelatin hydrolysis, tween-20, tween-60, tween-80 and esculin tests (Cappuccino and Sherman, 2011). Carbohydrates utilization was determined by growth on carbon utilization medium (ISP-9) supplemented with 1% carbon sources (Pridham and Gottlieb, 1948) and incubated at 25°C. Physiological characterization was carried out by performing the growth at different temperatures ranging from 15 to 50°C, pH ranges from 5 to 10, growth under anaerobic condition and sodium chloride concentration (1, 2, 3, 4, 5, and 6%). The condition allowing maximum growth was recorded.

4.2.6 Isolation of genomic DNA

Actinomycete genomic DNA was isolated by a protocol previously described (Magarvey et al., 2004) with some modifications. Cultures were grown in 10 ml of Luria Bertani broth (Merck) in McCartney bottles for 7 days and then centrifuged at 10,000 rpm (Universal Z300K model centrifuge; HERMLE Labortechnik Germany) for 5 min. The mycelial pellet was resuspended in 500 µl of 5 M NaCl and transferred to a 2-ml Eppendorf tube. The cells were centrifuged at 10,000 rpm for 5 min., and the pellet was resuspended in 1 ml of 10 mM Tris-HCl–1 mM EDTA (pH 7.5) (TE) containing 20 mg/ml of lysozyme and 20 mg/ml of RNase A and incubated at 37°C for 30 min. Following incubation, 250 µl of 0.5 M EDTA, 250 µl of TE containing 5 mg/ml of proteinase K, and 100 µl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37°C for 1 h. The tubes were mixed by inversion after the addition of 250 µl of 5 M NaCl. Immediately thereafter, 200 µl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was

added, and the tubes were heated in a 65°C water bath for 30 min. Cellular debris was removed by centrifugation at 8,000 rpm for 5 min and the supernatant solution was transferred to a new 2-ml microcentrifuge tube. Proteins and lipids were removed by the addition of 0.3 volume of phenol-chloroform, and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA was centrifuged, the pellet was rinsed with 70% ethanol to remove traces of salt. The supernatant was gently poured off and the pellets were dried under vacuum using a Tomy Micro VacTM mv-100 (Tomy Medico, Japan) vacuum dryer. The DNA was resuspended in 50 µl of TE and incubated at 65°C for 1 h to reconstitute the DNA, for immediate use or stored at -20°C.

4.2.7 PCR amplification

The 16S rDNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described primer StrepF (5'- ACGTGTGCAGCCCAAGAC-3') and StrepR (5'- ACAAGCCCTGGAAACGGGGT-3') (Rintala et al., 2001). PCR was performed in a total volume of 50 µl containing 30-50 ng DNA, 100 mM each primer, 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The amplification reaction was performed with a DNA Engine DYAD Peltier thermal cycler (BioRad, USA). The thermal cycling condition used was an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The PCR amplicons were analysed by electrophoresis in 1% (w/v) agarose gel and the size of the bands were determined using 1 kb molecular marker. The gel containing ethidium bromide (10 µg/ml) was viewed under Syngene Ingenius Bioimager (UK) to confirm the expected size of the PCR products. The remaining mixture was purified using NucleoSpin Gel and PCR Clean-up

kit (Macherey-Nagel, Germany). Both the forward and reverse primers were used in the sequencing of the purified PCR products which was conducted in facilities of Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems).

4.2.8 Sequence similarities and phylogenetic analysis

The resulting chromatograms were edited using Chromas Lite version 2.4 software (Technelysium Pty Ltd 2012). Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The obtained 16S rDNA sequences were compared to sequences in the NCBI GenBank database with the Basic Alignment Search Tool (BLAST) (Altschul et al., 1990). Multiple alignments of the sequences were carried out by Mafft program 7.050 (Katoh, 2013) against corresponding nucleotide sequences of the genus *Streptomyces* retrieved from GenBank. Phylogenetic and molecular evolutionary analyzes were conducted using software's in MEGA version 5.2.2 (Tamura et al., 2011). Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbor-joining data set. Manipulation and tree editing were carried out using TreeView (Page, 1996).

4.2.9 Nucleotide sequence accession numbers

The 16S rDNA gene sequences determined for the bacterial strains in this study were deposited in GenBank database, under the accession numbers: JX860342-JX860399.

4.2.10 Screening for antibiotic producing actinomycetes

Determination of antimicrobial activities of pure actinomycete cultures was performed by cross streak method as described previously (Duraipandiyan et al., 2010).

4.2.10.1 Test organisms

The test organisms used for the study include: *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* ATCC 12344, *Campylobacter coli* ATCC 43478, *Bacillus subtilis* ATCC 11774, *B. cereus* ATCC 11778, *Proteus mirabilis* ATCC 49132, *Enterococcus faecalis* ATCC 14506, *Shigella boydii* ATCC 9207, *Klebsiella pneumoniae* ATCC 8308, *Pseudomonas aeruginosa* ATCC 10145 and *Salmonella typhimurium* ATCC 14208 which were obtained from Davies Diagnostics (Pty) Ltd, South Africa. The sensitivity and resistant pattern of these test organisms was determined against standard antibiotics.

4.3 Results

4.3.1 pH value

Table 4.1 shows the average pH values which range between 7.19 and 8.32. This indicates that the soil samples were neutral to slightly alkaline.

Table 4.1: Isolation of *Streptomyces* from rhizosphere soil samples

Location	Plant	Scientific name	GRP Coordinates	Sample code	Average pH	Number of antibiotic producing *Streptomyces* isolates
Mahikeng	Onion	*Allium cepa*	S25° 53.318'	NRMO1	8.0±0.1	38
	Maize	*Zea mays*	S25° 53.296'	NRMM2	8.3±.04	52
	Cabbage	*Brassica oleracea*	S25° 53.276'	NRMC3	8.2±0.2	24
	Spinach	*Spinacia oleracea*	S25° 56.946'	NRMS4	8.0±0.1	35
	Sunflower	*Helianthus annuus*	S25° 56.657'	NRMM5	8.5±0.3	22
Tswaing	Maize	*Zea mays*	S26° 23.738'	NRTM1	7.5±0.2	12
	Pumpkin	*Cucurbita pepo*	S26° 23.731'	NRTP2	7.2±0.3	8
	Peach	*Prunus persica*	S26° 24.512'	NRTP3	7.6±0.5	15
	Sunflower	*Helianthus annuus*	S26° 24.506'	NRTM4	7.1±0.2	18
Ratlou	Maize	*Zea mays*	S26° 04.306'	NRRM1	7.9±0.1	26
	Maize	*Zea mays*	S26° 03.148'	NRRM2	7.7±0.1	22
	Sunflower	*Helianthus annuus*	S26° 03.036'	NRRS3	7.0±0.5	15
Ditsobotla	Sunflower	*Helianthus annuus*	S26° 09.633'	NRDS1	8.3±0.2	32
	Maize	*Zea mays*	S26° 09.628'	NRDM2	8.25±0.2	25
	Maize	*Zea mays*	S26° 09.840'	NRDM3	7.90±0.1	10
Ramotshere	Maize	*Zea mays*	S25° 54.558'	NRZM1	7.81±0.2	34
	Maize	*Zea mays*	S25° 54.289'	NRZM2	7.71±0.2	23

4.3.2 Isolation of actinomycete isolates

Using different isolation media, a total of 341 strains of actinomycetes were isolated, of which the maximum number of *Streptomyces* isolated was in AIA, followed by SCA with the least from LBA (Fig. 4.1). *Streptomyces* species were isolated from the different rhizosphere soil samples based on morphological characteristics. The isolates were tough, leathery and often whitish to greyish colonies.

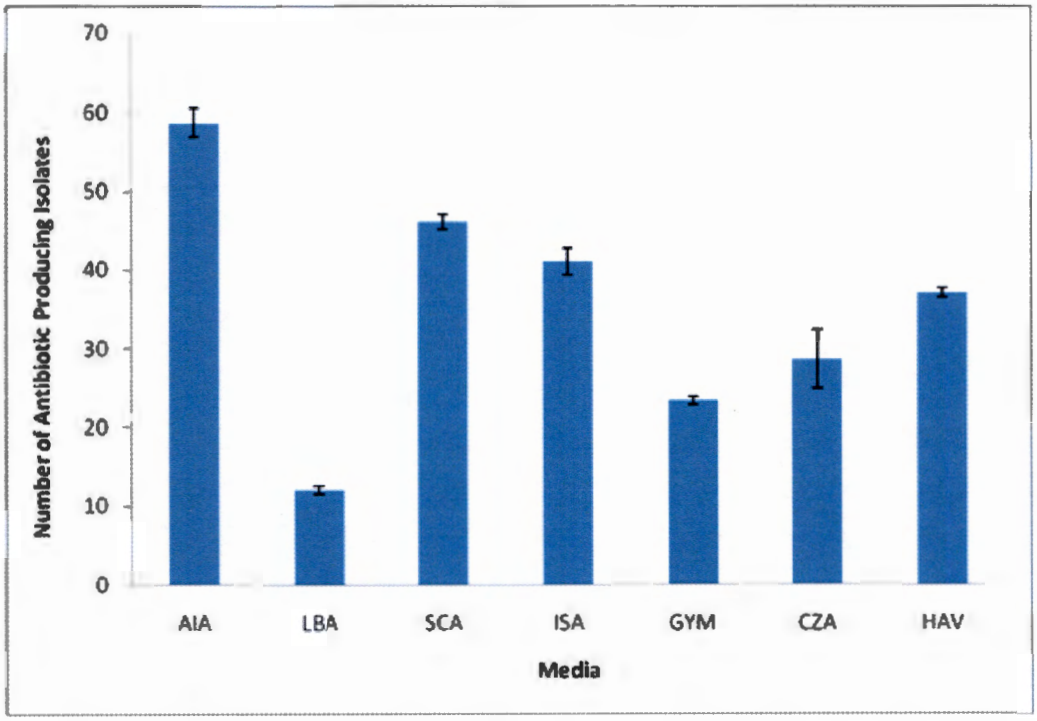


Figure 4.1: Number of antibiotic producing *Streptomyces* isolates from different media

4.3.3 Characteristics of *Streptomyces* isolates

Morphological characteristics of the *Streptomyces* isolates are shown in Table 4.2. The isolates exhibited varying degrees of growth from moderate to abundant on isolation media. Most of the bacterial colonies were convex, while some had either dome, flat or raised elevation. Various colony margins were exhibited and some part of the aerial mycelia could be observed around the colony. Aerial mycelia were initially white, becoming yellow to reddish purple, as the culture became older. Substrate mycelia ranged from yellow, grey, brown, orange to violet. Seven of the *Streptomyces* isolates produced diffusible pigment. The biochemical studies on Table 4.3 showed that all the isolates were Gram-positive, catalase positive and were able to hydrolyze starch. Twenty-seven isolates were able to produce enzyme oxidase. Eighteen of the isolates liquefied gelatin and hydrolyzed casein produced. Twenty-four isolates were able to degrade esculin, 19 isolates were able to utilize citrate, 25 utilized urea, 20 reduced nitrate and 22 were able to produce H_2S. Only 5 isolates tested positive for methyl red, all the other isolates were positive for Voges-Proskauer test. Seventy of the isolates were able to utilize tween 20 and 60, and 63 utilized tween 80. All the isolates grew on minimal media and utilize a good number of the carbon sources. Table 4.4 shows the carbon source utilization of the isolates; all the isolates were able to utilize glucose. Sucrose, fructose and mannose were utilized by the majority of the isolates while cellulose, xylose and sorbitol were utilized by a few of the isolates. Physiological properties showed in Table 4.5 indicated that the isolates were mesophilic, most of the isolates grew best at pH close to neutral or slightly alkaline but some exhibited optimal growth at slightly acidic and alkaline.

Table 4.2: Morphological characteristics of *Streptomyces* isolates on isolation media

Characteristic	Number of bacterial isolates
Growth	
a) abundant	3
b) good	65
c) moderate	5
Elevation	
a) dome	11
b) convex	44
c) flat	10
d) raised	9
Color of substrate mycelium	
a) brown	3
b) yellow	34
c) grey	20
d) yellowish-grey	14
e) violet	1
f) orange	1
Color of aerial mycelium	
a) yellow	11
b) yellowish-orange	4
c) green	3
d) greenish-yellow	2
e) reddish-purple	1
Pigmentation	
a) brown	5
b) light red	1
c) violet	1
Margin	
a) wavy	9
b) woolly	24
c) irregular	7
d) ciliate	17
e) smooth	16

Table 4.3: Biochemical characteristics of *Streptomyces* isolates

Characteristics	Number of strains	
	Positive	Negative
Gram staining	73	
Catalase	73	
Oxidase	27	46
Citrate utilization	19	54
Gelatin liquefication	18	55
Starch hydrolysis	73	
Casein hydrolysis	18	55
Esculin degradation	24	49
Nitrate reduction	20	53
H_2S production	22	51
Urea utilization	25	48
Methyl red	5	68
Voges-Proskauer	51	22
Tween 20	70	3
Tween 60	70	3
Tween 80	63	10

Table 4.4: Carbon source utilization of *Streptomyces* isolates

Carbon utilization	Number of strains	
	Positive	Negative
Glucose	73	
Sucrose	64	9
Sorbitol	10	63
Mannitol	50	23
Fructose	60	13
Lactose	53	20
Galactose	57	16
Rhamnose	53	20
Cellulose	31	32
Mannose	67	6
Inositol	57	16
Raffinose	53	20
Maltose	54	19
Xylose	29	44

Table 4.5: Physiological characteristics of *Streptomyces* isolates

Growth condition	Number of strain
Optimum temperature for growth (°C)	
a) 18-20	4
b) 20-25	9
c) 25-30	55
d) 30-35	5
Optimum pH for growth	
a) 6	7
b) 7	43
c) 8	15
d) 9	6
e) 10	3
NaCl tolerance (%)	
a) 1	22
b) 2	2
c) 3	13
d) 4	8
e) 5	8
f) 6	20
Growth under anaerobic conditions	10
Growth on MacConkey agar	1

4.3.4 Identification of the *Streptomyces* isolates

Figure 4.2 shows that PCR amplification of the genomic DNA with *Streptomyces* nucleotide signature sequences resulted in approximately 1.1 kb amplicons. The resultant nucleotides matched with 16S rDNA gene sequences of *Streptomyces* reference species available in the GenBank database library, which confirmed the identification of the potent bacterial isolates at the genus level. The sequence similarities ranged from 87 to 100% with members of the genus *Streptomyces*. These also revealed the phylogenetic affiliation to the genus *Streptomyces*. The phylogenetic tree was constructed with the 16S rDNA gene sequence data of members of the genus *Streptomyces* using neighbor joining method (Fig.4.3).

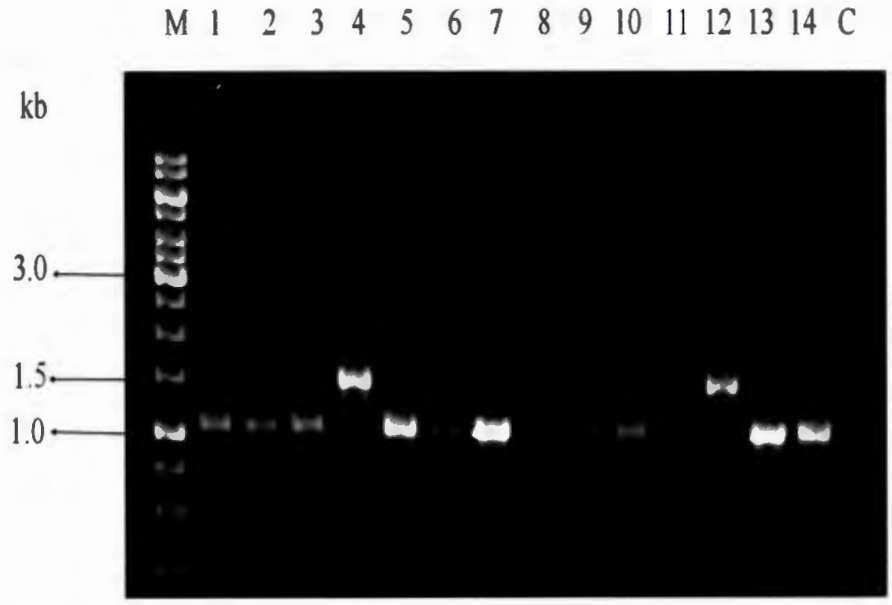


Figure 4.2: Agarose gel photograph indicating the positive band of approximately 1.1 kb for *Streptomyces* signatures nucleotide amplification from actinomycete isolates. M= 1 kb DNA Marker, 1-14= PCR amplification of *Streptomyces* isolates, C= Nuclease free water

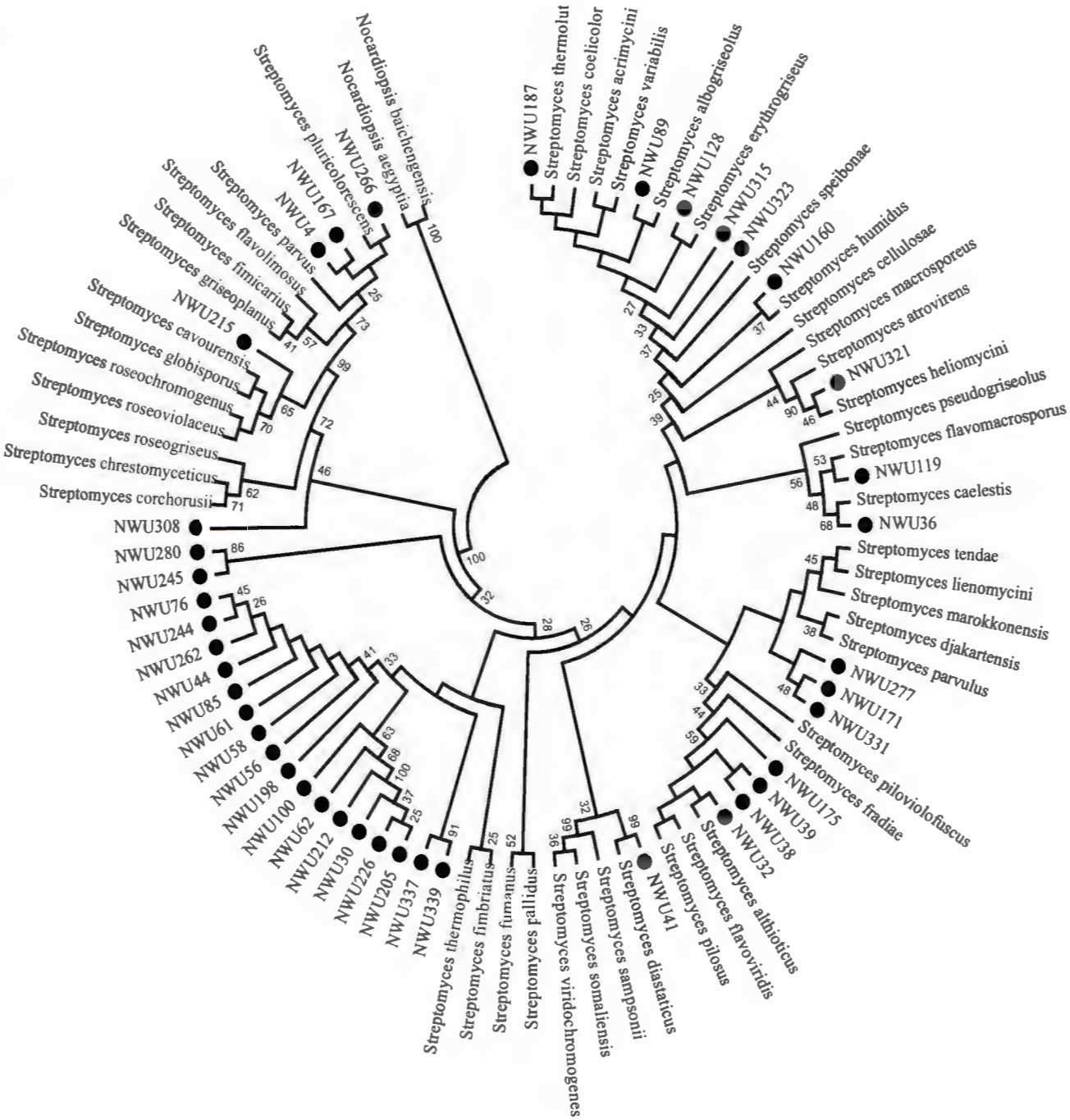


Figure 4.3: Neighbor-joining tree based on partial 16S rDNA sequences showing relationships between the *Streptomyces* isolates and the references strains of *Streptomyces*

4.3.5 Identification of the antibiotic producing *Streptomyces* isolates

The sensitivity and resistant patterns of the test organisms are presented in Table 4.6. *Bacillus subtilis* ATCC 11774 and *Sh. boydii* ATCC 9207 were the most sensitive while *Salm. typhimurium* ATCC 14208 and *Kl. pneumoniae* ATCC 8308 were the most resistant to the standard antibiotics. Seventy-three (21.40%) out of 341 bacterial isolates exhibited varying degrees of antimicrobial activity against at least three or more of the test organisms (Table 4.7). The inhibition action of the potent isolates was in the order: all the potent isolates suppressed the growth of *Staph. aureus* ATCC 29213 and *Strep. pyogenes* ATCC 12344, 90% suppressed the growth of *B. subtilis* ATCC 11774, 86% *B. cereus* ATCC 11778, 56% *Camp. coli* ATCC 43478, 49% *Sh. boydii* ATCC 9207, 40% *Ent. faecalis* ATCC 14506, 37% *Pr. mirabilis* ATCC 49132, 25% *Kl. pneumoniae* ATCC 8308, 12% *Ps. aeruginosa* ATCC 10145 and 8% *Salm. typhimurium* ATCC 14208. The soil sample collected from Mahikeng and plant crop maize has the highest number of potent isolates with respect to other soil samples.

Table 4.6: Sensitivity and resistant pattern of the test organisms

Microorganisms	Sensitive to	Resistance to
Staph. aureus ATCC 29213	CEP, CIP, COT, NEO, RIF, STR, TET, VAN	BAC, CXM, ERY, NOR, OX
Strep. pyogenes ATCC 12344	CEP, CIP, CXM, NEO, NOR, RIF, STR, TET, VAN	BAC, COT, ERY, OX
Camp. coli ATCC 43478	CIP, CXM, NEO, NOR, RIF, STR, TET, VAN	CEP, COT, ERY, OX
B. subtilis ATCC 11774	BAC, CEP, CIP, COT, CXM, ERY, NEO, NOR, RIF, STR, TET, VAN	OX
B. cereus ATCC 11778	CEP, CIP, CXM, ERY, NEO, NOR, RIF, STR, TET, VAN	BAC, COT, OX
Pr. mirabilis ATCC 49132	CEP, CIP, COT, CXM, NEO, NOR, RIF, STR, TET	BAC, ERY, OX, VAN
Ent. faecalis ATCC 14506	CEP, CIP, COT, CXM, ERY, NEO, RIF, STR, TET, VAN	BAC, NOR, OX
Sh. boydii ATCC 9207	BAC, CEP, CIP, CXM, NEO, NOR, RIF, STR, TET, VAN	ERY, OX
Kl. pneumoniae ATCC 8308	CIP, CXM, NEO, NOR, TET	BAC, CEP, COT, ERY, OX, RIF, STR, VAN
Ps. aeruginosa ATCC 10145	BAC, CIP, NEO, NOR, RIF, STR, TET, VAN	CEP, COT, CXM, ERY, OX
Salm. typhimurium ATCC 14208	CIP, CXM, NEO, TET	BAC, CEP, COT, ERY, NOR, OX, RIF, STR, VAN

CEP (Cephalothin), RIP (Rifampicin), VAN (Vancomycin), NEO (Neomycin), STR (Streptomycin), CIP (Ciproflaxacin), TET (Tetracycline), NOR (Norfloxacin), COT (Cotrimoxazole), OX (Oxacillin), CXM (Cefuroxime), ERY (Erythromycin), BAC (Bacitracin)

Table 4.7 Antibacterial activity of the antibiotic producing *Streptomyces* isolates to test organisms

	Number of strains producing antibiotics			
	Zone of inhibition (mm)			
Test organism	5-12	11-15	16-20	>20
Staph. aureus ATCC 29213	43	15	8	7
Strep. pyogenes ATCC 12344	62	4	4	3
Camp. coli ATCC 43478	20	12	2	7
B. subtilis ATCC 11774	50	9	5	9
B. cereus ATCC 11778	43	9	3	8
Pr. mirabilis ATCC 49132	19	7	1	1
Ent. faecalis ATCC 14506	19	4	5	1
Sh. boydii ATCC 9207	23	11	2	1
Kl. pneumoniae ATCC 8308	8	7		3
Ps. aeruginosa ATCC 10145	7	2		
Salm. typhimurium ATCC 14208	6			

4.4 Discussion

Screening for antimicrobial compounds is a continuous process to match the unending demand for novel compounds with potent activity, in order to curtail or eradicate pathogenic organisms affecting either humans or plants. It is important to screen the soil for actinomycetes because of their track record as prolific producers of secondary metabolites of high industrial value. This research was carried out with an aim to screen and isolate antibiotic producing Streptomycetes from rhizosphere soil, in order to harness their resources to the benefit of mankind.

The results from this present study revealed that a neutral to alkaline environment is more suitable for the isolation of *Streptomyces* spp, with NRMM5 being alkaline and having the highest number of the bacterial isolates. This correlates with similar results reported by Khamna et al. (2009) in their study. It was also noted that the number of *Streptomyces* isolated from maize (*Zea mays*) were higher compared to other rhizosphere soils. This might be due to differences in the composition and amount of root exudates and rhizodepositions in the 17 rhizosphere soil samples. The ability of certain rhizobacteria to colonize or occupy a niche in the rhizosphere (rhizosphere competence) might also be responsible for host-plant specificity (Babalola and Akindolire, 2011). Reports indicated that rhizosphere represent a unique biological niche that supports abundant and diverse microorganisms because of the presence of root exudates (Bais et al., 2006). It was also reported that the rhizosphere harbour more organisms that the bulk soil, this could be attributed to the presence of root exudates (Gonzalez-Franco et al., 2009).

Analysis of the different rhizosphere soils collected from Ngaka Modiri Molema district revealed the presence of Streptomycetes from all the samples. This revealed that *Streptomyces* are a widespread and prominent population in the soil. Similar results have been reported by other researchers, that Streptomycetes are widespread and indigenous

population in soil (Rifaat et al., 2006, Parthasarathi et al., 2010). The majority of the *Streptomyces* strains were isolated from the AIA compared to other isolation and cultivation media used. This agrees with the report of Thakur et al. (2007), that since it contains glycerol and sodium propionate, so it serves as a selective medium for actinomycetes. It is important to use the media that will support the growth of most actinomycetes in soil. It has been reported that only 1% of microorganisms are culturable due to isolation media composition, that either does not support the growth of most of the organisms or lacks particular substances essential for proliferation. The constituents of the media are crucial for the isolation of actinomycetes from the soil.

The *Streptomyces* present in this soil community is characterized through cultural, morphological, biochemical and physiological analyses. All the selected isolated are tough, leathery and filamentous colonies that were hard to pick from the culture plates, showing morphology typical of *Streptomyces.*

It is evident in this study, that the *Streptomyces* isolates were able to utilize quite a number of carbon sources. *Streptomyces sp* are saprophytes; this might be due to the capability to produce a large and diverse range of hydrolytic enzymes that help in degradation of different types of organic molecules (Gagnat et al., 1999). *Streptomyces species* have been identified as an industrial source of enzymes such as amylase, cellulase, lipase and xylanase. Previous research works have shown that the predominance of *Streptomyces* in the rhizosphere soils is due to their enzymatic activities and ability to produce a vast array of secondary metabolites (Kumar et al., 2012). These attributes help them to out-compete other microorganisms and even to strive in the soil.

Molecular approaches have led to additional advances in the identification of bacterial isolates, and especially in the differentiation of phenotypically close strains (Hong Nhung et al., 2007). Identification of the bacterial isolates was confirmed by computational analysis.

These bacterial isolates were identified as members of the genus *Streptomyces*. The sequences of the bacterial isolates used as query in BLASTn search were found to be closely related to *Streptomyces* spp that are producers of antimicrobial agents. As the number of reference organisms continues to grow in the GenBank library, the identification process is more accurate and precise. It has been reported that the 16S rDNA sequence analysis showed differences in the sequence diversity of bacteria (Clarridge, 2004). The 16S rDNA sequence has become a standard for the elucidation of phylogenetic relationships among microorganisms (Mitra et al., 2011, Satheeja and Jebakumar, 2011). Phylogenetic tree also serves as an important tool for identification and to show the evolutionary relationship among the bacterial isolates and that of the reference *Streptomyces* (Satheeja and Jebakumar, 2011). Identification of microorganisms to the species level is important (Van Hop et al., 2011); this helps to ascertain the type of possible secondary metabolites that could be produced by these *Streptomyces* isolates through their closely related species. This study showed that *Streptomyces* species exhibited broad spectrum antimicrobial activity. The bacterial isolates were screened for antibacterial activity, 21.41% of the bacterial isolates exhibited activity against at least three (*Staph. aureus* ATCC 29213, *Strep. pyogenes* ATCC 12344 *B. subtilis* ATCC 11774) of the test organisms. Soil has been considered excellent sources for the isolation of *Streptomyces* with diverse potential resources (Ting et al., 2009). The soil sample collected from Mahikeng and plant crop maize has the highest number of potent isolates with respect to other soil samples. Medicinal plants encourage the growth of actinomycetes especially *Streptomyces*, this might be due to the production of antimicrobial compounds. Maize has been reported to produce some antimicrobial compounds (Luo and Wang, 2012). *Streptomyces* capable of producing antimicrobial agents have been previously isolated from the rhizosphere. It has been reported that bioactive compounds from

Streptomyces exhibited different activities; these showed the biodiversity of the bioactive compounds present amongst the potent bacterial isolates (Valli et al., 2012).

4.5 Conclusion

Our studies have established that the rhizosphere is a goldmine of biodiversity of *Streptomyces* with antibacterial activity. Therefore, these bacterial isolates are likely to be the potential candidates for discovery of novel secondary metabolites for various biocontrol applications. Fermentation and chemical analysis of the extracts is concurrently being carried out in order to identify active compounds and to assess their novelty.

4.6 Acknowledgments

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CHAPTER 5

Isolation and Identification of Potential Antibiotic Producing Rare Actinomycetes from Rhizospheric Soils

Abstract

Background

The search for novel antibiotics producers and their characterization continues to be an important objective in the discovery of novel bioactive compounds. This work was carried out to isolate and identify bioactive secondary metabolite producing rare actinomycetes and to analyse the phylogenetic relationship.

Results

The rhizospheric soil samples were collected from different localities of Ngaka Modiri Molema district of North West Province, South Africa and screened for antibacterial potential. Molecular identification of the bacterial isolates by analysis of the 16S rDNA nucleotides sequences showed that the rare actinomycetes isolated include the following genera: *Actinomadura*, *Nocardiopsis*, *Promicromonospora*, *Nocardia*, *Arthrobacter*, *Pseudonocardia*, *Micrococcus*, *Nonomuraea*, *Rhodococcus*, *Streptosporangium* and *Saccharothrix* spp. Nineteen (21.6%) of the 88 rare actinomycetes isolates exhibited antibacterial activity against at least one of the test organisms. Phylogenetic analysis revealed that the bacterial isolates are the members of rare actinomycetes which are associated with the rhizosphere. Results from the phylogenetic analysis indicate that the 19 isolates could be sorted into 11 phylotypes. It was also inferred from the tree that the potent bacterial isolates

clustered with other antibiotic producing rare actinomycetes reference strains retrieved from the GenBank.

Conclusion

This study corroborates that rhizospheric soil harbours diverse actinomycetes which can be explored for antibacterial secondary metabolites.

Keywords: Antibacterial activity; antibiotic; characterization; phylogenetic; rare actinomycetes; rhizospheric soil

5.1 Background

Microbial natural products are the frontier in the discovery of bioactive compounds of pharmaceutical importance. The majority of the bioactive compounds in use today are derived from the secondary metabolites of actinomycetes (Baltz, 2007). The diversity of these bioactive secondary metabolites is unsurpassed in medicine and agriculture. Various bioactive compounds have been isolated and characterized from actinomycetes having great structural and functional diversity including antibacterial, antifungal, antiprotozoal, antiviral, anticholesterol, antihelminthic, anticancer, immunosuppressant agent, herbicides, and pesticides (Marinelli and Marcone, 2011, Newman and Cragg, 2012). These compounds do not only exhibit potent therapeutic activities but also possess the desirable pharmacokinetic properties. The order Actinomycetales is composed of approximately 140 genera, majority are free living organisms that are widely distributed in nature. They are aerobic, filamentous, Gram-positive bacteria and produce extensively branched substrate mycelium and aerial hyphae. Members of this order are characterized by high G+C in their genetic makeup and complex life cycle (Trujillo, 2001).

Among actinomycetes, *Streptomyces* is the most common actinomycete in terrestrial habitat, above 60% of the actinomycetes isolates (Goodfellow and Williams, 1983). The results of extensive screening show that more than 70% of known bioactive compounds are produced by *Streptomyces* (Demain and Sanchez, 2009). As more new antibiotics were discovered, the chances of finding novel bioactive compounds from *Streptomyces* have dwindled. The possibility of re-isolating already isolated compounds from *Streptomyces* is very high (Hayakawa, 2008). There is a need to screen the less exploited genera of rare actinomycetes such as *Actinomadura, Actinoplanes, Amycolatopsis, Dactylosporangium, Kibdelosporangium, Microbispora, Micromonospora, Nocardiopsis, Nonomuraea, Promicromonospora, Planobispora, Rhodococcus, Saccharothrix, Streptosporangium* and *Planomonospora* for novel antimicrobial agents (Lazzarini et al., 2000, Busti et al., 2006).

However new approaches for the isolation of soil actinomycetes have shown that other members of this order are also of importance. New species and genera have been identified, and most of them are able to produce novel bioactive compounds (Lazzarini et al., 2000). They have produced important classes of antibiotics such as macrolides, β–lactams, aminoglycosides, glycopeptides, lipopeptides, ansamycins, polyenes, anthracyclines, nucleosides, peptides, polyethers and tetracyclines.

As part of our ongoing research to find bioactive secondary metabolites that may have application in medicine, this research work is designed to isolate and identify antibiotic producing rare actinomycetes from rhizospheric soils. We isolated and characterized actinomycete strains, and screened them for antibacterial activity. Of the actinomycetes isolated, the rare actinomycetes strains were also subjected to phylogenetic analysis using comparison of their 16S rDNA gene sequences.

5.2 Results

5.2.1 Isolation of rare actinomycetes

Actinomycetes were isolated from the 17 rhizospheric soil samples plated based on the morphological appearance of the isolates: tough, leathery, powdery, often whitish to greyish colonies and some with pigmentation. Out of 341 isolates, 88 (26.6%) were identified as rare actinomycetes.

5.2.2 Screening for antibiotic producing actinomycetes

Among the 88 rare actinomycetes isolated from the different soil samples, 19 (22%) isolates exhibited antibacterial activities against at least one of the test organisms (Table 5.1). Most of the isolates exhibited broad spectrum activities against test organisms. The following percentage of selected isolates exhibited an inhibitory effect against the test organisms including: *Staph. aureus* (100%), *Strep. pyogenes* (89%), *Camp. coli* (79%), *B. subtilis* (57%), *B. cereus* (73%), *Pr. mirabilis* (73%), *Ent. faecalis* (42%), *Sh. boydii* (16%), *Kl. pneumoniae* (47%), *Ps. aeruginosa* (10%) and *Salm. typhimurium* (5%).

Table 5.1: Antibacterial activity of selected isolates against pathogenic organisms

	Isolate Code: NWU																		
Test organism	60	73	80	88	98	101	121	126	146	166	183	208	239	252	255	284	296	299	336
Staph. aureus ATCC 29213	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Strep. pyogenes ATCC 12344	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+
Camp. coli ATCC 43478	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+
B. subtilis ATCC 11774	+	+	+	-	+	-	-	-	+	+	-	+	+	+	-	-	+	+	-
B. cereus ATCC 11778	+	+	+	+	-	+	+	-	+	+	+	+	+	-	-	-	+	+	+
Pr. mirabilis ATCC 49132	+	+	+	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+
Ent. faecalis ATCC 14506	-	-	+	-	+	-	+	-	+	-	+	-	-	+	+	+	-	-	-
Sh. boydii ATCC 9207	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-
Kl. pneumoniae ATCC 8308	+	-	+	-	+	+	+	+	-	-	-	-	-	+	-	+	-	+	-
Ps. aeruginosa ATCC 10145	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
Salm. typhimurium ATCC 14208	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Inhibition of growth; -= No inhibition of growth

5.2.3 Morphological characteristics of selected isolates

Results of morphological characteristics of the isolates revealed that the growth of the isolates was few to good on the ISP-medium 2. The isolates of NWU60, NWU80, NWU98, NWU146, NWU208, NWU255, NWU284, NWU299 and NWU336 produced aerial mycelium while only NWU73 did not produce substrate mycelium. Isolates NWU73 and NWU166 were observed with yellowish pigment and NWU282 with brown pigment (Table 5.2). The reverse colony color observed ranges from cream (NWU183), yellowish cream (NWU60, NWU73, NWU80, NWU98, NWU166, NWU239, NWU284 and NWU299), peach (NWU88), orange (NWU146), light brown (NWU126, NWU255 and NWU336) to brown (NWU101, NWU121, NWU208, NWU252 and NWU296).

Table 5.2: Morphological characteristics of selected isolates on isolation medium

Isolate code	Growth	Aerial mycelium	Substrate mycelium	Pigmentation	Reverse colony color
NWU60	Good	Dull white	Grey	None	Yellowish cream
NWU73	Moderate	None	None	yellow	Yellowish cream
NWU80	Good	White	Yellow	None	Yellowish cream
NWU88	Good	None	Pink	None	Peach
NWU98	Good	White	White	None	Yellowish cream
NWU101	Good	None	White	None	Brown
NWU121	Moderate	None	Army green	None	Brown
NWU126	Moderate	None	Light yellow	None	Light brown
NWU146	Good	White	Pink	None	Orange
NWU166	Few	None	White	yellow	Yellowish cream
NWU183	Good	None	White	None	Cream
NWU208	Good	White	White	None	Brown
NWU239	Moderate	None	White	None	Yellowish cream
NWU252	Good	None	White	Brown	Brown
NWU255	Moderate	White	White	None	Light brown
NWU284	Good	White	White	None	Yellowish cream
NWU296	Good	None	White	None	Brown
NWU299	Good	White	Creamish white	None	Yellowish cream
NWU336	Good	White	Dull white	None	Light brown

5.2.4 Biochemical and physiological characteristic of selected isolates

Biochemical characteristics results indicate that all isolates are Gram positive, catalase positive and have the ability to hydrolyze starch. Isolates NWU98, NWU121, NWU126, NWU166, NWU208, NWU239, NWU252, NWU255 and NWU296 were able to produce the enzyme catalase. None of the isolates were able to liquefy gelatin. Only isolates NWU166, NWU183, NWU299 and NWU336 were able to utilize citrate; NWU60 and NWU239 were able to utilize urea; and NWU60, NWU88, NWU239, NWU284 and NWU336 were able to hydrolyze casein. The positive utilization of esculin degradation was recorded in NWU73, NWU80, NWU98, NWU101, NWU166, NWU183, NWU208, NWU255, NWU284 and NWU336. Isolates NWU60, NWU88, NWU101, NWU126, NWU252, NWU255, NWU284, NWU296, NWU299 and NWU336 were able to reduce nitrate. Only isolates NWU73 and NWU239 were able to produce hydrogen sulphide (Table 5.3). The isolates were able to utilize a wide range of carbon sources (Table 5.4). All the isolates were able to utilize glucose; sucrose, mannitol, fructose, rhamnose, mannose inositol and maltose were utilized by the majority of the isolates while sorbitol, lactose, galactose, cellulose, raffinose and xylose were utilized by few of the isolates. It is also evident that different physiological characteristics influenced the growth rate of the isolates. The optimum temperature for the growth of the isolates was between 25-30°C. The optimum pH growth is between 7.0 and 8.0 which are neutral to slightly alkaline. The isolates were able to tolerate sodium chloride up to 5-7% concentration. Isolates NWU121, NWU146, and NWU166 were not able to grow under anaerobic conditions. Only NWU126 and NWU284 were able to grow on MacConkey agar (Table 5.5).

Table 5.3: Biochemical characteristics of selected isolates

Test	Isolate Code (NWU)																		
	60	73	80	88	98	101	121	126	146	166	183	208	239	252	255	284	296	299	336
Gram Staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	+	-	+	+	-	+	-	+	+	+	+	-	+	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+
Gelatin liquefication	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin degradation	-	+	+	-	+	+	-	-	-	+	+	+	-	-	+	+	-	-	+
Casein hydrolysis	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+
Nitrate reduction	+	-	-	+	-	+	-	+	-	-	-	-	-	+	+	+	+	+	+
H_2S production	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Urea utilization	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	-	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	-	+
Tween 20	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	-	-	+
Tween 60	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	-	-	-	+
Tween 80	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	-	-	-	+

+ = Positive; - = Negative

Table 5.4: Carbon source utilization of selected isolates

Carbon source	Isolate Code: NWU																		
	60	73	80	88	98	101	121	126	146	166	183	208	239	252	255	284	296	299	336
Glucose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Sucrose	++	++	++	++	±	-	++	-	-	++	±	++	++	+	++	++	++	++	++
Sorbitol	-	±	-	±	-	-	±	-	+	+	+	-	-	-	-	+	++	+	-
Mannitol	++	++	++	+	++	±	+	-	-	+	±	-	++	-	±	++	+	++	-
Fructose	+	++	++	++	-	++	++	+	++	++	++	++	+	+	++	++	+	++	+
Lactose	-	+	+	++	++	-	-	±	-	-	±	-	+	+	+	++	+	-	+
Galactose	-	++	+	++	+	-	-	-	+	++	±	-	++	-	±	++	-	-	-
Rhamnose	+	++	±	++	+	-	+	+	+	++	±	-	+	+	-	+	-	-	++
Cellulose	±	-	±	-	-	-	±	-	-	±	-	-	-	-	-	±	-	±	±
Mannose	-	++	+	+	+	+	++	+	+	++	+	+	++	±	++	++	++	+	+
Inositol	-	-	++	-	+	+	++	+	+	+	+	±	±	-	=	++	++	-	±
Raffinose	++	+	++	++	+	-	+	-	-	+	-	-	+	+	-	+	+	-	+
Maltose	++	++	++	++	+	-	++	-	+	++	+	+	++	+	++	++	+	+	-
Xylose	+	+	+	++	-	-	++	-	-	-	+	-	+	-	-	+	+	-	-

Results recorded by the method of the International Streptomyces Project (ISP): ++, strongly positive utilization, i.e., growth on the tested carbohydrate in basal medium is equal to or greater than the growth on basal medium plus glucose; +, positive utilization, i.e., growth on the tested carbohydrate is significantly better than that on the basal medium without carbon source but somewhat less than that on glucose; ±, utilization doubtful, i.e., growth on the tested carbon source is only slightly better than that on the basal medium without carbon source and significantly less than that with glucose; -, utilization negative, i.e., growth is similar to or less than the growth on basal medium without carbon source

Table 5.5: Physiological characteristics of selected isolates

Growth condition	Isolate Code: NWU																		
	60	73	80	88	98	101	121	126	146	166	183	208	239	252	255	284	296	299	336
Optimum temperature for growth (°C)	25	25	30	30	25	25	30	30	30	25	25	30	25	25	25	30	30	25	25
Optimum pH for growth	7	7	8	8	7	7	7	7	7	8	7	8	7	7	7	8	7	7	7
NaCl tolerance (%)	7	7	5	7	5	5	7	7	7	7	5	5	5	5	7	6	5	7	5
Growth under anaerobic conditions	+	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+
Growth on MacConkey agar	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-

+ = Positive; - = Negative

5.2.5 Molecular identification of selected isolates

A 1.5 kb fragment was amplified from the genomic DNA with the bacterial universal primers (F1R5) (Figure 5.1). Identification of the isolates was confirmed by computational analysis. The generic identification of rare actinomycetes was performed by analysis of partial sequences of their 16S rDNA gene. The partial nucleotide sequences of the 16S rDNA gene of the isolates were compared with the nucleotide database of NCBI web server through the BLAST tool. The BLAST search inferred that the isolates were members of the GC-rich actinomycetes. The 16S rDNA gene nucleotide sequence of different actinomycetes was obtained by BLASTn search; however 58 strains of actinomycetes were selected based on high identity (%) with good E value. Table 5.6 results show that query sequences were best pairwise aligned with 16S rDNA gene sequence of actinomycetes with sequence similarity, and identity ranged between 79-100%, with E value of 0.

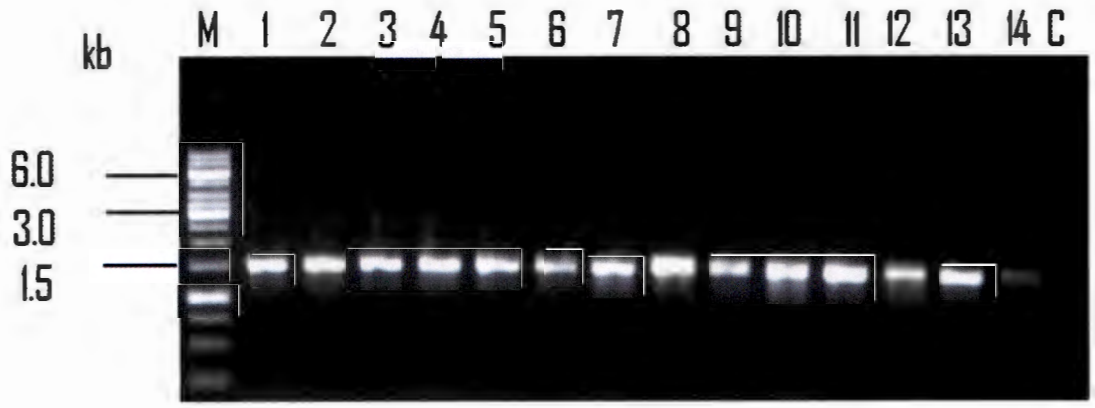
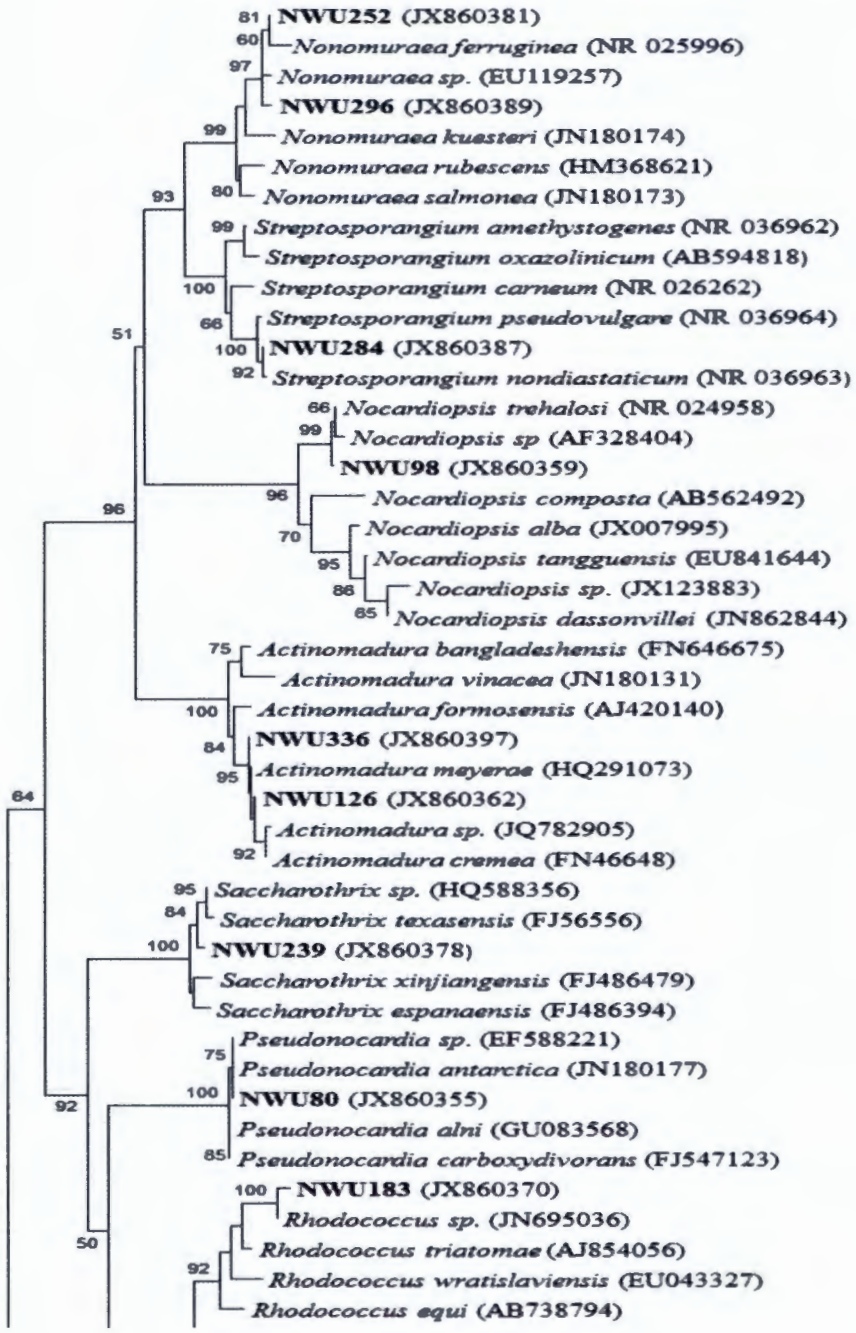


Figure 5.1: Agarose gel photograph indicating the positive band of approximately 1.5 kb for 16S rDNA gene amplification from actinomycete isolates. **M=** 1 kb DNA Marker, **1-14=** PCR amplification of isolates, **C=** Nuclease free water

5.2.6 Phylogenetic analysis of selected isolates

The 19 isolates were subjected to sequencing and phylogenetic analysis. The 16S rDNA sequences of the 19 isolates were aligned with 58 actinomycetes sequences obtained from GenBank; and *Bacillus* spp. as the out-group. The phylogenetic position of the isolates was evaluated by constructing a phylogenetic tree using neighbor-joining methods (Fig. 5.2). This method placed the bacterial isolates in different clades encompassing members of the order Actinomycetales with bootstrap support. Bootstrap values based on 1000 replications were listed as percentages at the branching points. The tree shows completely resolved, well-supported phylogeny of the 19 bacterial isolates with high resolution of all inner branches.


NWU252 (JX860381)
Nonomuraea ferruginea (NR 025996)
Nonomuraea sp. (EU119257)
NWU296 (JX860389)
Nonomuraea kuesteri (JN180174)
Nonomuraea rubescens (HM368621)
Nonomuraea salmonea (JN180173)
Streptosporangium amethystogenes (NR 036962)
Streptosporangium oxazolinicum (AB594818)
Streptosporangium carneum (NR 026262)
Streptosporangium pseudovulgare (NR 036964)
NWU284 (JX860387)
Streptosporangium nondiastaticum (NR 036963)
Nocardiopsis trehalosi (NR 024958)
Nocardiopsis sp (AF328404)
NWU98 (JX860359)
Nocardiopsis composta (AB562492)
Nocardiopsis alba (JX007995)
Nocardiopsis tangguensis (EU841644)
Nocardiopsis sp. (JX123883)
Nocardiopsis dassonvillei (JN862844)
Actinomadura bangladeshensis (FN646675)
Actinomadura vinacea (JN180131)
Actinomadura formosensis (AJ420140)
NWU336 (JX860397)
Actinomadura meyerae (HQ291073)
NWU126 (JX860362)
Actinomadura sp. (JQ782905)
Actinomadura cremea (FN46648)
Saccharothrix sp. (HQ588356)
Saccharothrix texasensis (FJ56556)
NWU239 (JX860378)
Saccharothrix xinjiangensis (FJ486479)
Saccharothrix espanaensis (FJ486394)
Pseudonocardia sp. (EF588221)
Pseudonocardia antarctica (JN180177)
NWU80 (JX860355)
Pseudonocardia alni (GU083568)
Pseudonocardia carboxydivorans (FJ547123)
NWU183 (JX860370)
Rhodococcus sp. (JN695036)
Rhodococcus triatomae (AJ854056)
Rhodococcus wratislaviensis (EU043327)
Rhodococcus equi (AB738794)

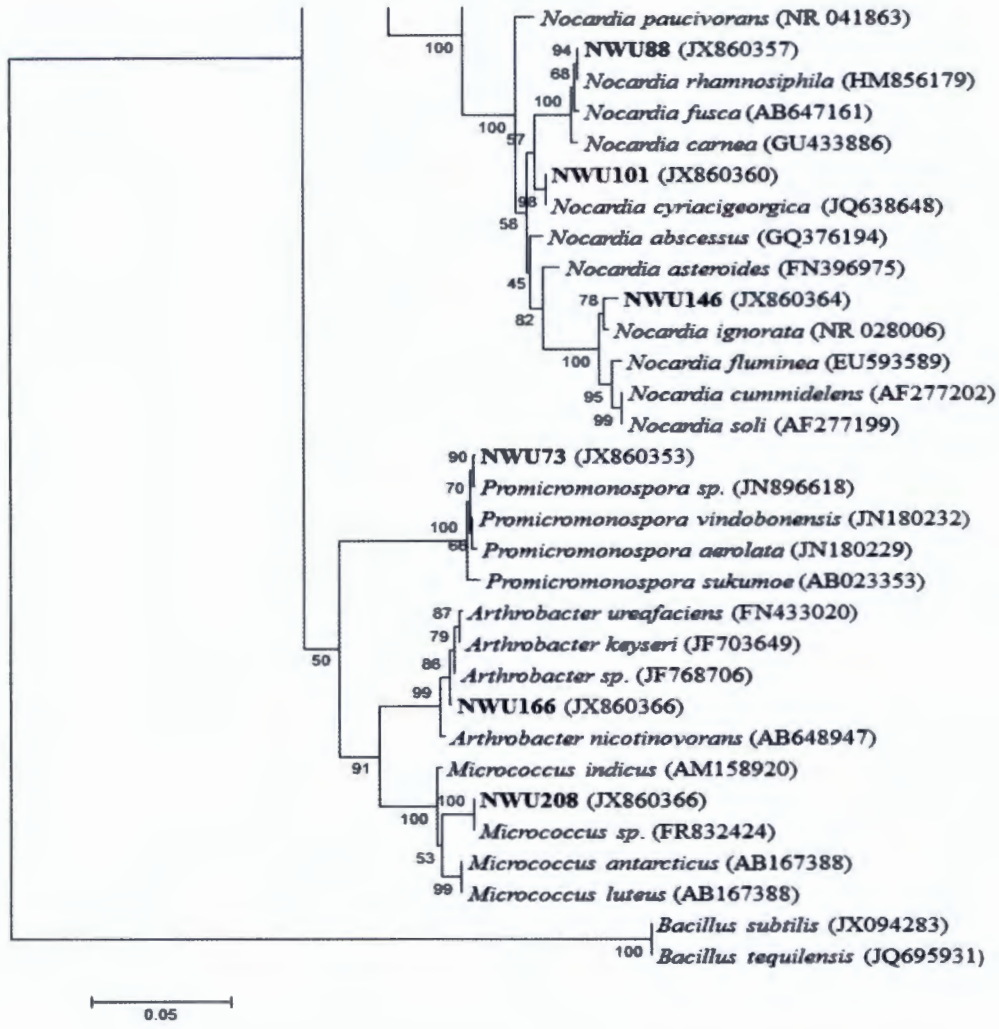


Figure 5.2: Neighbor-joining tree of the selected isolates and representative species of actinomycetes based on partial 16S rDNA gene sequences. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.05 substitutions per nucleotide position.

5.3 Discussion

The discovery of novel bioactive compounds from actinomycetes has marked an era in antibiotic research and succeeding developments in antibiotic chemotherapy. In the course of searching for possible novel antimicrobial agents against the spread of antibiotic resistant pathogens, antibiotic producing actinomycetes were isolated from rhizospheric soil samples collected from Ngaka Modiri Molema district in the North West Province, South Africa. Actinomycetes have been explored for many bioactive compounds of high clinical importance and are still routinely screened for novel bioactive compounds (Gebreyohannes et al., 2013). Considerable attention is currently given to the isolation and characterization of rare actinomycetes, due to evidence that screening such organisms raises the prospect of discovering novel bioactive compounds that can be developed as a resource for biotechnology (Hong et al., 2009). Although several research works have reported that soil is an excellent source of actinomycetes with diverse potential, it has not been fully explored, and there is tremendous potential to isolate rare actinomycetes with biological activities. Rare actinomycetes are difficult to isolate by conventional culturing methods due to their very slow growth on culture plates, and hence their inability to compete with fastidious microorganisms. Because actinomycetes are at a competitive disadvantage when grown on agar media in association with other soil inhabiting microbes, isolation media must be designed to reduce the development of competing microbes without adversely affecting actinomycetes.

In this study, 88 rare actinomycetes were isolated by pretreatment of soil samples, which stimulates the isolation of actinomycetes by eliminating most unwanted organisms. It was previously reported that pretreatment of the soil samples decreased the growth of fast growing bacteria and fungi, allowing the rare actinomycetes to dominate the culture plate (Seong et al., 2001). In one of the studies, research was carried out by Hayakawa and co-

workers to isolate *Streptosporangium* spp from soil samples. They reported that the soil samples were subjected to both physical and chemical pretreatments, and cultured on selective medium supplemented with specific antibiotics. These treatments drastically eliminate unicellular bacteria and unwanted actinomycetes, including *Streptomyces* spp. from the isolation plates, thereby facilitating the selective isolation of *Streptosporangium* spp. For isolations from various soils, this method achieved 20% selective isolation of *Streptosporangium* spp. (Lazzarini et al., 2000). The specific isolation of *Actinomadura* spp was achieved by air-drying, heating soil and using selective media supplemented with antibiotics such as streptomycin and rifamycin. More than 250 known antibiotics have been produced by *Actinomadura* strains (Lazzarini et al., 2000). Other methods such as the enrichment method have also been reported to increase the discovery of rare actinomycetes such as *Actinoplanes*, *Amycolatopsis*, *Planobispora*, *Dactylosporangium*, *Catenuloplanes* and *Virgosporangium*. This study indicates that rare actinomycetes can be successfully recovered from soil samples, once pretreatment and selective isolation methods are applied.

The combination of pretreatment methods and selective media supplemented with specific antibiotics, have been found to stimulate the isolation of diverse rare actinomycete genera that were only recovered incidentally by conventional methods. This approach helps to answer the question: are these less exploited actinomycetes less abundant in the environment or are they just more difficult to isolate and cultivate? The appropriate pretreatment methods and various selective isolation media that met several nutritional requirements with specific antibiotics increased the range of strains isolated. The pretreatment methods will contribute to isolating novel rare actinomycetes which will help to improve knowledge concerning the occurrence, distribution, ecology, taxonomy and evolution of rare actinomycetes. The isolation of rare actinomycetes is valuable for the discovery of novel bioactive secondary

metabolites. The development of selective isolation methods, have led to the discovery of novel bioactive secondary metabolites of industrial importance.

The rhizosphere is a unique biological niche that supports an abundant diversity of microorganisms, and the combination of strains found in the rhizosphere is governed by physical and chemical characteristics of the soil and by plant species (Zhao et al., 2012). The presence of relatively large populations of rare actinomycetes in the rhizospheric soil samples indicates that it is a suitable ecosystem that promotes the growth of the microorganisms. Rare actinomycetes from the rhizosphere produce secondary metabolites with novel molecules and pharmaceutical properties suggesting that the rhizosphere can be an interesting source for bioprospecting (Qin et al., 2009). The presence of actinomycetes in the rhizosphere is consistent with other reports which showed that actinomycetes are prominent in the plant root system (Yilmaz et al., 2008). Hong and co-workers reported a higher number of bioactive compounds producing actinomycetes from the rhizosphere than from corresponding soil, this might be due to the secretion of exudates from the plant root that can serve as food for the organisms (Babalola, 2010).

As the aim of this study was to isolate rare actinomycete genera, we focused on the isolates that according to the morphological criteria did not seem to belong to the genus *Streptomyces*, 88 of such isolates were analysed by macro- and micromorphology, biochemical, and physiological criteria in order to preliminarily identify the genera. These characteristics can serve as markers by which individual strains can be distinguished. The morphological, biochemical and physiological characteristics varied from one isolate to another depending on the required growth conditions. The isolates utilized a wide range of carbon sources due to their ability to produce extracellular enzymes that metabolised the polymeric components of the nutrient mixture to monomeric forms for their growth. The

utilization of various carbon sources serve as additional criteria for classification of actinomycetes.

The 16S rDNA gene sequence analysis by molecular methods resulted, in identification of the genera. A good agreement between preliminary genera identification of the isolates based on their morphology and characteristics and subsequent identification based on 16S rDNA gene sequence analysis was observed. The 16S rDNA sequencing analysis is routinely used to identify actinomycete isolates after comparing with reference organisms in nucleotide sequence database (Franco-Correa et al., 2010). The phylogenetic relationship between isolates was determined by 16S rDNA nucleotide sequence analysis of representative strains of each identified genus. As shown on the phylogenetic tree, depicting also bootstrap values, the 19 selected isolates were sorted into 11 clusters with highest similarity to the genera *Nocardia* spp., *Nocardiopsis*, *Streptosporangium*, *Rhodococcus*, *Actinomadura*, *Promicromonospora*, *Arthrobacter*, *Micrococcus*, *Saccharothrix*, *Pseudonocardia* and *Nonomuraea*. A good agreement between the BLAST search and clustering in the phylogenetic tree of the selected isolates was observed. These isolates clustered with known strains that are producers of bioactive compounds. Some of the closest neighbors of our isolates were found to produce bioactive compounds. This data indicates a considerable biodiversity of actinomycetes in the soil samples. Even though these isolates may be strains of known species, they are still considered to be important sources of novel bioactive compounds, as it has been reported that strains of the same species might produce different secondary metabolites depending on their isolation sources (Jensen et al., 2007).

Actinomycetes are known as prolific producers of secondary metabolites that are of importance in medicine and agriculture. The rare actinomycetes isolates obtained in this study were screened for their antibiotic production potential. Out of the 88 rare actinomycetes tested for activity against pathogens, 19 (21.6%) exhibited broad spectrum antibacterial

activity suggesting their ability to produce bioactive secondary metabolites. The fact that the isolates exhibited broad spectrum antimicrobial activity, this signifies possible production of several antimicrobial compounds and/or production of compounds with multiple microbial targets. Isolates NWU60, NWU80, NWU121, NWU146, NWU252 and NWU284 exhibited broad spectrum antibacterial activities against the test organisms. These isolates showed potential as sources of antimicrobial agents, and there is a need to explore them. These isolates have been identified as *Nocardiopsis sp* (NWU60), *Pseudonocardia sp* (NWU80), *Actinomadura sp* (NWU121), *Nocardia sp* (NWU146), *Nonomuraea sp* (NWU252) and *Streptosporangium sp* (NWU284), all of which have been reported as novel producers of bioactive compounds (Lazzarini et al., 2000, Liu et al., 2011).

Several researchers reported that rare actinomycetes have biocontrol activity against pathogenic organisms (Bredholdt et al., 2007, Gebreyohannes et al., 2013). Many of the rare actinomycetes produced antibiotic complex ranging from two to ten structurally related components (Gastaldo and Marinelli, 2003). These bioactive compounds exhibit broad spectrum of diverse chemical classes making the chemotherapy potential expressed by members of this group very attractive to industrial screening programmes (Vaishnav and Demain, 2011). It is possible that the activities observed are due to already known antibiotics such as aminoglycosides, polyenes, macrolides, tetracyclines and glycopeptides, which are commonly produced by many actinomycetes.

5.4 Conclusion

This present study provides further evidence of significant biodiversity of actinomycetes in rhizospheric soils that present strains that can be a valuable source of bioactive compounds with antibiotic activity. The rare actinomycetes are targeted as they have proven to be a valuable source of bioactive secondary metabolites, notably antibiotics, even if a relatively

low number of strains have been exploited in comparison with *Streptomyces.* Fermentation and chemical analysis of the extracts is concurrently being carried out in order to identify active compounds and to assess their novelty.

5.5 Methods

5.5.1 Actinomycetes isolation

The sampling area and details of soil samples collected have been previously described (Adegboye and Babalola, 2013). The soil samples were subjected to various physical and chemical pretreatment methods in order to facilitate isolation of actinomycetes (Hayakawa, 2008). Actinomycetes were isolated by serial dilution method from soil samples (Gebreyohannes et al., 2013). Various selective media used in this study were previously described (Adegboye and Babalola, 2013). The plates were observed periodically for the growth of actinomycetes. The pure colonies were selected, isolated and maintained in starch casein agar slants at 4°C for subsequent studies.

5.5.2 Screening for antibiotic production

Antagonistic activity of isolates against Gram-negative and Gram-positive bacteria was screened by using the perpendicular streak method. The experiment was carried out in triplicate.

5.5.3 Morphological characterization

Pure bacterial isolates were characterized culturally following the protocol given by the International Streptomyces project (ISP) (Shirling and Gottlieb, 1966), growth on sterile ISP-medium 2 was recorded after incubation at 25°C for 14 days. Morphological characters such as colony characteristics, pigment production, absence or presence of aerial and substrate mycelia were observed.

The arrangement of spores and sporulating structures were examined microscopically using cover the slip culture method by inserting a sterile cover slip at an angle of 45°C in the starch casein agar medium (Gebreyohannes et al., 2013).

5.5.4 Biochemical and physiological characterization

Various biochemical and physiological tests were performed for the characterization of the bacterial isolates using standard methods previously described (Adegboye and Babalola, 2013).

5.5.5 Isolation of genomic DNA

The genomic DNA was extracted from all the actinomycetes isolates using the CTAB method as previously described (Adegboye and Babalola, 2013).

5.5.6 PCR amplification

The 16S rDNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al., 1991, Adegboye and Babalola, 2013).

5.5.7 Sequence similarities and phylogenetic analysis

The chromatograms were edited using Chromas Lite version 2.4 software (Technelysium Pty Ltd 2012) Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The obtained 16S rDNA sequences were compared to sequences in the NCBI GenBank database with the Basic Alignment Search Tool (BLAST) (Altschul et al., 1990). Multiple alignments of the sequences were carried out by Mafft program 7.050 (Katoh, 2013) against corresponding nucleotide sequences of the genus *Streptomyces* retrieved from GenBank. Phylogenetic analyses were conducted using software's in MEGA version 5.2.2 (Tamura et al., 2011). Evolutionary distance matrices were generated as described by (Jukes

and Cantor, 1969) and a phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbor-joining data set. Manipulation and tree editing were carried out using TreeView (Page, 1996).

5.5.8 Supporting data

The 16S rDNA gene sequences determined for the bacterial isolates in this study were deposited in GenBank database and assigned accession numbers (Table 6.6).

5.6 Conflict of interest statement

We declare that we have no conflict of interest.

5.7 Author contribution

MFA conceived the study, carried out the molecular studies, sequence alignment and drafted the manuscript. OOB participated in the study design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

5.8 Acknowledgements

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CHAPTER 6

Phylogenetic Characterization of Culturable Antibiotic Producing *Streptomyces* from Rhizospheric Soils

Abstract

Streptomyces spp were isolated from rhizospheric soils collected from Ngaka Modiri Molema District, North West Province, South Africa. Ten of these bacterial isolates were found to exhibit broad spectrum antimicrobial activity against test organisms with varying degrees of activity. The cultural characteristics of the bacterial isolates were consistent with that of the members of the genus *Streptomyces*. Molecular identification of the potent bacterial isolates was carried out by amplifying the 16S rDNA gene; this gave the expected size of 1.5 kb and was sequenced. Further computational analysis including BLAST search and phylogenetic analysis were performed to correlate the bacterial isolates with other species of the genera in the database library. The computational analysis of the amplified 16S rDNA gene confirmed that the bacterial isolates are members of the genus *Streptomyces* with 89-100% sequence similarity. The phylogenetic analysis showed that the ten bacterial isolates were divided into 3 major clusters with varying bootstrap values. The strain NWU195 formed a distinct phyletic line in the *Streptomyces* 16S rDNA gene tree suggesting a new strain. The 16S rDNA sequences of the bacterial isolates were submitted to the Genbank under the accession numbers JX284398-JX284407. The 16S rDNA gene sequence analysis is a significant tool for phylogenetic analysis of *Streptomyces spp.*

Keywords: *Streptomyces*; rhizosphere; phylogenetic, antimicrobial; 16S rDNA

6.1 Introduction

Soil is a habitat for microorganisms and also serves as a reservoir for their metabolites (Chin et al., 2006). The genus *Streptomyces* is one prominent soil inhabitant, comprising up to 90% of actinomycetes isolated from soil samples. *Streptomyces* is the largest and the most important genus in the order actinomycetales. The genus *Streptomyces* is a prolific producer of bioactive secondary metabolites that have important applications both in medicine and agriculture (Demain and Sanchez, 2009, Atta et al., 2010). In the history of drug discovery, the majority of novel substances of microbial origin are isolated from *Streptomyces*, over two-thirds of all microbial antimicrobial agents are derived from them (Newman and Cragg, 2007, Ganesan, 2008). Many novel drugs have been developed from *Streptomyces* spp including *S. griseus*, *S. hygroscopicus*, *S. coelicolor*, *S. avermitilis*, *S. rochei*, *S. plicatus*, *S. fungicidicus*, *S. flaveus* and *S. globisporus*; belonging to different classes of antibiotics such as aminoglycosides, ansamycins, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines (Watve et al., 2001, Baltz, 2007, Marinelli and Marcone, 2011). These organisms are of high biotechnological and commercial value; and continue to be routinely screened for new bioactive compounds (Chen et al., 2007, Hozzein et al., 2011, Zotchev, 2011). *Streptomyces* spp are aerobic, filamentous, Gram-positive soil dwelling bacteria with high G+C content in their genomic composition.

Pathogenic organisms are gaining resistance to existing clinical drugs either through acquiring resistance (chromosomal mutation) or acquisition of genetic materials from other bacteria (vertical or horizontal transfer of genes) (Alanis, 2005, Skippington and Ragan, 2011). This uprising has rendered many onetime drugs of choice ineffective against the pathogens (Arias and Murray, 2009). Escalating numbers of antibiotics futile against pathogenic organisms is a worldwide scenario. There is a need to develop novel antibiotics with different mechanisms of action.

Various researches are taking place all over the world searching for antimicrobial agents to combat the menace of infectious disease agents. Despite the fact that soils have been continuously screened over 50 years for potent organisms there is still a possibility of isolating novel antibiotics from terrestrial Streptomycetes. It has been reported that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered (Busti et al., 2006). Some *Streptomyces*, spp possess more than 20 gene clusters devoted to the synthesis of secondary metabolites (Guerra et al., 2012). Determination of the nucleotide sequence of the 16S rDNA gene is a well established standard method for the identification and phylogenetic classification of unknown organisms up to the species level. Our investigation aimed to isolate and screen antibiotic producing *Streptomyces* from rhizospheric soils and evaluate their evolutionary linage for possible novel antimicrobial agent(s).

6.2 Materials and Methods

6.2.1 Sampling area

The study area covered the Ngaka Modiri Molema District in North West Province of South Africa (Figure 6.1). The latitude and longitude of the district is 25°55'N and 25°50'E respectively. It covered a total of 28,206 km^2 area. Temperatures range from 17° to 31°C (62° to 88°F) in the summer and from 3° to 21°C (37° to 70°F) in the winter. The average rainfall is 360 mm.

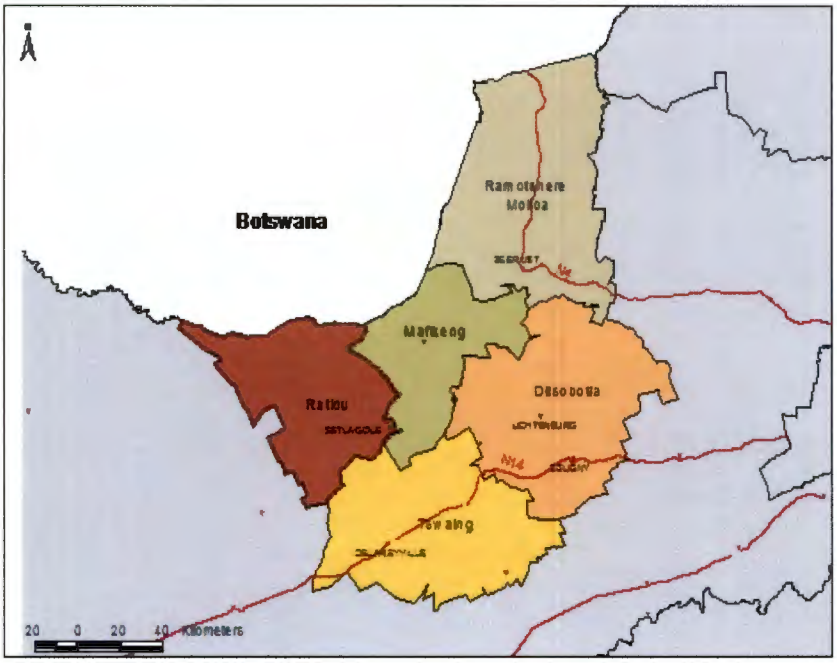

Figure 6.1: A map of Ngaka Modiri Molema district showing different localities where soil samples were collected.

6.2.2 Isolation of actinomycetes

Isolation and enumeration of actinomycetes present in the soil sample was performed by serial dilution plate technique using starch casein agar, as described previously (Morakchi et al., 2009).

6.2.3 Screening for antibiotic producing actinomycetes

Determination of antimicrobial activities of pure actinomycete cultures was performed by cross-streak method as described previously (Oskay, 2009).

6.2.3.1 Test organisms

The test organisms were *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* ATCC 12344, *C. coli* ATCC 43478, *Bacillus subtilis* ATCC 11774, *B. cereus* ATCC 11778, *Proteus mirabilis* ATCC 49132, *Enterococcus faecalis* ATCC 14506, *Shigella boydii* ATCC 9207, *Klebsiella pneumoniae* ATCC 8308, *Pseudomonas aeruginosa* ATCC

10145 and *Salmonella typhimurium* ATCC 14208, which were obtained from Davies Diagnostics (Pty) Ltd, South Africa.

6.2.4 Cultural characteristics

Pure bacterial isolates were characterized culturally following the protocol given by the international Streptomyces project (ISP) (Shirling and Gottlieb, 1966); growth on ISP-2 medium was recorded after incubation at 25°C for 14 days.

6.2.5 Isolation of genomic DNA

Actinomycete genomic DNA was isolated by a protocol previously described (Magarvey et al., 2004) with some modification. Cultures were grown in 10 ml of Luria Bertani broth (Merck) in McCartney bottles for 7 days and then centrifuged at 10,000 rpm (Universal Z300K model centrifuge; HERMLE Labortechnik, Germany) for 5 min. The mycelial pellet was resuspended in 500 µl of 5 M NaCl and transferred to a 2-ml Eppendorf tube. The cells were centrifuged at 10,000 rpm for 5 min., and the pellet was resuspended in 1 ml of 10 mM Tris-HCl–1 mM EDTA (pH 7.5) (TE) containing 20 mg/ml of lysozyme and 20 mg/ml of RNase A and incubated at 37°C for 30 min. Following incubation, 250 µl of 0.5 M EDTA, 250 µl of TE containing 5 mg/ml of proteinase K, and 100 µl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37°C for 1 h. The tubes were mixed by inversion after the addition of 250 µl of 5 M NaCl. Immediately thereafter, 200 µl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was added, and the tubes were heated in a 65°C water bath for 30 min. Cellular debris was removed by centrifugation at 8,000 rpm for 5 min and the supernatant solution was transferred to a new 2-ml microcentrifuge tube. Proteins and lipids were removed by the addition of 0.3 volume of phenol-chloroform, and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA

was centrifuged, the pellet was rinsed with 70% ethanol to remove traces of salt. The supernatant was gently poured off and the pellets were dried under vacuum using a Tomy Micro VacTM mv-100 (Tomy Medico, Japan) vacuum dryer. The DNA was resuspended in 50 µl of TE and incubated at 65°C for 1 h to reconstitute the DNA, for immediate use or storage at -20°C.

6.2.6 PCR amplification

The 16S rDNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with previously described primer F1 (5'-AGAGTTTGATCCTGGCTCAG-3') and R5 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisbury et al., 1991). PCR was performed in a total volume of 50 µl containing 30-50 ng DNA, 100 mM of each primer, 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP. The amplification reaction was performed with a DNA Engine DYAD Peltier thermal cycler (BioRad, USA). The thermal cycling condition used was an initial denaturation at 96°C for 5 min, followed by 30 cycles of denaturation at 96°C for 45 s, annealing at 56°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. The PCR amplicons were analysed by electrophoresis in 1% (w/v) agarose gel. The gel containing ethidium bromide (10 µg/ml) was viewed under Syngene Ingenius Bioimager (UK) to confirm the expected size of the product. The remaining mixture was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany).

6.2.7 Nucleotide sequence determination

PCR purified products of the 16S rDNA of the strains were analysed for nucleotide sequence determination by using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. Nucleotide sequence of the 16S rDNA of the strains were determined and compared for similarity level

with the reference species of bacteria contained in genomic database banks, using the 'NCBI Blast' available at the ncbi-nlm-nih.gov website (Altschul et al., 1990).

6.2.8 Molecular taxonomy determined by sequences and Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using softwares. Nucleotide sequences were analyzed and edited by using BioEdit software (Hall, 1999). The partial 16S rDNA gene sequences were used to search the GenBank database with the BlastN algorithm to determine relative phylogenetic positions (Altschul et al., 1990). Multiple alignments of the sequences were carried out by Mafft program 6.864 (Katoh and Toh, 2010) against corresponding nucleotide sequences retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969). Phylogenetic analyses were conducted using MEGA version 5.10 (Tamura et al., 2011) and neighbor-joining (Saitou and Nei, 1987); minimum evolution (Rzhetsky and Nei, 1992); maximum likelihood; UPGMA and maximum parsimony (Fitch, 1986) trees was constructed. The methods were used in order to expatiate on the phylogeny and for better comparison. The robustness of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Putative chimeric sequences were identified using the ChimeraBuster 1.0 software. Manipulation and tree editing were carried out using TreeView (Page, 1996).

6.2.9 Nucleotide sequence accession numbers

The 16S rDNA gene sequences obtained in this study have been submitted to the GenBank database and assigned accession numbers indicated in parentheses, NWU4 (JX284398), NWU233 (JX284399), NWU49 (JX284400), NWU91 (JX284401), NWU110 (JX284402), NWU195 (JX284403), NWU204 (JX284404), NWU339 (JX284405), NWU100 (JX284406), and NWU14 (JX284407).

6.3 Results

6.3.1 Isolation of actinomycetes

Streptomyces spp. was isolated from the soil samples collected from different localities in Ngaka Modiri Molema district. The colonies of actinomycetes are recovered from different serial decimal dilution Petri dishes. The bacterial isolates have morphological characteristics that were consistent with members of the genus *Streptomyces*. The colonies appear dry, rough, colored or not, adhering to the medium and presence of aerial and/or substrate mycelia.

6.3.2 Cultural characterization

The *Streptomyces* isolates were studied for cultural characteristics (Table 6.1). Cultural characteristics of the bacterial isolates were derived on the basis of observations made after 21 days of incubation on ISP-2. All the isolates growth varied from good to few. The colors of the mycelia varied from greyish to brown. These characteristic morphological properties strongly were suggested that the isolates belonged to the genus *Streptomyces*.

Table 6.1: Cultural characteristics of bacterial isolates on ISP-2 medium

Isolate	Growth	Aerial mycelium	Substrate mycelium	Reverse colony color	Pigmentation
NWU4	Good	Grey	Yellowish brown	Yellowish brown	Brown
NWU14	Good	Grey	Light brown	Brown	None
NWU49	Good	Dark green	Light brown	Green	None
NWU91	Moderate	White	Yellow	Orange	None
NWU100	Few	Grey	Greyish yellow	Brown	None
NWU110	Moderate	Greyish green	Dark brown	Brown	None
NWU195	Good	White	Yellowish grey	Reddish orange	None
NWU204	Good	Grey	Light brown	Brown	None
NWU233	Moderate	Cream	Yellow	Brown	None
NWU339	Few	Greyish green	Brown	Green	None

6.3.3 Screening of *Streptomyces* isolates

Among them, ten isolates were distinguished due to their antibacterial activity against test organisms. These *Streptomyces* isolates exhibited broad spectrum antimicrobial activities. The results was tabulated in Table 6.2. The following percentage of *Streptomyces* isolates exhibited inhibitory effect against the test organisms including: *S. aureus* (100%), *S. pyogenes* (100%), *C. coli* (90%), *B. subtilis* (100%), *B. cereus* (90%), *P. mirabilis* (40%), *E. faecalis* (90%), *S. boydii* (70%), *K. pneumoniae* (40%), *P. aeruginosa* (30%) and *S. typhimurium* (50%). Three isolates exhibited high antibacterial activities against all the test organisms and appeared promising.

Table 6.2: Antibacterial activity of potent actinomycetes isolates against pathogenic organisms

Test organisms	Isolate codes									
	NWU4	NWU14	NWU49	NWU91	NWU100	NWU110	NWU195	NWU204	NWU233	NWU339
Pseudomonas aeruginosa ATCC 10145	-	+	-	+	-	-	-	+	-	-
Klebsiella pneumoniae ATCC 8308	-	+	+	+	-	-	-	+	-	+
Enterococcus faecalis ATCC 14506	-	+	+	+	+	+	+	+	+	+
Shigella boydii ATCC 9207	-	+	-	+	-	+	+	+	+	+
Proteus mirabilis ATCC 49132	-	-	-	+	-	-	-	+	+	+
Bacillus subtilis ATCC 11774	+	+	+	+	+	+	+	+	+	+
Bacillus cereus ATCC 11778	-	+	+	+	+	+	+	+	+	+
Streptococcus pyogenes ATCC 12344	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus ATCC 29213	+	+	+	+	+	+	+	+	+	+
Campylobacter coli ATCC 43478	+	+	+	+	-	+	+	+	+	+
Salmonella typhimurium ATCC 14208	+	+	-	+	-	+	-	+	-	+

+ = Activity; - = No activity

6.3.4 Molecular identification of *Streptomyces* isolates

A 1.5 kb fragment was amplified from the genomic DNA with the bacterial universal primers (F1R5) (Fig. 6.2). Identification of the isolates was confirmed by computational analysis. The generic identification of Streptomycetes was performed by analysis of partial sequences of their 16S rDNA gene. The partial nucleotide sequences of the 16S rDNA gene of the isolates were compared with nucleotide database of NCBI web server through BLAST tool. The BLAST search inferred that the isolates were members of the GC-rich Actinomycetales. The 16S rDNA gene sequence of different *Streptomyces species* was obtained by BLASTn search; however 24 strains of *Streptomyces* were selected based on high identity (%) with good E value. Table 6.3 results show that query sequences were best pairwise aligned with 16S rDNA gene sequence of *Streptomyces* spp with sequence similarity and identity ranged between 89-100%, with E value of 0.

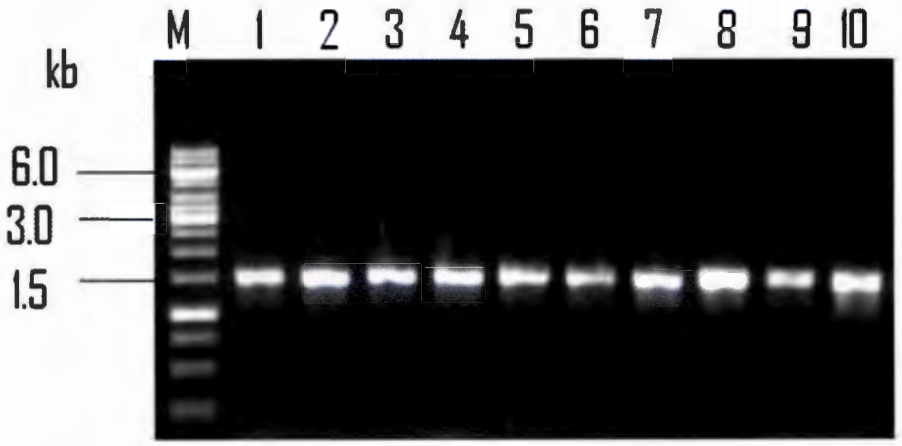

Figure 6.2: Amplified fragment of 16S rDNA gene of the potent bacterial isolates 1-10 (1.5 kb); M: 1 kb Marker

Table 6.3: Results of 16S rDNA gene sequence similarities of *Streptomyces* isolates and GenBank accession numbers using BLASTn algorithm

Isolate code	Sequence length (bp)	Closest related strain in database	Accession number	Similarity (%)	E-value
NWU4	1338	*Streptomyces globisporus*	HQ995504	99	0
NWU14	1341	Uncultured *Streptomyces sp.*	JQ358574	95	0
NWU49	1306	*Streptomyces viridosporus*	NR 0438575	99	0
NWU91	1335	*Streptomyces rochei*	JF486442	100	0
NWU100	1363	*Streptomyces hirsutus*	AB184844	89	0
NWU110	1295	*Streptomyces emelensis*	NR 043869	98	0
NWU195	1397	*Streptomyces hygroscopicus*	FJ406123	95	0
NWU204	1308	*Streptomyces fungicidicus*	AB184529	99	0
NWU233	1373	*Streptomyces espinosus*	X80826	98	0
NWU339	1362	*Streptomyces griseus*	AB184821	92	0

6.3.5 Phylogenetic analysis and *Streptomyces sp.* diversity

The ten potent *Streptomyces* isolates were subjected to sequencing and phylogenetic analysis. The 16S rDNA sequences of the ten isolates were aligned with 24 Streptomycetes sequences obtained from GenBank data library; and *Kitasatospora sp* as the out-group. The phylogenetic position of the isolates was evaluated by constructing phylogenetic trees using neighbor-joining (NJ), minimum evolution (ME), maximum likelihood (ML), maximum parsimony (MP) and UPGMA methods (Fig. 6.3-7). These methods consistently placed the bacterial isolates in different clades encompassing members of the genus *Streptomyces* with bootstrap support. Bootstrap values based on 1000 replications were listed as percentages at the branching points. The results from the different approaches show completely resolved, well-supported phylogeny of the ten bacterial isolates with high resolution of all inner branches. Bacterial isolate NWU100 shows a low sequence similarity (89%) with the closest related stain (*S. prasinopilosus*); this suggests that it is a novel *Streptomyces sp.* Phylogenetic analysis also revealed that the isolate NWU195 forms a distinct clade and the low sequence similarity values further suggest that NWU195 possibly belongs to a novel species with *S. hygroscopicus* as its closest relative. This is further supported by its taxonomic positions, confirmed by phylogenetic analyzes (Fig. 6.3-7). Overall, the high-level branching in the phylogenetic trees agrees well with traditional systematic divisions, which groups organisms that belong taxonomically to the same family or the same genus into different species.

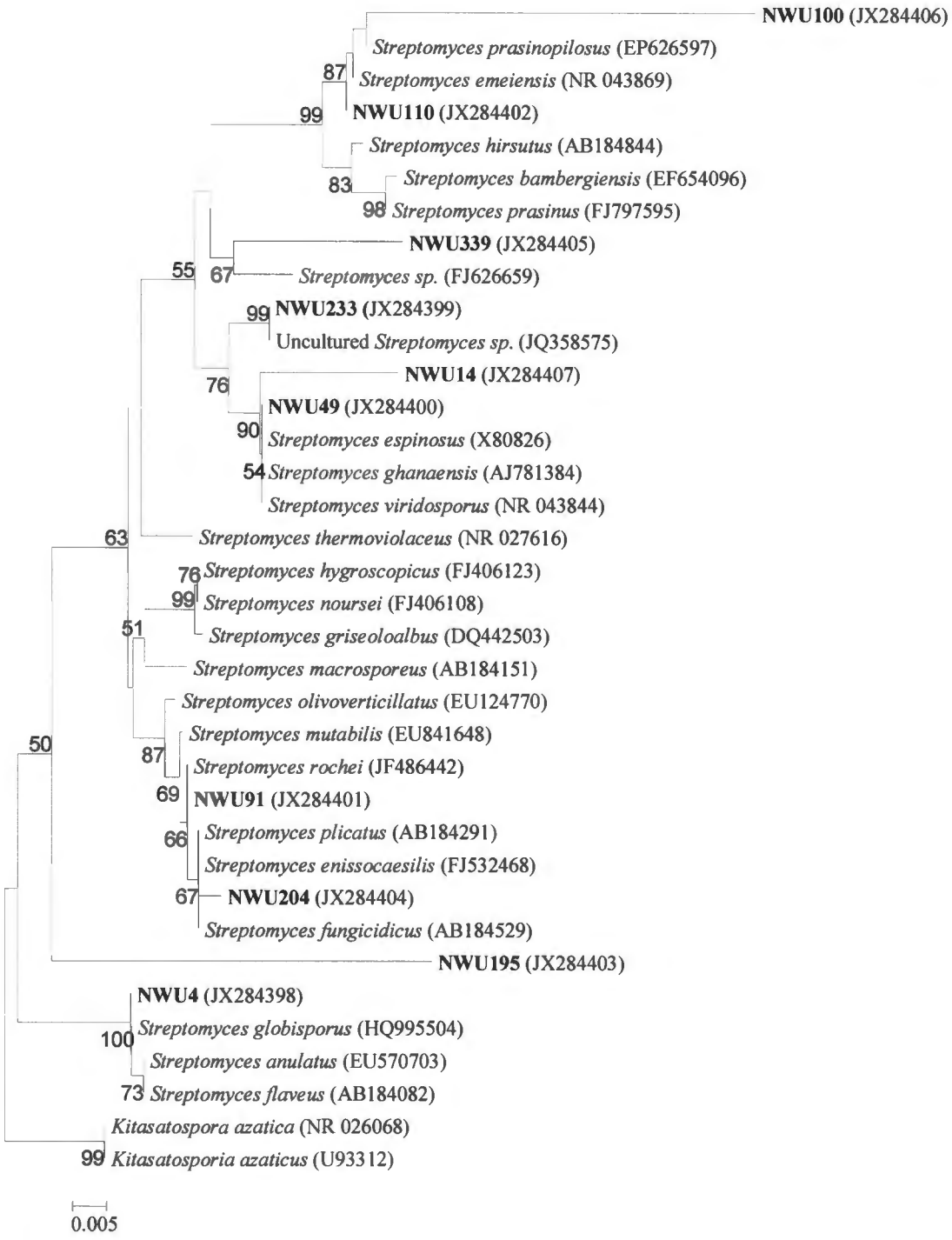

Figure 6.3: Minimum evolution phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between bacterial isolates and the most closely related type strains of the genus Streptomyces. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

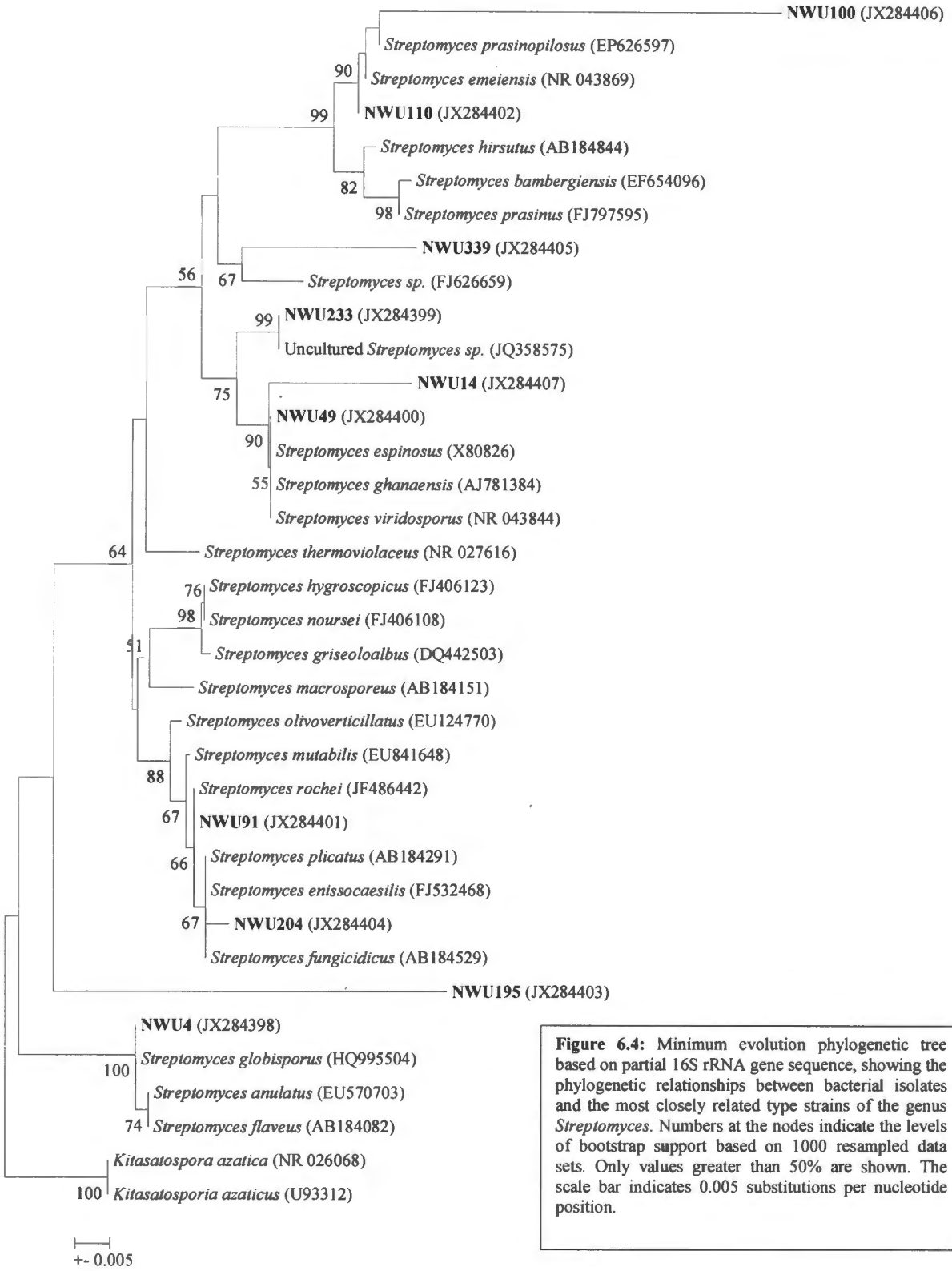

Figure 6.4: Minimum evolution phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between bacterial isolates and the most closely related type strains of the genus *Streptomyces*. Numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. Only values greater than 50% are shown. The scale bar indicates 0.005 substitutions per nucleotide position.

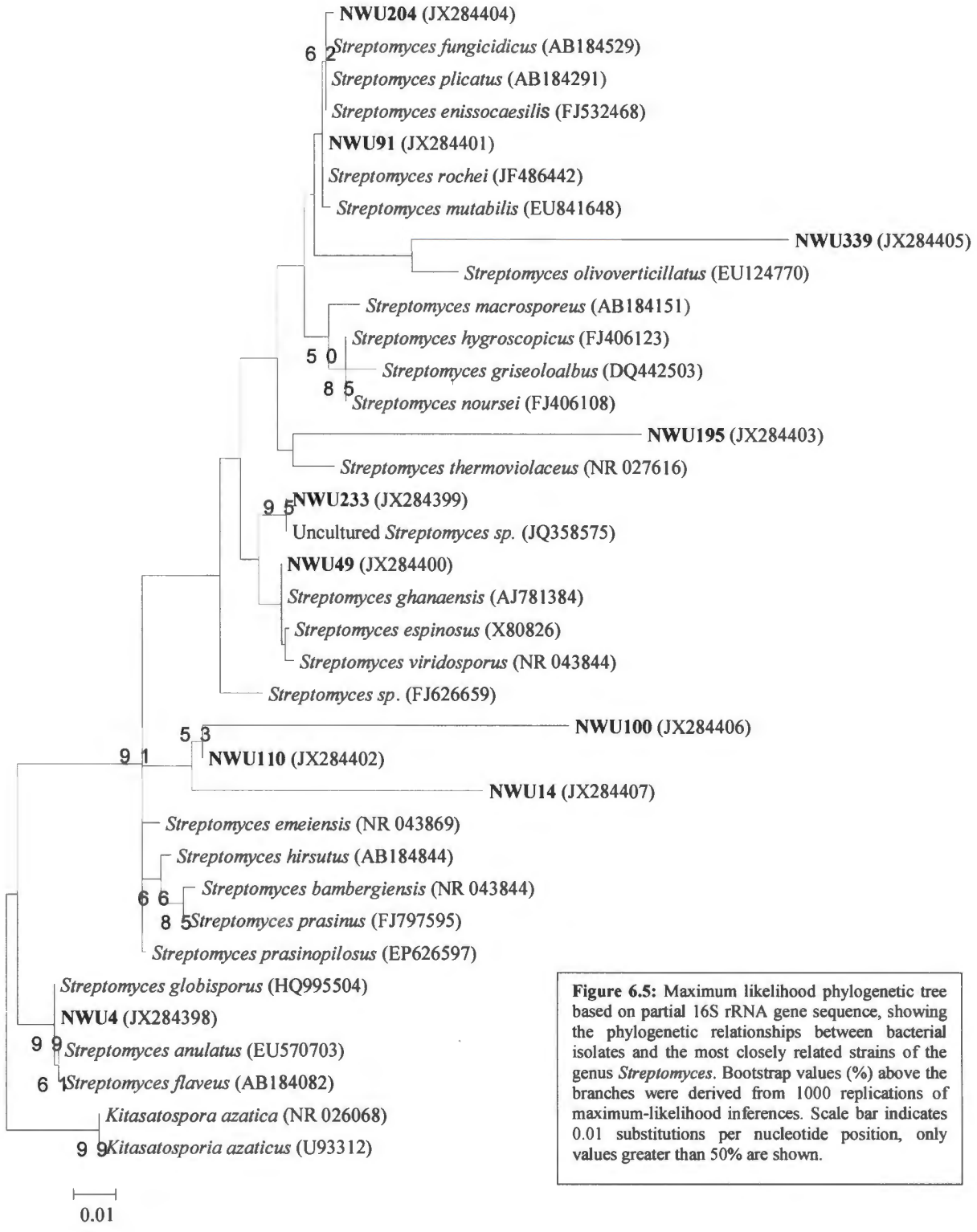

Figure 6.5: Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between bacterial isolates and the most closely related strains of the genus *Streptomyces*. Bootstrap values (%) above the branches were derived from 1000 replications of maximum-likelihood inferences. Scale bar indicates 0.01 substitutions per nucleotide position, only values greater than 50% are shown.

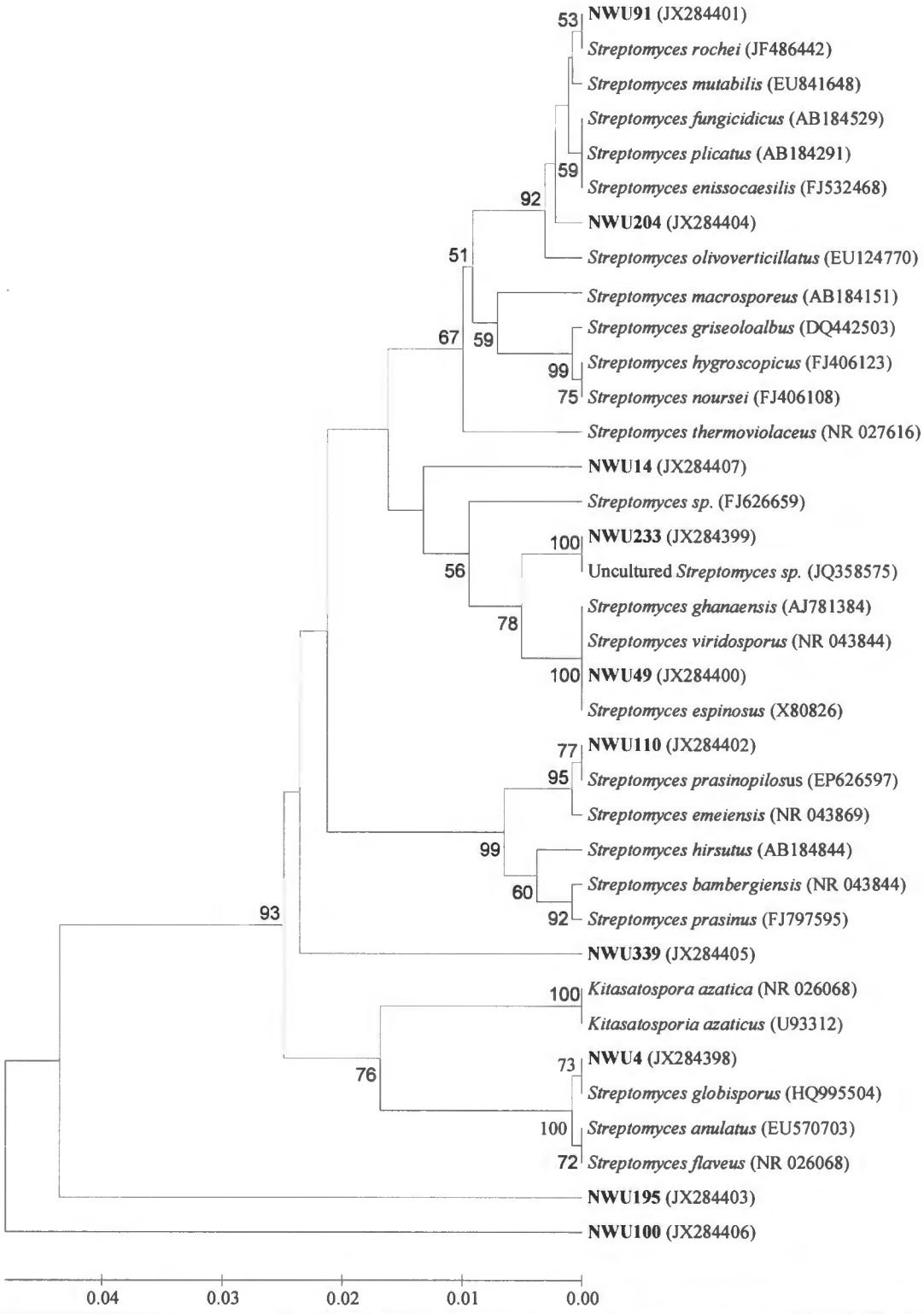

Figure 6.7: UPGMA phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between bacterial isolates and the most closely related type strains of the genus *Streptomyces*. Bootstrap values (%) above the branches were derived from 1000 replications of maximum-likelihood inferences. Scale bar indicates 0.01 substitutions per nucleotide position, only values greater than 50% are shown.

6.4 Discussion

Screening of nature for novel antimicrobial agents is a continuous process to match unending demand for bioactive substances in order to curtail the issues of infectious diseases. Nature has been a renowned source of bioactive compounds; this can be through screening of plants or microorganisms (Chin et al., 2006). Microorganisms have proven to be an attractive source of bioactive compounds of industrial importance (Marinelli and Marcone, 2011). The unearthing of microbial secondary metabolites has a great capability in the development of industrial microbiology. The order Actinomycetales, especially the genus *Streptomyces* are known to be inexhaustible producers of important microbial metabolites of medical and agricultural importance (Lam, 2006, Raja and Prabakarana, 2011). The terrestrial habitat is considered an excellent source for the exploration of *Streptomyces* with substantial potential. Several studies have previously reported on the isolation and diversity of *Streptomyces* from the terrestrial environment (Oskay et al., 2005, Rifaat et al., 2006). It was evident in this study that the genus *Streptomyces* is the dominant actinomycetes in rhizosphere. Similar studies carried out by other researchers' also show that *Streptomyces* spp are predominant in the rhizosphere (Ting et al., 2009, Khamna et al., 2010). Previous research works have shown the number and diversity of the genus *Streptomyces* in the rhizospheric soils is in relation to the type and amount of exudates, and plant species (Bais et al., 2006). It has been reported that root exudates stimulates the growth of actinomycetes in the rhizosphere (Bertin et al., 2003). The cultural characteristics of the bacterial isolates were similar to those described by other researchers, this reveals that the isolates under study belong to the genus *Streptomyces* (Rifaat et al., 2006, Arunachalam et al., 2010).

The majority of the novel antimicrobial agents are derived from soil borne Actinomycetales. *Streptomyces* spp are an important group of organisms in the production of antimicrobial agents against pathogenic organisms. The bacterial isolates from the

rhizospheric soils showed broad spectrum antimicrobial activity against pathogenic organisms. The result of the screening reveals that most of the potent bacterial isolates were more active against Gram-positive organisms than Gram-negative organisms. This can be attributed to the cell wall structure of the Gram-negative organisms having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes (Willey et al., 2010). The Gram-positive organisms are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. The fact that the bacterial isolates exhibited broad spectrum antimicrobial activity signifies possible production of several antimicrobial compounds and/or production of compounds with multiple microbial targets. Several researchers have already reported *Streptomyces* to have biocontrol activity against pathogenic organisms (Hozzein et al., 2011, Srividya et al., 2012). It has been shown that the principle mechanism of this biological activity involves the production of secondary metabolites (Marinelli and Marcone, 2011).

The identification of the bacterial isolates to the species level is vital since this provides informative insight about the organism, possible kind of bioactive compounds and if it is novel or not (Adegboye and Babalola, 2012). The identification of the potent bacteria isolates in this study was based on 16S rDNA gene sequence analysis. The sequence comparison of the bacterial isolates showed 89-100% identification similarities with 16S rDNA gene sequence of the genus *Streptomyces.* Analysis of 16S rDNA gene sequences has been proved to be a powerful method for phylogenetic characterization of microorganisms (Thenmozhi and Kannabiran, 2010). It helps to elucidate the evolutionary relationship among microorganisms. The phylogenetic relationship of the potent bacterial isolates to known *Streptomyces* spp was first estimated through a BLAST search of the GenBank database. For a more robust analysis, the closest related strains were chosen for pairwise sequence

comparison and construction of the phylogenetic trees. The bacterial isolates were grouped in distinct branches from each other. It was reported that strains which are clustered in different groups might produce different antimicrobial agents (Intra et al., 2011).

All the closest strains to the bacterial isolates have been linked to the production of one bioactive compound or more. *S. globisporus* has been described as a soil dwelling Gram-positive bacteria with antibacterial, antifungal and antitumor activities (Ostash et al., 2003, Li et al., 2010). Mutanolysin was isolated from *S. globisporus*, it is a muralytic enzyme that cleaves the β-N-acetylmuramyl-(1→4)-N-acetylglucosamine linkage of the bacterial cell wall polymer peptidoglycan-polysaccharide (Brönneke and Fiedler, 1994, Shiba et al., 2000). Volatiles from *S. globisporus* have been reported to act as antifungal agents against *Penicillium italicum* that cause blue mould infection on citrus (Li et al., 2010).

A wide range of antimicrobial substances produced by *Streptomyces* spp isolated from the rhizosphere have been described, including *Streptomyces sp.* NRRL 30566 which bears 99% 16S rDNA sequence similarity to *S. griseus*. *Streptomyces sp.* NRRL 30566 was reported to produce a novel antibiotic, kakadumycins which are DNA intercalating antibiotics that act by inhibiting DNA directed enzymatic RNA synthesis (Castillo et al., 2003). A novel compound faeriefungin (polyene macrolides), was isolated from *S. griseus*, it was reported to have antibacterial, antifungal and insecticidal activities (Nair et al., 1989). Moenomycin A is a novel antibiotic produced by *S. ghanaensis*, and it is a direct inhibitor of the enzyme peptidoglycan glycosyltransferases (transglycosylases); thereby inhibiting cell wall synthesis (Ostash et al., 2009).

6.5 Conclusion

Our results suggest that *Streptomyces* spp in the rhizosphere are diverse and these strains are suitable for natural product screening. The current molecular techniques seem to

be a powerful tool in the identification of bacterial isolates based on the characterization of the 16S rDNA genes. Computational analyses are effective and reliable tools used to envisage the relatedness between the bacterial isolates and those in the GenBank database towards identification of antimicrobial compounds. It can be concluded that the use of phylogenetic analysis gives a better picture of the evolutionary relationship in the species level identification.

6.6 Acknowledgement

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CHAPTER 7

Evaluation of Antibiotic Biosynthetic Potential of Actinomycetes Isolates to Produce Novel Antimicrobial Agents

Abstract

The order actinomycetales contains a distinct group of medically, industrially and economically important bacteria due to their ability to produce bioactive secondary metabolites. Biosynthetic gene clusters are responsible for the biosynthesis of bioactive secondary metabolites produced by actinomycetes. The analysis of biosynthetic gene clusters presents a useful foundation for the discovery of novel bioactive compounds. Through PCR-based approach 341 actinomycete isolates were screened for PKS-I, PKS-II and NRPS genes. The amplification of the genes from some of the actinomycete isolates is an indication of their potential as antibiotic producers. Sixteen isolates (4.6%) were identified as PKS-I gene positive strains, 15.2% for PKS-II and 26% for NRPS gene. Through the screening, it was found that *Streptomyces* have higher prevalence of PKS-I, PKS-II and NRPS genes compared to others genera. Phylogenetic analysis of the nucleotide sequences from the amplified biosynthetic genes confirmed that the isolates formed a close phylogenetic relationship with known antibiotic producers. PCR-based approach using degenerative primers to screen for the presence of biosynthetic gene clusters responsible for the biosynthesis of bioactive secondary metabolites, is an effective approach for discovering novelty and diverse antibiotics from actinomycetes.

Keyword Actinomycetes secondary metabolites biosynthetic gene cluster PCR screening PKS NRPS

7.1 Introduction

Terrestrial actinomycetes are well known as prolific producers of secondary metabolites. These secondary metabolites exhibit pharmaceutical properties, such as antibacterial, antifungal, antiprotozoal, anticancer, anticholesterol and immunosuppressant. These bioactive compounds were synthesized through biosynthetic metabolic pathways. Polyketides (PKS) and nonribosomal peptides (NRPS) pathways are synthesized by various specific enzymatic steps (Wenzel and Müller, 2005). These enzymes perform the repetitive chemical condensation of monomeric units, such as carboxylic acid and/or amino acid monomers. The vast structural complexity and functional diversity of PKS, NRPS or hybrid compounds are as a result of complex enzyme organization of the assembly lines and specific tailoring enzymes responsible for post-assembly modifications (Gontang et al., 2010). Recent advances in the areas of small molecule detection, isolation and structure elucidation, genome sequencing and molecular techniques has helped improve the understanding of the molecular genetics of secondary metabolite biosynthesis, rekindling interest in discovery platforms aimed at realizing the biosynthetic capacity of antibiotic producing microorganisms.

The PCR based approaches have been used successfully to amplify genes associated with secondary metabolite biosynthesis and therefore it is possible to predict whether secondary metabolite pathways are present within an organism (Wood et al., 2007). It becomes feasible to envisage or estimate the number and novelty of bioactive compounds following phylogenetic analyses of the amplified and sequenced secondary metabolite genes. Secondary metabolites through PKS and NRPS pathways have proven to be one of the primary sources of new chemical scaffolds from which novel pharmaceutical agents have been developed (Gontang, 2008).

The order Actinomycetales are renowned producers of bioactive metabolites with a track record of over 10,000 antimicrobial agents in clinical use (Baltz, 2007). Biosynthesis of

these secondary metabolites is catalyzed by specific enzymes usually encoded by gene clusters. Polyketide synthases and non-ribosomal peptide synthetases are the major enzymes of secondary metabolites synthesis (Ichikawa et al., 2013). Examples of classes of antibiotics produced through this biosynthesis include asamycins, tetracyclines, polyenes and glycopeptides.

In an effort to isolate novel bioactive compounds, degenerate primer sets were used to screen for the presence of biosynthetic gene clusters associated with PKS-I, PKS-II and NRPS pathways in genomic DNA of 341 actinomycetes strains. The isolation of antibacterial actinomycetes strains was based on the amplification of the expected size of the gene of interest. The PCR screening of the genomic DNA for specific antibiotic genes of interest allows for the rapid determination of the antibiotic biosynthetic potential of the isolates.

7.2 Materials and Methods

7.2.1 Bacterial strains and cultivation

All actinomycete strains isolated and identified from rhizospheric soil samples (Adegboye and Babalola, 2013) were grown in Luria Bertani broth (Merck) at 30°C with agitation in shaker incubator for 7 days.

7.2.2 Antibacterial assays

Antagonistic activity of isolates against Gram-negative and Gram-positive bacteria was screened by using perpendicular streak method (Parthasarathi et al., 2010). In perpendicular streak method, Mueller Hinton agar (Merck) was used and each plate was streaked with individual actinomycete isolates at the center/diameter of the plate and incubated at 30°C for 7 days. Later, 24 h fresh sub-cultured test bacteria were prepared and streaked perpendicular to the isolates and incubated at 37°C for 24 h. The experiment was carried out in triplicate.

7.2.3 DNA extraction

The genomic DNA was extracted from all the actinomycetes isolates using the CTAB method as previously described (Adegboye and Babalola, 2013).

7.2.4 PCR primers

Four sets of primers were used: F1: 5′-AGAGTTTGATCITGGCTCAG-3′ and R5: 5′-ACGGITACCTTGTTACGACTT-3′ targeting 16S rDNA gene (Weisburg et al., 1991). AHBA-F: 5′-CCSGCSTTCACSTTCATCTC-3′ and AHBA-R: 5′-AISYGGAICATIGCCATGTAG-3′ targeting 3-amino-5-hydroxyl-benzoic acid (AHBA) synthase gene (Wood et al., 2007). ARO-PKS-F: 5′-GGCAGCGGITTCGGCGGITTCCAG-3′ and ARO-PKS-R: 5′-CGITGTTIACIGCGTAGAACCAGGCG-3′ targeting the ketosynthase alpha (KS_{α}) and ketosynthase beta (KS_{β}) gene pair (Wood et al., 2007). NRPS-A3F: 5′-GCSTACSYSATSTACACSTCSGG-3′ and NRPS-A7R: 5′-SASGTCVCCSGTSCGGTAS-3′ targeting the targeting NRPS adenylation (A) domains (Ayuso-Sacido and Genilloud, 2005). All the oligonucleotide primers were synthesized by IDT (South Africa).

7.2.5 PCR amplification conditions

PCR was performed in a total volume of 50 µl containing 30-50 ng DNA, 100 mM of each primer, 0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$, and 0.4 mM of each dNTP (Fermentas, USA). The amplification reaction was performed with a DNA Engine DYAD Peltier thermal cycler (BioRad, USA). The PCR cycling programme was as follows: initial denaturation (96°C for 2 min); 30 cycles of denaturation (96°C for 45 s), annealing (56°C for 16S rRNA and AHBA synthase genes/64°C for the KS_{α}–KS_{β} gene pair/57°C for NRPS gene, for 30 s), and extension (72°C for 2 min); and a final extension (72°C for 5 min). The PCR amplicons were analyzed by electrophoresis on 1% (w/v) agarose gel. The gel containing ethidium bromide (10 µg/ml) was viewed under Syngene Ingenius Bioimager (UK) to

confirm the expected size of the PCR products. The remaining mixture was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Germany).

7.2.6 DNA sequencing

The Sequencing of the purified PCR products were conducted at the facilities of Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa using ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, USA).

7.2.7 Phylogenetic analysis

All nucleotide sequences for the 16S-rDNA, PKS and NRPS phylogenetic trees were obtained from GenBank, except for the sequences that were determined in this study. Nucleotide sequences were analyzed and edited using BioEdit software (Hall, 1999). The obtained sequences were compared to sequences in the NCBI GenBank database with the Basic Alignment Search Tool (BLAST) (Altschul et al., 1990). Multiple alignments of the sequences were carried out by Mafft program 7.050 (Katoh, 2013) against corresponding nucleotide sequences retrieved from GenBank. Phylogenetic and molecular evolutionary analyzes were conducted using software in MEGA version 5.2.2 (Tamura et al., 2011). Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by neighbor-joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings of the neighbor-joining data set. Manipulation and tree editing were carried out using TreeView (Page, 1996).

7.2.8 Nucleotide sequence accession numbers

The GenBank accession numbers of the nucleotide sequences generated in this study are shown in parentheses beside the isolate codes on the phylogenetic trees.

7.3 Results

7.3.1 Bioactivity of actinomycete isolates

All the actinomycete isolates were tested for antibacterial activity using cross streak method. The result shows that 54% of the isolates exhibited antibacterial activity against one or more of the test organisms. Analysis of the bacterial isolates showed that the genus *Streptomyces* is the most active in exhibiting broad spectrum antimicrobial activities.

7.3.2 Detection of biosynthetic gene clusters in actinomycete isolates

Degenerate primers targeting genes encoding for polyketide synthases (PKS-I and PKS-II) and nonribosomal peptide synthetase (NRPS) were used to screen the biosynthetic potential of 341 actinomycete isolates, as identification of these genes provides indirect evidence of potential chemical diversity among these actinobacteria in terms of natural product drug discovery. The antibiotic producing actinomycete isolates were the subject of 16S rDNA gene sequencing which is used for the identification of the strains by BLAST search program. It is apparent that the actinomycete isolates can be assigned to the genera *Streptomyces*, *Nocardia*, *Rhodococcus*, *Pseudonocardia*, *Saccharothrix*, *Promicromonospora*, *Arthrobacter*, *Micrococcus*, *Nocardiopsis*, *Streptosporangium*, *Nonomuraea* and *Actinomadura*.

PCR screening for the biosynthetic gene clusters involved in the production of antimicrobial agents depends on the amplification of the predicted size of the DNA fragments. As expected, PCR screening of the genome of the actinomycete isolates for the biosynthetic gene cluster yielded DNA fragments of the predicted size from the potential producers. PCR screening for the type I polyketide synthase gene, yielded DNA fragments of approximately 750 bp (Fig. 7.1). The results indicated 4.6% (16) positive strains for the presence of type I polyketide biosynthetic gene cluster from the 341 actinomycete isolates screened. Molecular identification of the actinomycete isolates using 16S rDNA shows that *Streptomyces* are the

most prominent genus containing the AHSA biosynthetic gene cluster. A standard nucleotide BLAST search was carried out using the partial nucleotide sequences of the positive strains against reference sequences in the GenBank database. This showed that the actinomycete isolates were most similar to other type I polyketide producers such as *Amycolatopsis mediterranei*, *Micromonospora* spp, and *Streptomyces* spp. Fig. 7.2 shows the results of the PCR screening for the type II polyketide synthase gene pair, KS_{α} and KS_{β} with an expected amplified DNA fragment of approximately 500 bp. Out of 341 actinomycte isolates screened 15.2% were found to contain the biosynthetic gene cluster for type II polyketide. A BLAST search against the GenBank database using the nucleotide sequences generated from this study showed that the positive strains were most similar to the corresponding sequences from aromatic polyketide producers. An approximately 750 bp DNA fragment was amplified from the positive strains for NRPS gene (Fig. 7.3). Of all the 341 actinomycete isolates screened, 26% (46) were positive for the presence of the NRPS pathway. The NRPS gene sequences were generated and compared to sequences in the GenBank by BLAST analysis. The result showed that majority of the strains belong to the genus *Streptomyces*.

Figure 7.1: Agarose gel electrophoresis of PCR products from selective amplification of 500 bp fragment using AHBA-F/AHBA-R specific for PKS-I sequences

Figure 7.2: Agarose gel electrophoresis of PCR products from selective amplification of 500 bp fragment using ARO-PKS-F/ARO-PKS-R specific for PKS-II sequences

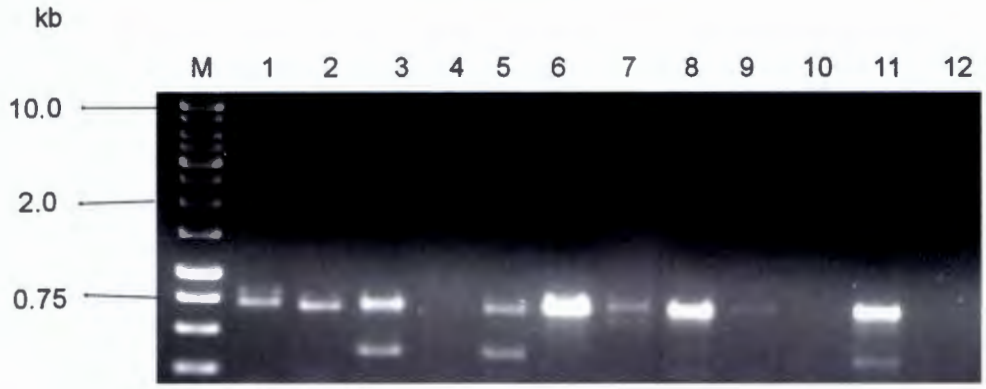

Figure 7.3: Agarose gel electrophoresis of PCR products from selective amplification of 750 bp fragment using NRPS-A3F/NRPS-A7R specific for NRPS adenylation sequences

7.3.3 Phylogenetic analysis of antibiotic producing actinomycete isolates

The phylogenies based on the 16S rDNA and biosynthetic gene nucleotide sequences from the actinomycete isolates constructed by the neighbor-joining method are shown in Fig. 7.4-7. Trees constructed by the UPGMA and maximum parsimony method gave similar topology (data not shown). Actinomycete isolates for the 16S rDNA were picked for the phylogenetic tree based on the exhibition of inhibitory activities against at least one test organism. Analysis based on the 16S rDNA gene alignments showed that the actinomycete isolates shared between 89-100% similarities with their closest relatives in the GenBank database that are known antimicrobial agent producers. It was also found that the actinomycete isolates formed a clade or cluster with already known antibiotic producing actinomycete reference sequences from the GenBank. As seen from the phylogenetic tree (Fig. 7.4), depicting also bootstrap values, the 23 antibiotic producing actinomycete isolates were sorted into 14 main clusters with highest similarity to already known antibiotic producing actinomycete reference sequences from the GenBank. The isolates selected for the phylogenetic tree were representative samples of the antibiotic producing actinomycetes from this study. A scrutiny of the 16S rRNA phylogenetic trees showed that isolate NWU195 formed a distinct phyletic line.

Further phylogenetic analysis was carried out on the positive strains for the biosynthetic gene cluster with similarity to known antibiotic producing actinomycetes. The neighbor-joining phylogenetic tree was constructed using the nucleotide sequences of AHSA synthase gene obtained in this study and the gene segment from other PKS-I producers reference sequences from the GenBank (Fig. 7.5). The nucleotide sequences of the AHSA synthase gene from the positive strains previously identified as *Streptomyces* isolates formed a distinct clade from other known antibiotic producer of the genus *Streptomyces*. The *Micromonospora* spp cluster with NWU337 forming a phylogenetically distinct lineage. Figure 7.6 shows the phylogenetic

position of the isolates with known aromatic polyketide producers based on the analysis of the PKS-II gene nucleotide sequences. The nucleotide sequences of NRPS gene from the positive isolates and selected ones from the GenBank were used in constructing the phylogenetic tree (Fig. 7.7). Clustering with other known producers is evident. The lineage relationships of branches composed of the individual isolates are supported by bootstrap values that are based on neighbor-joining analyses.

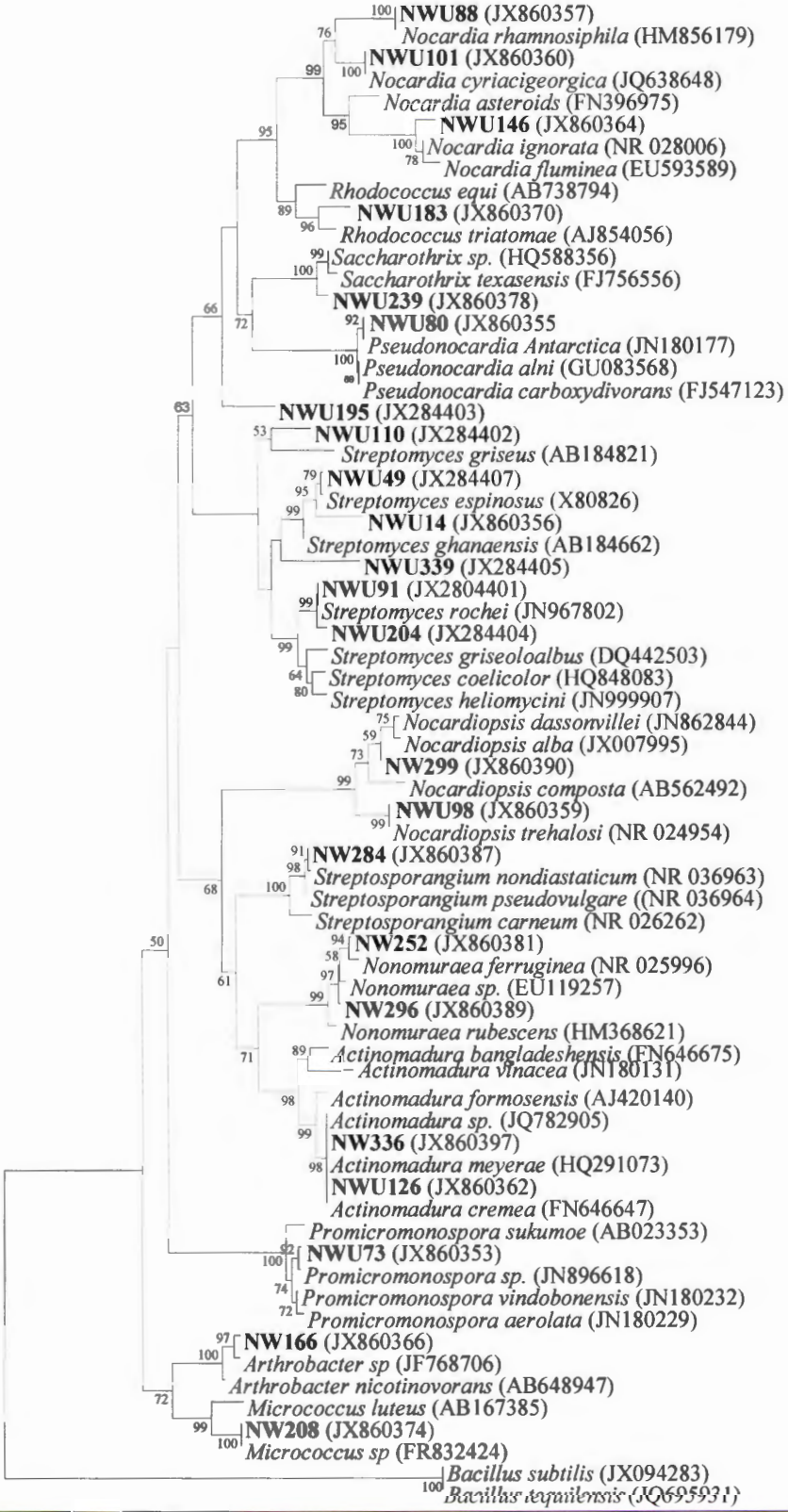

Figure 7.4: Neighbor-joining phylogenetic representation of cultured actinomycetes and their closest NCBI (BLASTn) relatives based on the 16S rRNA gene sequences. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater

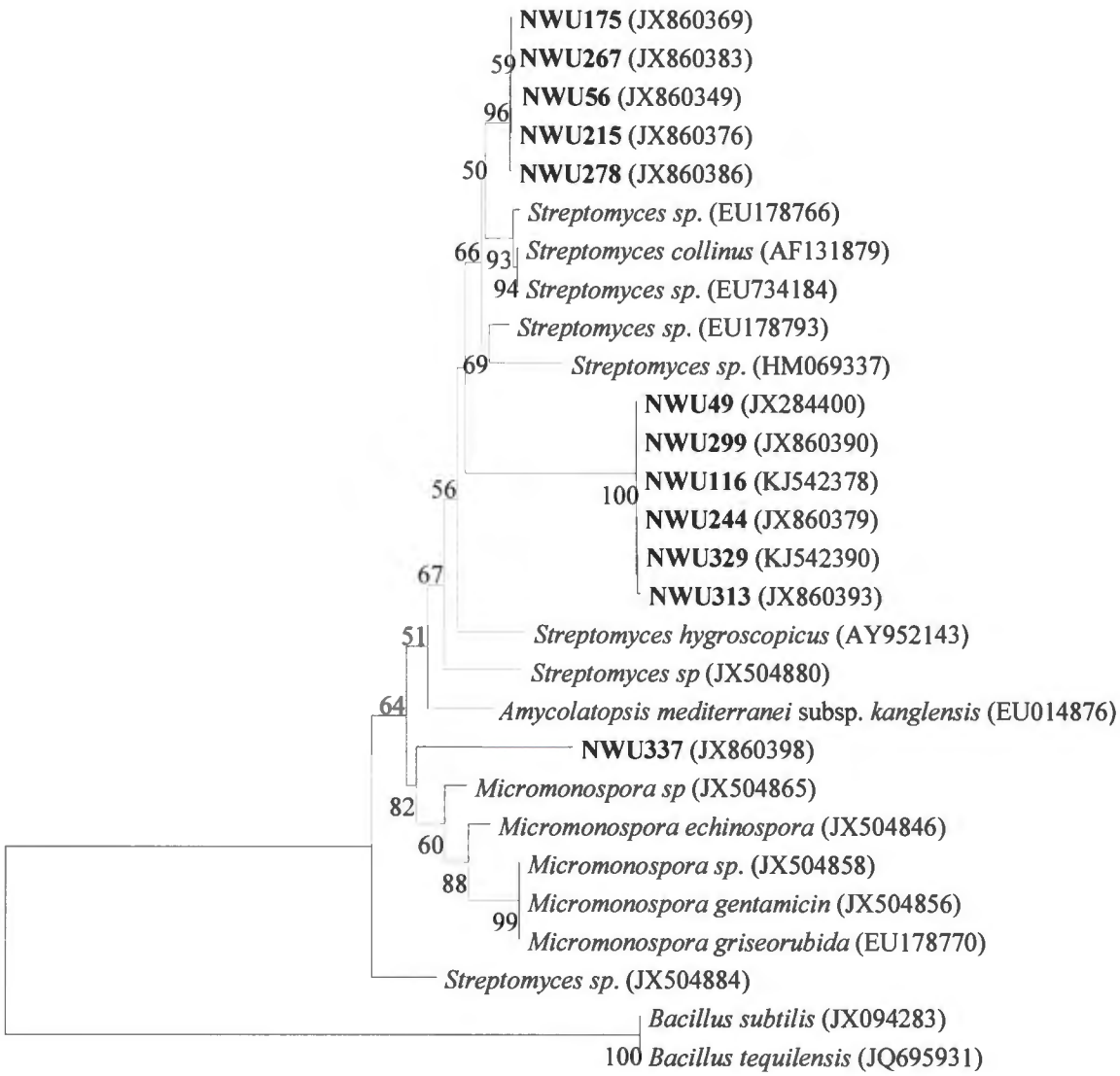

Figure 7.5: Neighbor-joining phylogenetic representation of cultured actinomycetes and their closest NCBI (BLASTn) relatives based on the AHSA gene sequences. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater

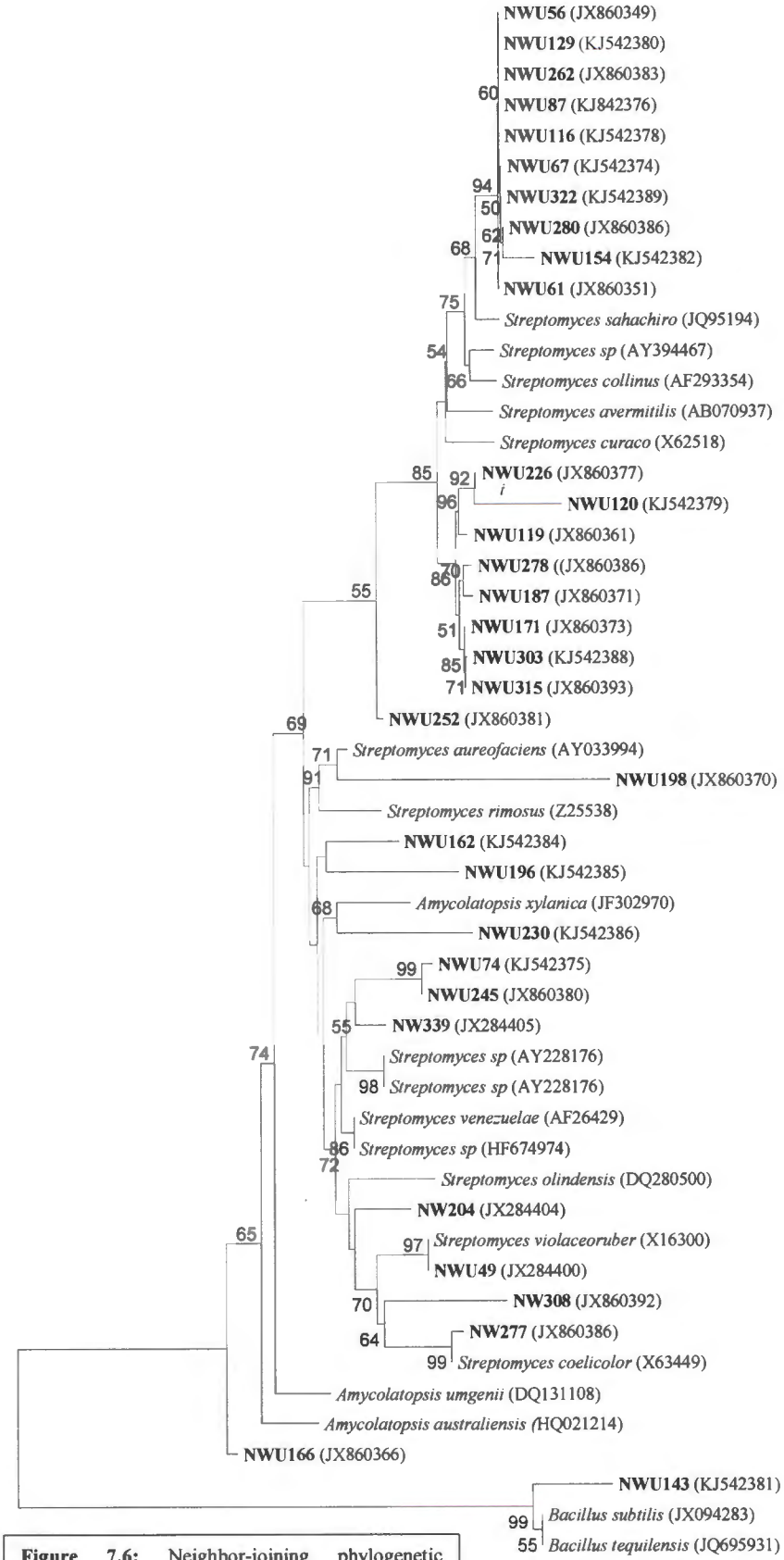

Figure 7.6: Neighbor-joining phylogenetic representation of cultured actinomycetes and their closest NCBI (BLASTn) relatives based on the PKS-II gene sequences.

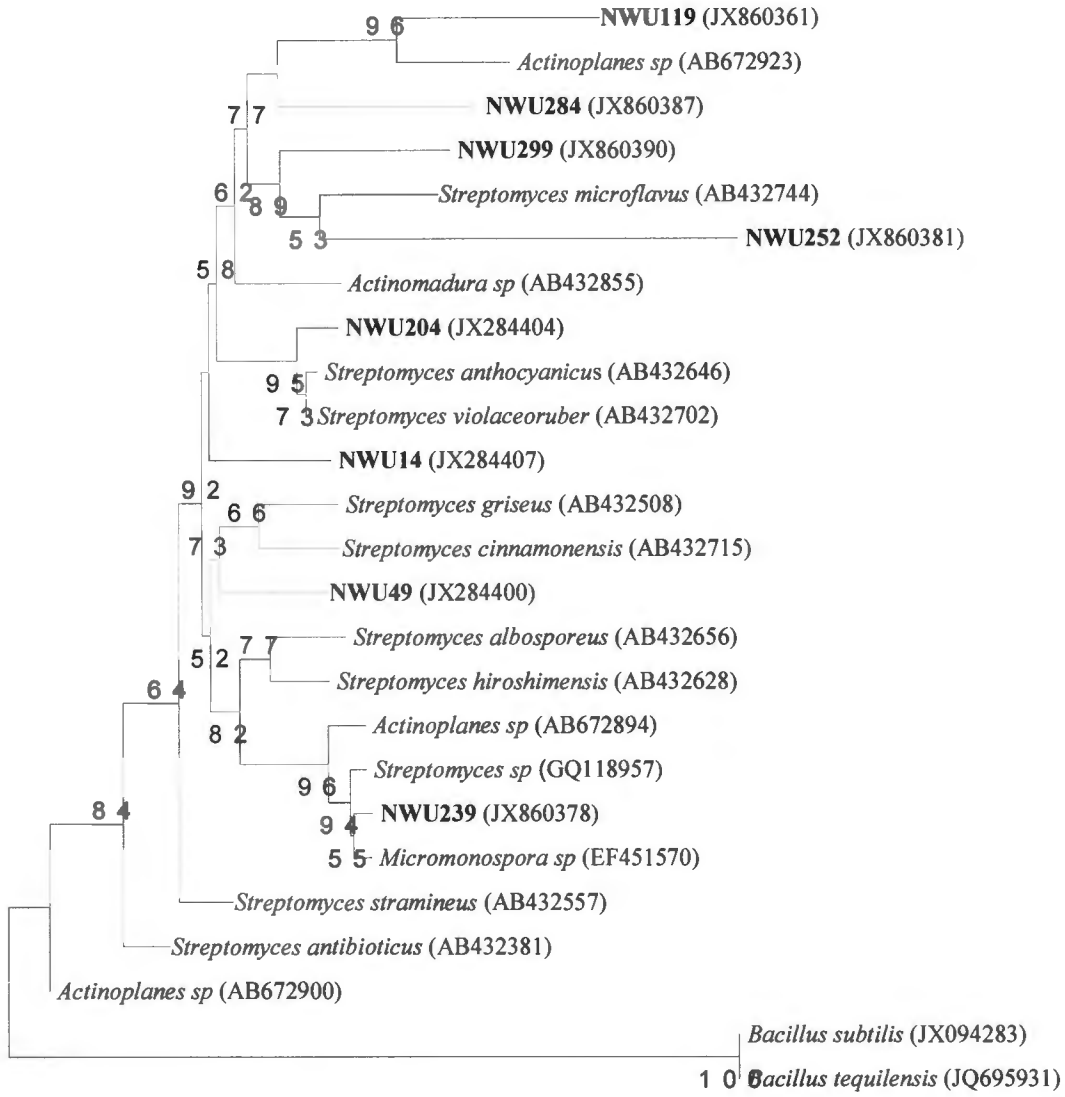

Figure 7.7 Neighbor-joining phylogenetic representation of cultured actinomycetes and their closest NCBI (BLASTn) relatives based on the NRPS gene sequences. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater

7.4 Discussion

As part of our bioprospecting programme actinomycete isolates from rhizospheric soil samples were screened in order to assess their biosynthetic potential, using degenerate primers to detect the presence of PKS-I, PKS-II and NRPS genes. The PCR-based approach offers a method by which to quickly screen a large collection of isolates in order to identify strains with the potential to produce specific structural classes of secondary metabolites (Gontang et al., 2010). The potential of actinomycetes to produce bioactive compounds has been exploited for years. Recent genomic sequence analyses of the biosynthetic gene clusters have revealed a previously unrecognized biosynthetic potential and diversity in actinomycetes (Ayuso-Sacido and Genilloud, 2005).

The PCR-based approach can only indicate when a gene is present or absent through the amplification of the gene or not. Some isolates such as NWU (14, 49, 119, 204, 220 and 339) exhibited antibacterial activity against selected test organisms and these corrected well with successful amplification of either one or more of the targeted genes from the genomes of the isolates. The PCR-based approach helps to expedite the screening of a large collection of isolates in order to identify strains with the potential to produce bioactive secondary metabolites (Gontang et al., 2010). It can be inferred that these isolates contain at least one complete biosynthetic gene cluster for bioactive secondary metabolite production. Some actinomycete isolates such as NWU (73, 91, 110, 208, 252, 284 and 336) did exhibit antimicrobial activity but there were no amplification products. The absence of PCR amplicons in some of the isolates suggests the lack of the biosynthetic genes although it cannot be concluded that the isolates in question lack such a gene. While it is possible that the isolates do not harbor any PKS or NRPS genes, this might also be as a result of the less conserved domain sequences sharing low homology with the primers in strains less well known to produce secondary metabolites of pharmaceutical importance. Also according to

Wood et al. (2007) this might be as result of variations in primer target sequences preventing the primers from binding efficiently. A limitation in this kind of study is that the design of degenerative primers is based on the available sequences in the GenBank of more abundant and well-studied organisms such as *Streptomyces*, unlike the rare actinomycetes that have few available sequences on their biosynthetic gene clusters in database library. As the number of whole genome sequences for rare actinomycetes increases, this will help in designing primers to amplify diverse biosynthetic genes. Some isolates such as NWU (87, 154, 196, 229, 267 and 322) also did not exhibit activity but there is amplification of the biosynthetic gene cluster. According to Finking and Marahiel (2004) not all biosynthetic gene clusters are involved in the biosynthesis of bioactive secondary metabolites. It is also possible that the genes detected by the PCR are non-functional or the isolates in question might have different nutritional requirements for the production of bioactive secondary metabolites. Also Wood et al. (2007) concluded that positive PCR amplification does not indicate that the genes are expressed nor does it show that the strain possesses the full suite of biosynthetic gene cluster for the biosynthesis of that class of antibiotic. The detection of PKS-I, PKS-II and NRPS genes in some isolates such as NWU (119, 204, 230 and 299) are indicators of their potential natural product diversity and divergent genetic evolution. Those isolates with amplification of at least 2 genes but no antimicrobial activity will be induced for the production of bioactive secondary metabolites. This might lead to the discovery of novel compounds especially among the rare actinomycetes

A phylogenetic approach using the nucleotide sequences generated from the DNA fragment of the biosynthetic gene clusters (PKS-I, PKS-II and NRPS) of the positive actinomycete isolates elucidated how nucleotide sequence information can be used to predict the number and novelty of possible bioactive compounds through the phylogenetic relatedness to known antibiotic producers. Phylogenetic analysis showed that most isolates have high bootstrap

values with known antibiotic producers. The study correlates with the finding of other researchers (Bai et al., 2006, Bredholdt et al., 2007, Hwang et al., 2007) who isolated and screened for biosynthetic genes from actinomycetes.

As seen in Fig. 7.5, the isolates formed 2 distinct clades from other organisms on the phylogenetic tree; this indicates the possibility of novel bioactive compounds biosynthesis from the isolates. Although NWU337 is identified as *Streptomyces sp*, it clustered with *Micromonospora* spp which are known producers of aminoglycoside antibiotics. In Fig. 7.6, NWU198 clustered with *S. aureofaciens* and *S. rimosus* with bootstrap value of 91, these organisms are known producers of tetracycline. Isolates NWU (49, 204, 277 and 308) clustered with *S. olindensis, S. violaceoruber* and *S. coelicolor*, known producers of antibiotics. NWU277 has a bootstrap value of 99 with *S. coelicolor*, a prolific producer of antibiotics such as actinorhodin, methylenomycin, undecylprodigrosin, and perimycin in Fig. 7.7, isolate NWU14 formed a distinct phyletic line suggesting it might a produce novel bioactive compound. Isolate NWU49 clustered together with *S. griseus* and *S. cinnamonensis* with a bootstrap value of 77, showing that it is close relative to these organisms which are known producers of streptomycin and monensin respectively. Isolates in close phylogenetic relationships with known antibiotic producers might also be involved in the biosynthesis of the same or similar bioactive compounds since they share similar structure features (Wang et al., 2013).

In conclusion, the PCR-based approach helps identify actinomycete isolates with biosynthetic gene clusters for the production of bioactive secondary metabolites. The presence of a particular gene gives an indication of the type of biosynthetic pathway to be explored. From the result generated in this study, there is a clear relationship between the occurrence of biosynthetic gene clusters and production of antimicrobial agents. The advantage of the PCR-based approach for novel drugs is that it provides knowledge about the type of prospective

compounds that can be produced by the strain having the biosynthetic gene. While the information is expedient, phylogenetic analysis of biosynthetic genes associated with the production of secondary metabolites can give an insight into both the novelty and diversity of pathways present within an isolate. All these give an insight about the applicable extraction and purification technique to be applied for that class of bioactive compound. Fermentation extracts from some of the actinomycete isolates are currently being analysed chemically in order to identify the bioactive compounds and assess their novelty.

7.5 Acknowledgements

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CHAPTER 8

Optimization of Fermentation Conditions for Antibiotic Production by Actinomycete Isolates

Abstract

For optimizing the bioactive secondary metabolite production from three strains of *Streptomyces* spp (NWU14, NWU49 and NWU91), experiments with the supplementation of nutrients were conducted based on the method of one-factor-at-a-time. The chemical and physical parameters affecting the production of bioactive secondary metabolite were optimized. The isolates exhibited broad spectrum antibacterial activity against *Enterococcus faecalis* ATCC 14506 and *Klebsiella pneumoniae* ATCC 8308; and produced bioactive secondary metabolites under the conditions of fermentation. The results indicated that the most suitable carbon source was glucose at a concentration of 1% for NWU49 and 2% w/v for NWU14 and NWU91. The best source of nitrogen was oatmeal for NWU14 and NWU91 and yeast extract for NWU49. The results of this study showed that the temperature, pH, inoculum volume, salt concentration and fermentation period directly influenced the production of bioactive secondary metabolites. The results obtained allow an efficient production of bioactive secondary metabolites by the isolates of actinomycete used in this study.

Keywords: *Streptomyces*; optimization; fermentation; antibacterial activity; secondary metabolites

8.1 Introduction

Exploration of microbial bioactive secondary metabolites is of great significance and an area of active research that has received much attention due to their medical and industrial

applications. To date thousands of bioactive compounds have been isolated and identified. Most of these have been discovered during soil screening from actinomycetes which constitute an inexhaustible reservoir of these bioactive compounds (Berdy, 2005, Newman and Cragg, 2012). The bacteria belonging to the group actinomycetes are ubiquitous, however they mostly found in soil. Their occurrence is greatly influenced by availability of nutrients, humidity, temperature, pH, and type of vegetation. They play a crucial role in the recycling of nutrients and decomposition of recalcitrant compounds. Actinomycetes can utilize a wide variety of substrates due to their ability to produce extracellular enzymes such as amylase, lipase, protease and cellulase.

The biosynthesis of bioactive secondary metabolites apart from biosynthetic gene clusters depends upon the conditions of fermentation. In order to achieve the optimal production of antimicrobial agents, the optimization of the fermentation process is an important step (Ravi and Dasari, 2011). This can be accomplished by systematic study of the different fermentation parameters such as nutrient limitation, pH, temperature, incubation time, agitation and aeration (da Silva et al., 2012). The fermentation strain is greatly influenced by the combination of media components and conditions involved in the fermentation process (Wang et al., 2010). The one-factor-at-a-time approach for optimal antibiotic production by the isolates was used in this study. This technique is based on the classical method of changing one independent variable while fixing all others at a certain level using the fermentation process (Singh and Rai, 2012). As a result of the ease and convenience of one-factor-at-a-time, this method has been most widely used for improving the fermentation medium and conditions.

In this study, antibiotic producing actinomycetes isolated from rhizospheric soil samples were studied to determine ideal conditions of fermentation for optimal production of bioactive compounds. Optimization of fermentation conditions is fundamental in getting high

yields of the bioactive compounds produced by the culture strain. Thus, this study gives an insight into the optimal condition for the production of microbial bioactive metabolites from selected antibiotic producing actinomycete isolates.

8.2 Materials and Methods

Among the active isolates recognised in the preliminary screening programme by cross-streak method, promising isolates were selected for extracellular antibiotic production studies by submerged cultivation using cup-plate method (Adegboye and Babalola, 2013). The most sensitive organisms which gave sharp inhibition zones were selected as test organisms (*Enterococcus faecalis* ATCC 14506 and *Klebsiella pneumoniae* ATCC 8308). Well sporulated selected isolates (7-10 days old) were used for the antibiotic production studies. Five ml of sterile water was transferred aseptically into each slant and the growth of the isolate on the surface of the medium was scraped with a sterile inoculating needle and transferred each into 45 ml of basal fermentation medium (composition (%): glucose, 0.5 yeast extract, 0.5; sodium chloride, 0.01; calcium carbonate, 0.01; 1000 ml sterile distilled water with pH 7.0) and incubated at 25°C on a rotary shaker at 220 rpm for 5 days.

8.2.1 Optimization of fermentation parameters

Optimization is a process by which the ideal condition for the growth of the organism and maximal production of bioactive secondary metabolites by it is attained. In order to select a medium for optimal antibiotic production, the following important parameters are investigated (a) effect of carbon source, (b) effect of nitrogen source, (c) effect of temperature, (d) effect of pH, (e) effect of salt concentration, (f) effect of inoculum, (g) effect of fermentation period. The experiments were carried out in such a way that one independent variable was studied at a time. Experiments were conducted in triplicate and the results were expressed as mean ± SE (standard error).

8.2.2 Effect of carbon source

Different carbon sources used in this study include: glucose, starch, sucrose, fructose, lactose, maltose, galactose, glycerol, mannitol, sorbitol, and mannose. Each carbon source was incorporated separately at 1.0% w/v into the basal production medium in order to study its effect on the production of antimicrobial compound. A 10% v/v level of inoculum was transferred to the production medium and the fermentations were run at 25°C for 5 days on a rotary shaker at 220 rpm. Finally at the end of the fermentation, the crude extracts from each sample were evaluated for their antibacterial activity using agar plate diffusion method.

8.2.3 Antimicrobial activity studies

The samples of the crude extract were collected in sterile centrifuge tubes and then centrifuged at 8000× *g* rpm for 20 min, at 8°C and the clear culture filtrate was separated. The clear supernatant was used for antibiotic assay using agar-plate diffusion method. The antibacterial activity against the bacterial organisms was tested on Mueller-Hinton agar (Sigma Aldrich). The molten sterile Mueller-Hinton agar medium was cooled to 40-45°C, inoculated with the test organism, mixed thoroughly, poured into sterile Petri dishes and allowed to solidify. The agar was perforated using a sterile cork borer. The clear supernatant fermentation broth was added to each cup (50 µl) by using a micropipette. The procedure was repeated for all the crude extracts and was tested for antibacterial activity. The plates were left for 2 h on the bench for antibiotic diffusion and then incubated at 37°C for 24 h. After 24 h the inhibition zones were recorded. Experiments were conducted in triplicate and results were expressed as the mean values along with standard error.

8.2.4 Determination of optimum concentration of glucose

During preliminary tests, glucose was found to be the best carbon source for the antibiotic production at one uniform concentration. The next step was therefore, to study the effect of various concentrations of glucose on antibiotic production. The following

concentrations were investigated to determine the optimum concentration of glucose for maximum antibiotic production (% w/v): 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. Each of the above concentrations was incorporated into the basal production medium with a 10% v/v level inoculum and incubated at 25°C for 5 days at 220 rpm. The antimicrobial activity was determined as described earlier.

8.2.5 Effect of nitrogen source

Different nitrogen sources were also tested for optimal growth conditions which included malt extract, yeast extract, beef extract, peptone, ammonium sulfate, potassium nitrate, oatmeal, corn meal, urea and casein. Each nitrogen source was incorporated at 1.0% w/v level into the basal production medium. The fermentation and evaluation of their antibacterial activities were carried out as per the general procedure.

8.2.6 Determination of optimum concentration of oat meal and yeast extract

Among the nitrogen sources employed, oat meal for NWU14 and NWU91 and yeast extract for NWU49 were found to be the best nitrogen source for the antibiotic production. Hence they are selected to study the effect of various concentrations on antibiotic production. The following concentrations were investigated to determine the optimum concentration for maximum antibiotic production (% w/v): 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. Each of the above concentrations was incorporated into the basal production medium and fermentations were allowed. The antimicrobial activity was determined as described earlier.

8.2.7 Effect of temperature

Temperature is also one of the important factors affecting growth and overall formation of microorganisms which is paramount to the production of secondary metabolites. Its effect on secondary metabolites production was studied at different temperatures ranging

from °C: 15, 20, 25, 30, 35, 40, 45 and 50. The fermentation was carried out and the antibiotic produced was assayed.

8.2.8 Effect of pH

The optimum initial pH of the medium is one the factors effecting growth, product formation of microorganisms and the character of their metabolism. The H^+ or OH^- ion concentrations may have a direct effort on the cell or it may act indirectly. The effect of initial pH of the medium was studied with pH values 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. The fermentation was carried out and the antibiotic produced was assayed. The pH of the medium was adjusted with 1M HCl and 1M NaOH by using a pH meter (Crison Basic 20+, Spain).

8.2.9 Effect of salt concentration

The effect of sodium chloride was studied at different concentrations (% w/v) such as: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0. The fermentation was carried out and the antibiotic produced was assayed.

8.2.10 Effect of inoculum size

In all our previous experiments, a 10% v/v level of the fresh culture was used as the inoculum. In the present experiment different inoculum volumes (% v/v) such as: 2.5, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 were used. The fermentation and evaluation of their antibacterial activities were carried out as per the general procedure.

8.2.11 Effect of fermentation period

With all the optimized parameters, the selected actinomycete isolates (10% v/v, 48 h inoculum) were subjected to fermentation with 500 ml flask containing 250 ml production medium. The fermentation was conducted at 30°C for 2 weeks and the samples (10 ml) were

drawn at regular intervals. The antibacterial activities were observed and the optimized fermentation period was determined.

8.2.12 Statistical analysis

Data obtained on the antimicrobial activity under different culture conditions were statistically analyzed and expressed as mean ± standard error with one-way analysis of variance (ANOVA) using IBM SPSS statistics 22.

8.3 Results

8.3.1 Effects of carbon source

In this study the effect of carbon sources for the production of bioactive secondary metabolites by the selected antibiotic producing actinomycetes are presented in Figure 8.1a (I-VI). The selected isolates were able to grow in all the carbon sources tested. The result showed that glucose is the most effective carbon source, as all the three isolates used in this study exhibited the highest antimicrobial activity against the test organisms. The values of glucose were significantly different from that of other carbon sources. The zone of inhibition ranges between 10.00 mm and 35.33 mm, with the highest being with NWU49 against *K. pneumoniae* ATCC 8308.

Glucose concentration was optimized, since it was found to be the most suitable carbon source for antibiotic production. The result indicated that concentration of 1% w/v for NWU49 and 2.0% w/v for NWU14 and NWU91 of glucose into the fermentation medium was found to be the optimum concentration for antibiotic production (Fig. 8.1b I-VI). The optimal concentration of glucose in the medium is statistically proven to have a significant effect on antibiotic production. Further increase of glucose above the optimal concentration in the fermentation medium results in decrease in antimicrobial activity of the isolates.

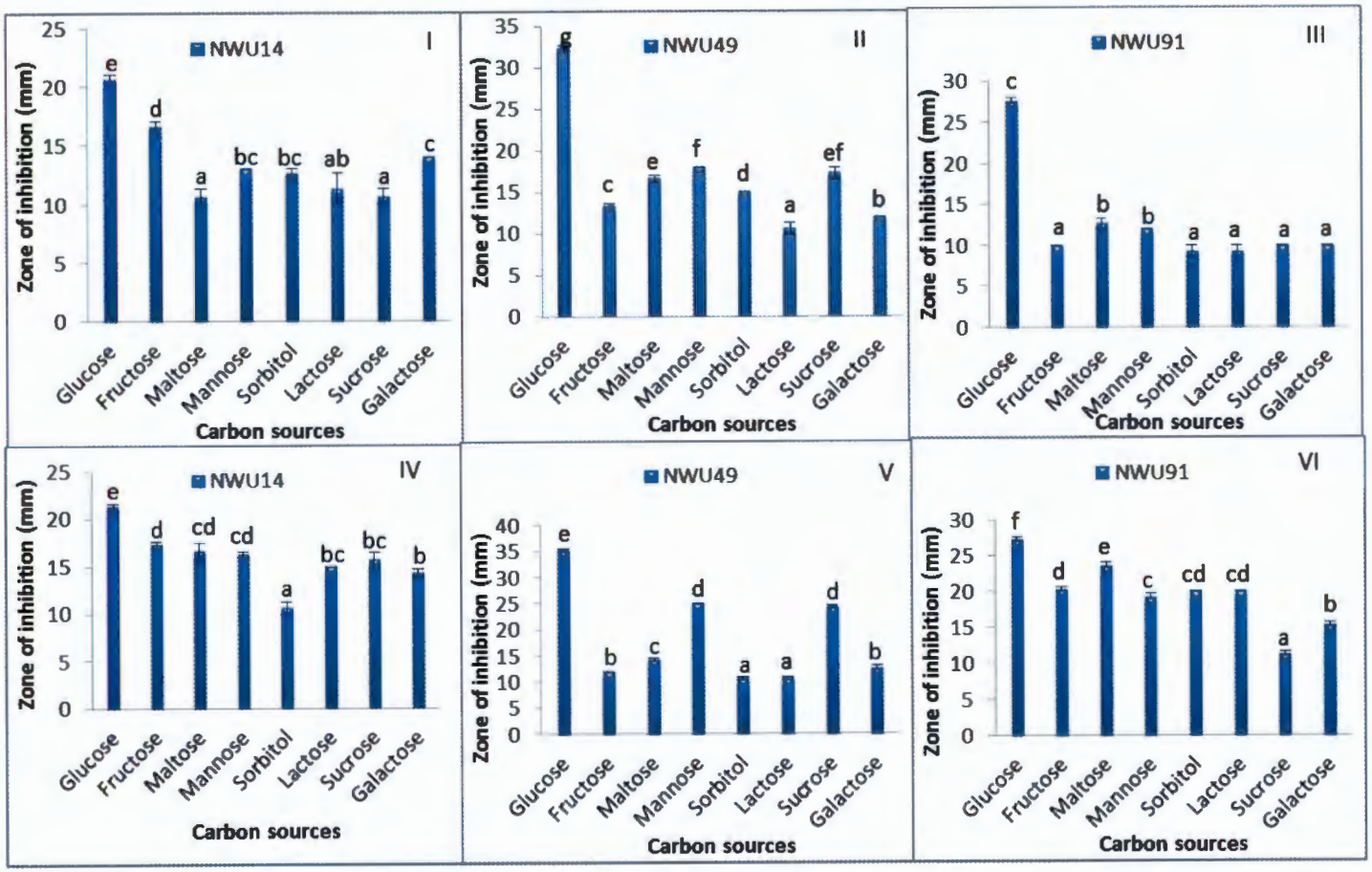

Figure 8.1a: Effects of different carbon sources on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

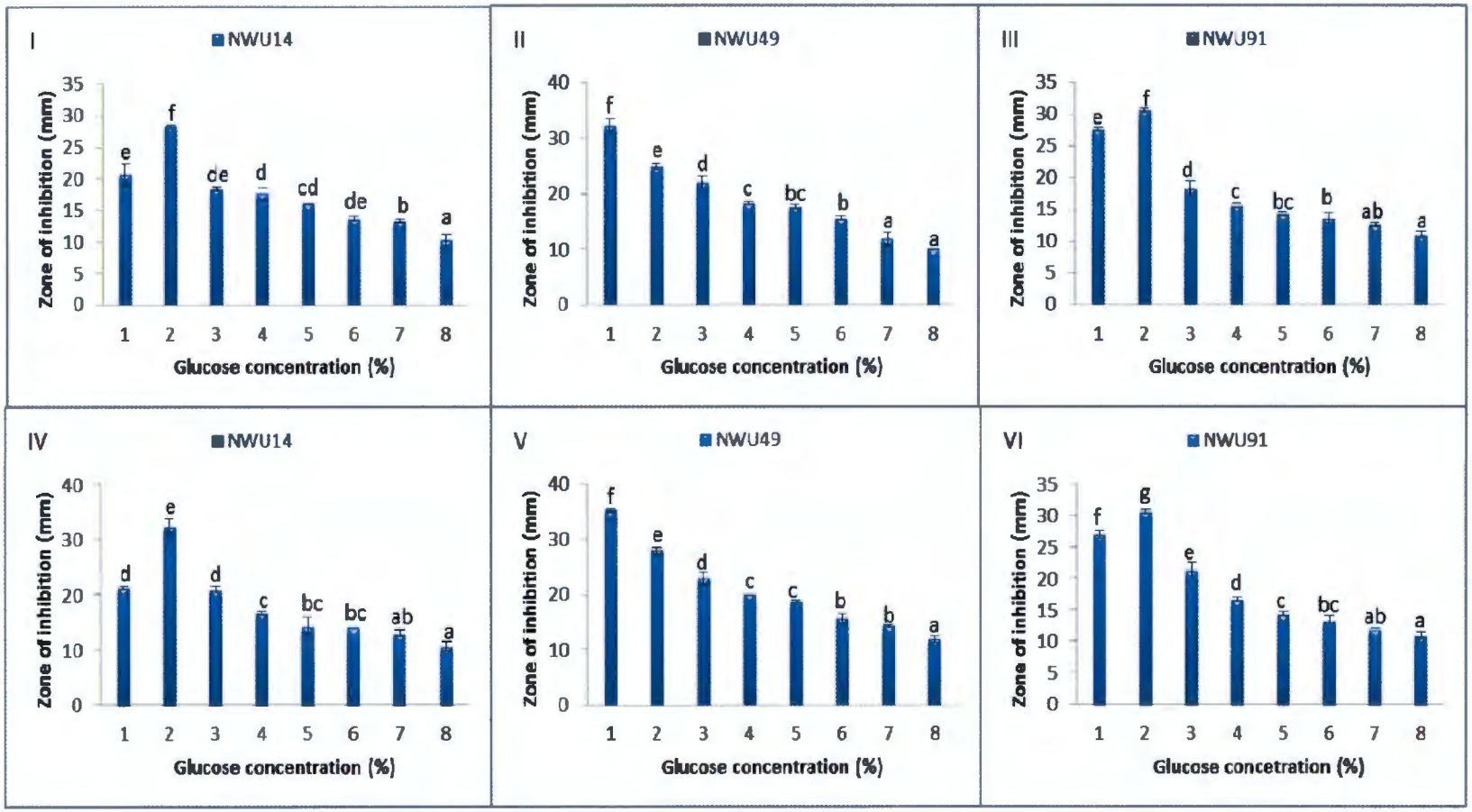

Figure 8.1b: Optimum concentration of glucose for the production of bioactive secondary metabolites by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506, and (IV-VI) against *K. pneumoniae* ATCC 8308.

8.3.2 Effect of nitrogen sources

The effect of nitrogen sources on antibiotic production is shown in Figure 8.2a (I-VI). The selected isolates were able to grow in all the 9 nitrogen sources tested with bioactive secondary metabolites production. It was statistically significant that oatmeal was found to be the best nitrogen source for NWU14 and NWU91 while yeast extract was the best for NWU49. The zone of inhibition ranges between 10.00 mm and 32.66 mm against the test organisms, with the highest value exhibited by NWU49 against *E. faecalis* ATCC 14506.

For optimal production of the bioactive secondary metabolites, the results statistically analyzed showed the highest antibacterial activity at 4% w/v concentration of the oatmeal for NWU14 and NWU91 with little significant difference from other concentrations. One percentage (1%) w/v concentration of the yeast extract was the optimal concentration for NWU49 (Fig. 8.2b I-VI), also with little significant difference. Further increase in concentrations resulted in reduction of the antibacterial activity.

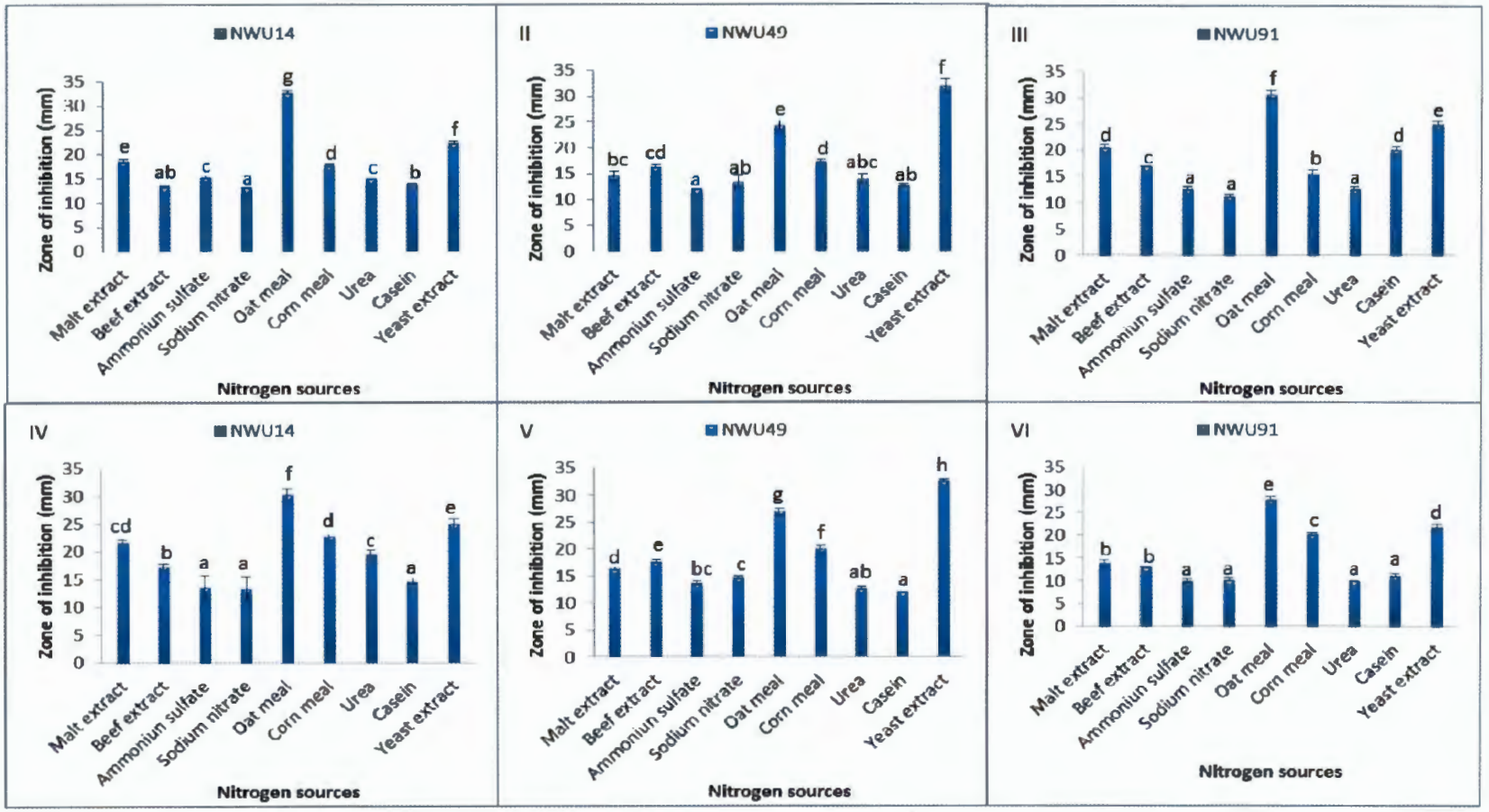

Figure 8.2a: Effects of different carbon sources on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

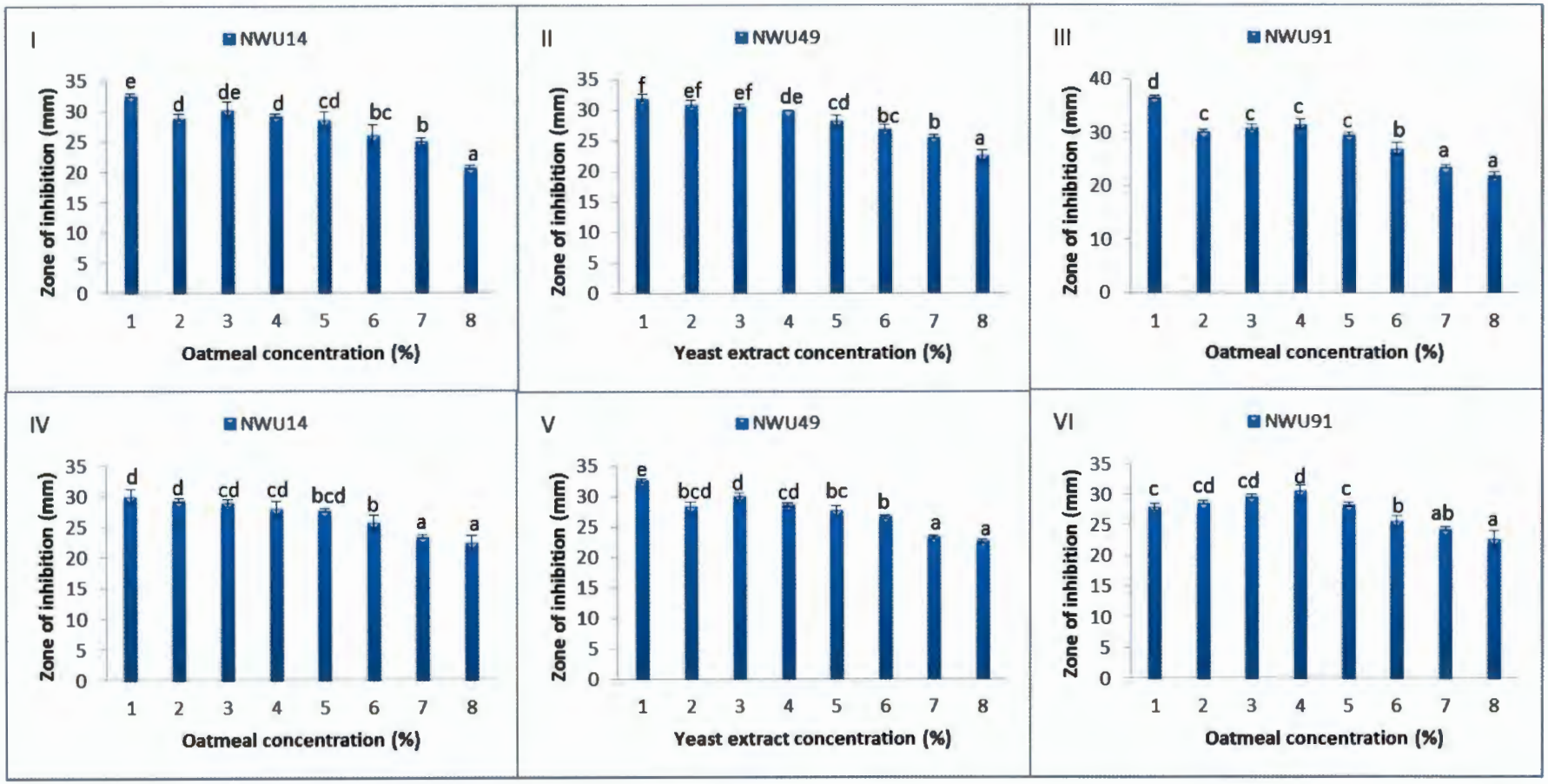

Figure 8.2b: Optimum concentration of oatmeal for NWU14 and NWU91, and yeast extract for NWU49 for the production of bioactive secondary metabolites (I-III) against *E. faecalis* ATCC 14506, and (IV-VI) against *K. pneumoniae* ATCC 8308..

8.3 Effect of temperature

The effect of temperature on the biosynthesis of antimicrobial agents by the antibiotic producing isolates of actinomycetes was studied in detail (Fig 8.3 I-VI). The isolates showed optimal antibiotic production at 30°C, which is significantly different from other values. Temperature above 30°C tends to have an adverse effect on antibiotic production. No antibacterial activity was observed at temperature 15°C and 50°C against *E. faecalis* ATCC 14506, although there was activity at those temperatures against *K. pneumoniae* ATCC 8308. The highest antimicrobial activity observed was 35.66 mm against *E. faecalis* ATCC 14506 exhibited by NWU49 at 30°C.

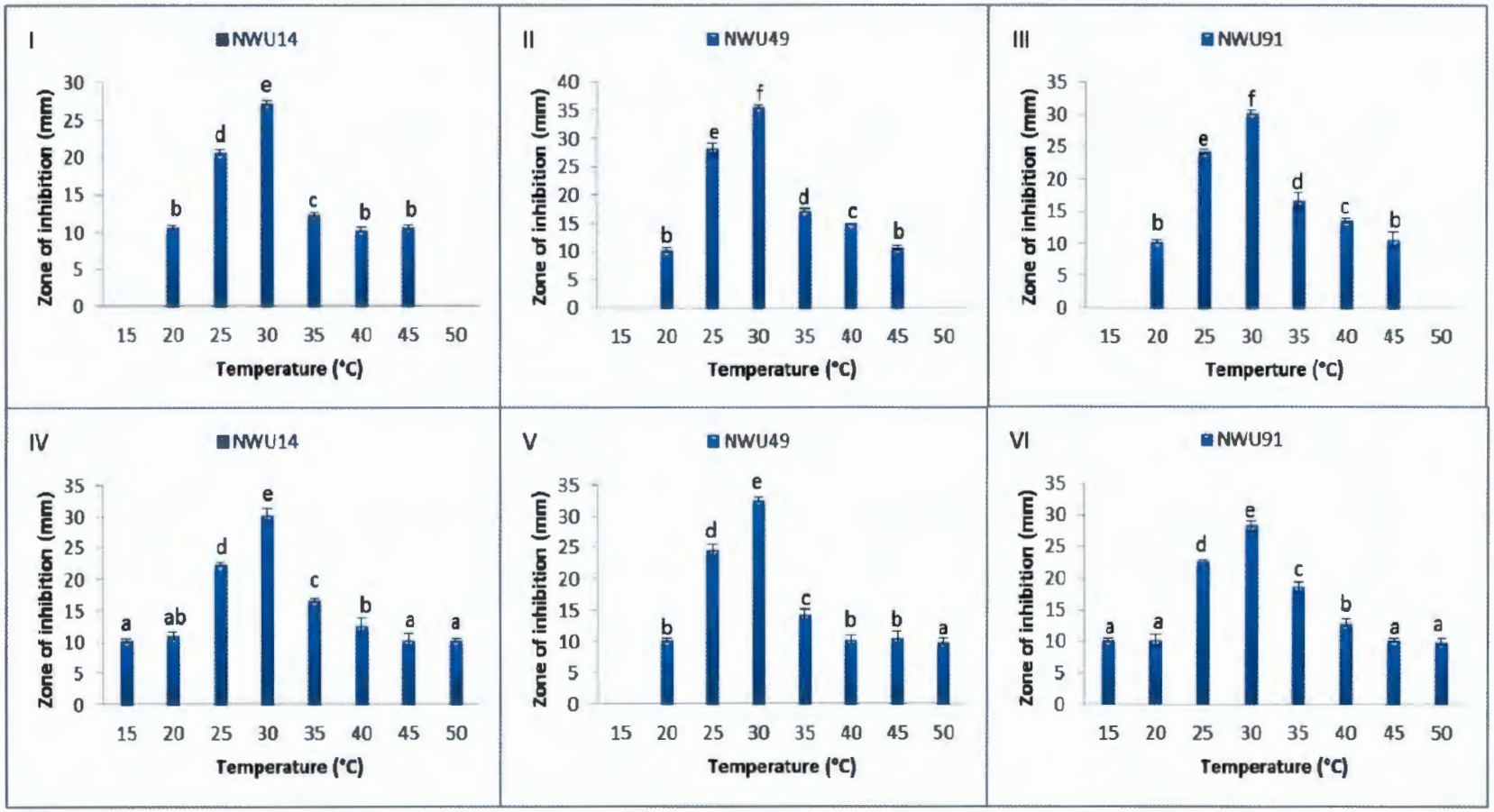

Figure 8.3: Effects of temperarure on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

8.4 Effect of pH

The effect of pH on antibiotic production by the selected isolates of actinomycetes is presented in Figure 8.4 (I-VI). It is clear from the results that the optimal pH for the production of bioactive secondary metabolites by the selected isolates of actinomycetes was at pH 7.0. Towards the extreme acidic and alkaline conditions antibiotic production was greatly affected with its production being negligible. Increase and decrease in the pH beyond 7 resulted in decrease in the antibacterial activity. The highest antibacterial activity was exhibited by strain NWU49 against *K. pneumoniae* ATCC 8308 with a zone of inhibition of 32.33 mm at pH 7.0.

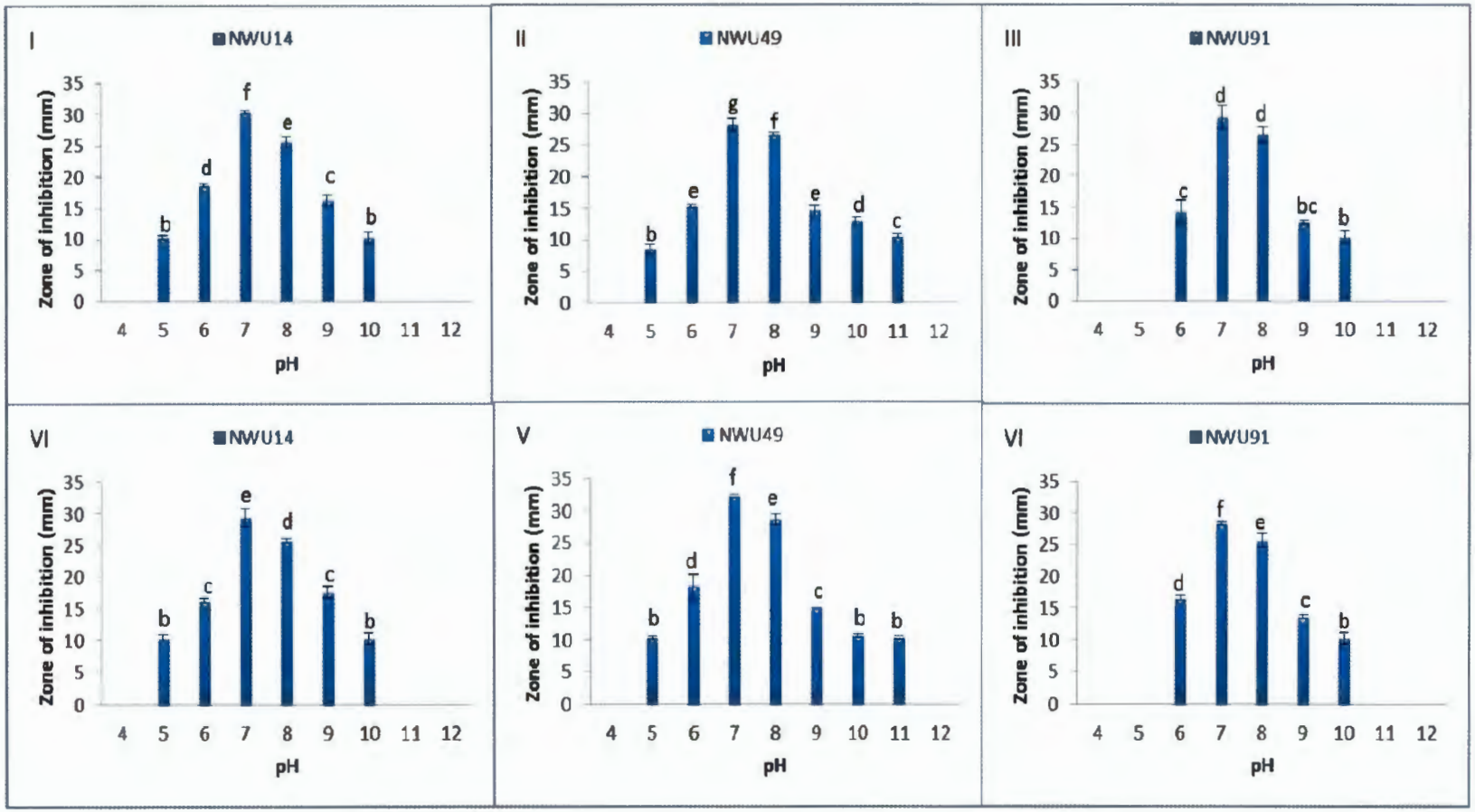

Figure 8.4: Effects of pH on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

8.5 Effect of salt concentration

The statistical analysis showed that production of antibiotic was at its maximum in the presence of 2% NaCl (Fig. 8.5 I-VI) and further increase in the salt concentration gradually reduced antibacterial activity. The highest antibacterial activity was 30.66 mm exhibited by NWU49 against *E. faecalis* ATCC 14506.

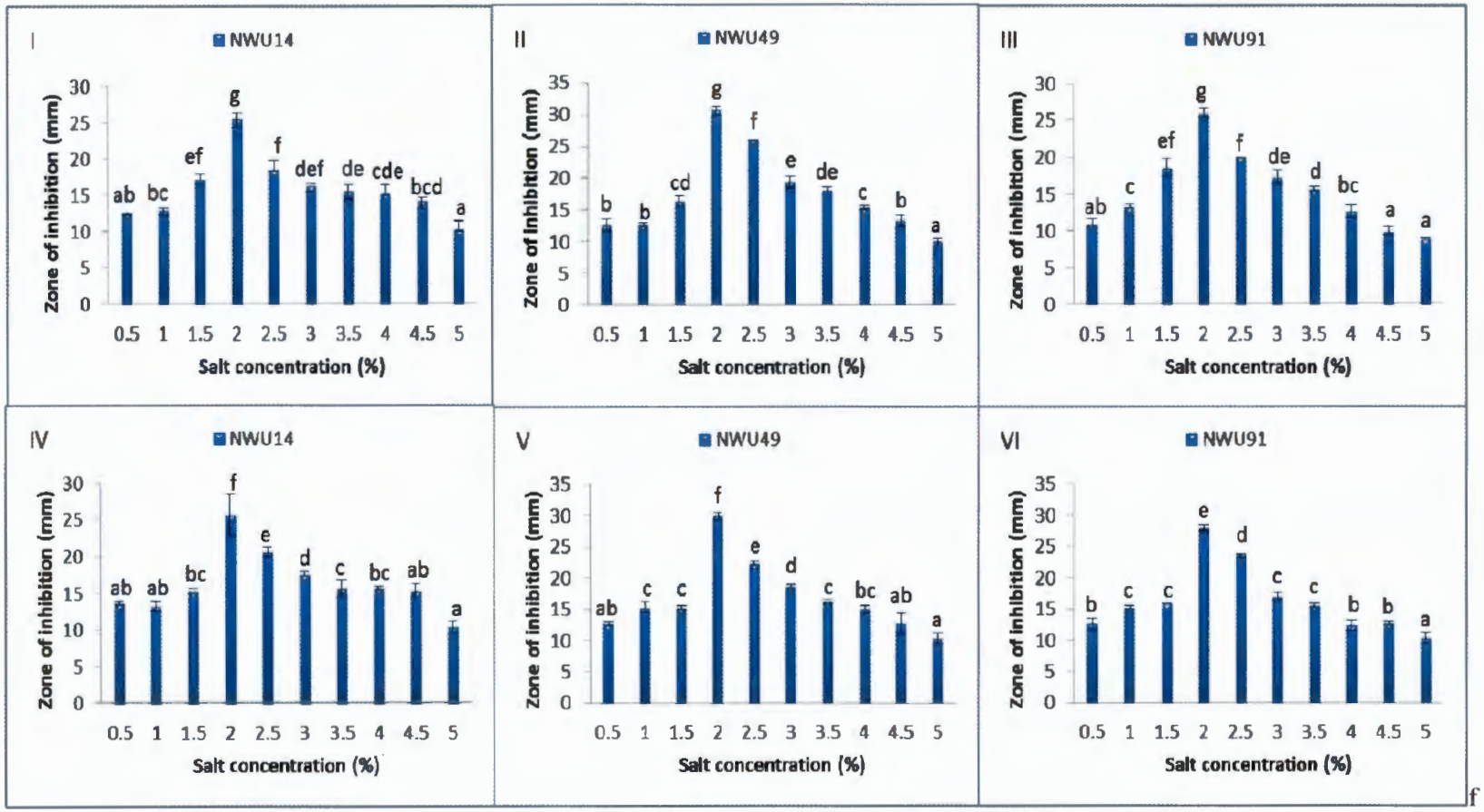

Figure 8.5: Effects of salt concentration on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

8.6 Effect of inoculum size

The results indicate that the inoculum size had a significant effect on the antibiotic production (Fig. 8.6 I-VI). The optimum inoculum for antibiotic production was found to be 10% v/v; and further increases in the inoculum size resulted in decreased production of the antimicrobial metabolites. The highest antibacterial activity was 32.00 mm exhibited by NWU14 against *K. pneumoniae* ATCC 8308.

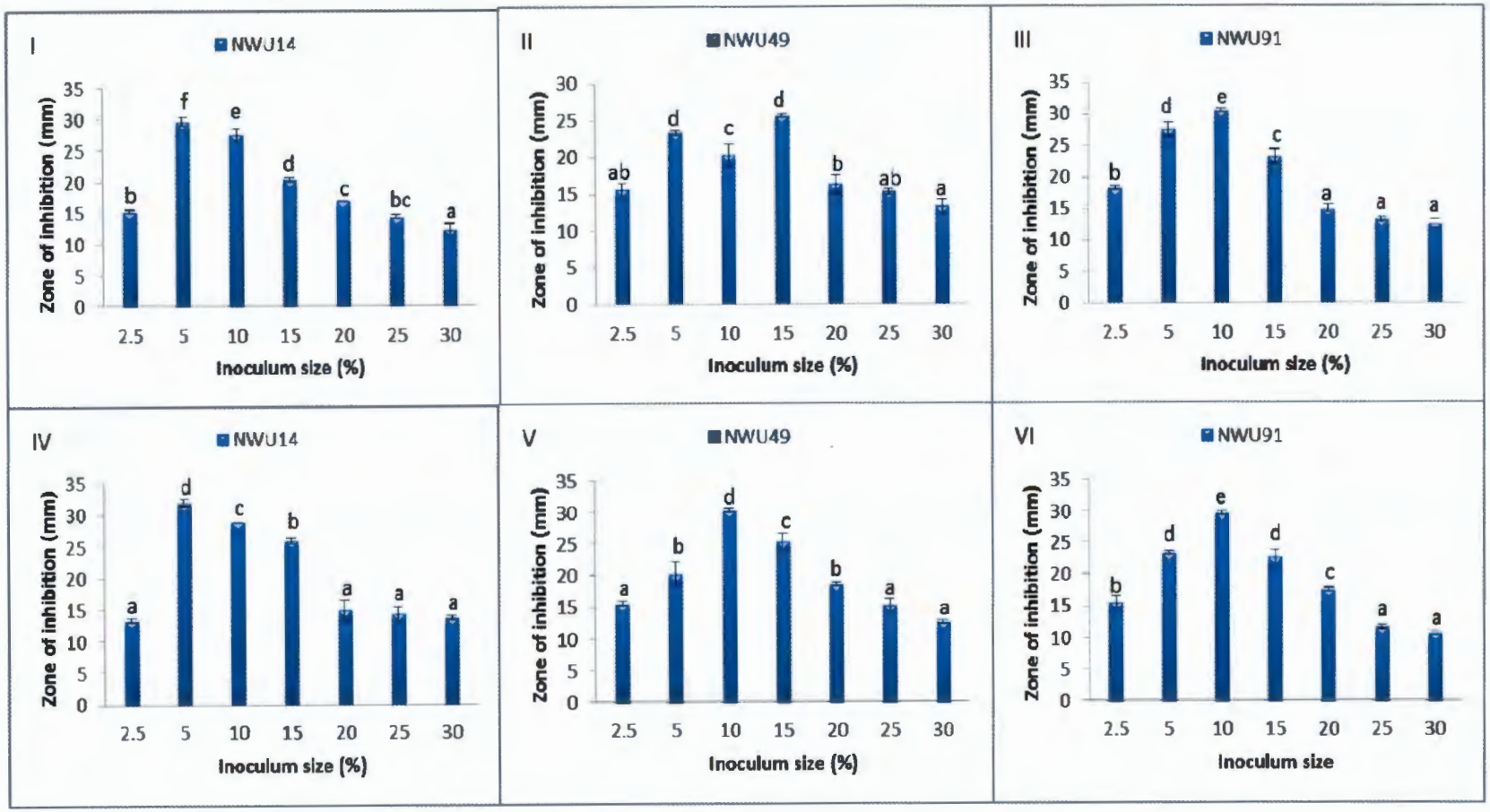

Figure 8.6: Effects of inoculum size on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

8.7 Effect of fermentation period

The duration of fermentation is an important factor affecting the production of antibiotic. In Figure 8.7 (I-VI), the fermentation period had a positive effect on the antibiotic production up to day 10 and then it decreased. The antibiotic production was at its optimum on day 10, the highest antibacterial activity was 45.33 mm exhibited by NWU49 against *E. faecalis* ATCC 14506. Statistically there is a significant difference between the 10^{th} day and the other days during the fermentation period.

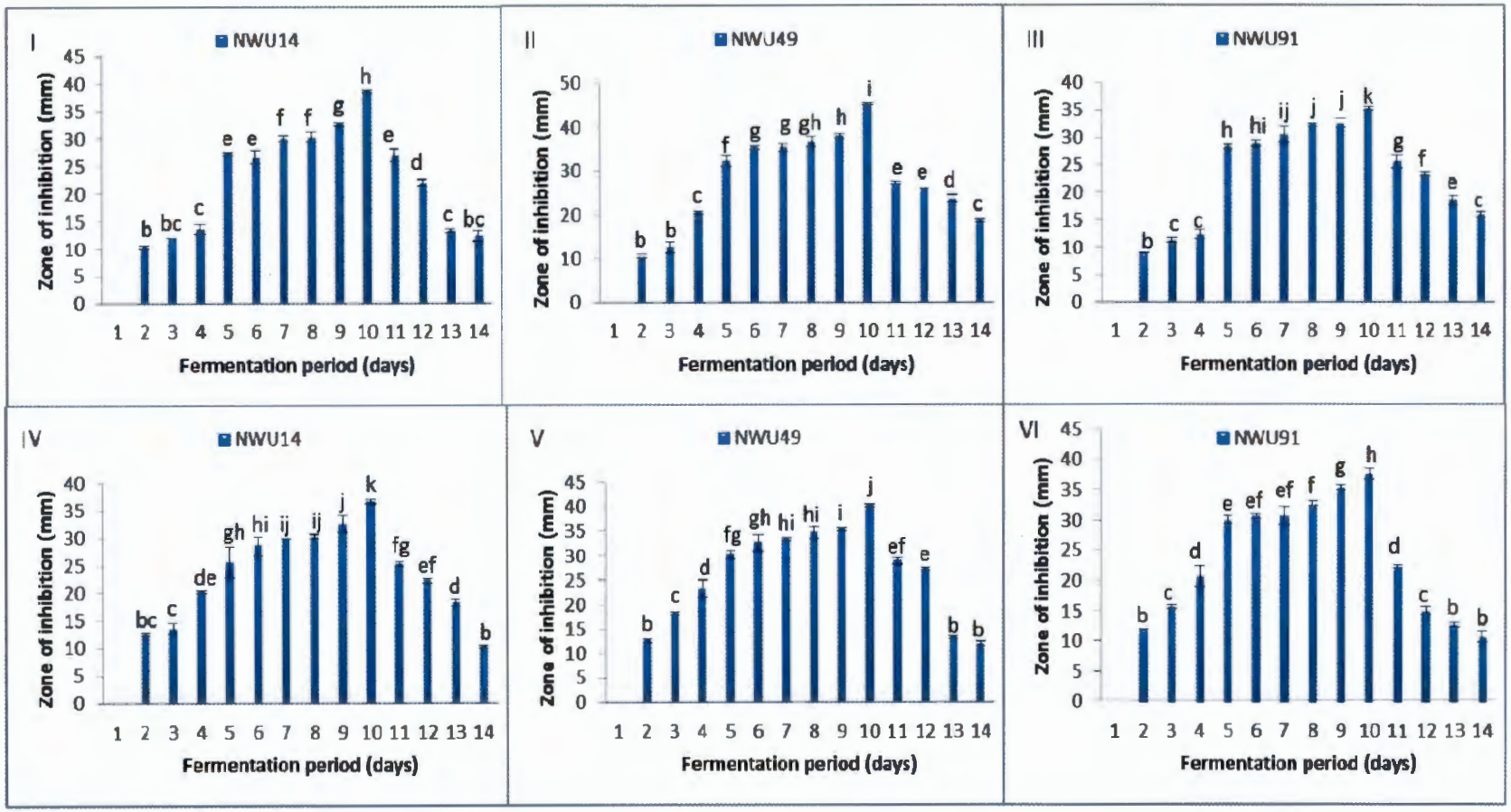

Figure 8.7: Effects of fermentation period on bioactive secondary metabolites production by selected antibiotic producing actinomycetes (I-III) against *E. faecalis* ATCC 14506 and (IV-VI) against *K. pneumoniae* ATCC 8308.

8.4 Discussion

The fermentation in actinomycetes is a complex process. It is not only dependent upon the performance of the fermentation medium, but inevitably requires suitable environmental conditions which include temperature, pH, inoculum size and fermentation period besides others. These factors are known to affect the production of bioactive secondary metabolites (da Silva et al., 2012). Thus, the development of efficient fermentation medium and conditions for the production of bioactive secondary metabolites is fundamental.

Nutrition plays a vital role in the onset and intensity of both primary and secondary metabolism. Among various other nutrients, carbon and nitrogen sources are vital parts of fermentation medium and they play a crucial role as structural and energy compounds in cells (da Silva et al., 2012). Therefore, in order to determine the optimal fermentation medium for the production of various bioactive secondary metabolites, different sources of carbon and nitrogen were tested. The production of antibiotics varies significantly in response to different carbon sources used in the fermentation medium. Among different carbon sources, the results showed that antibacterial activity was higher in a fermentation medium having glucose as carbon source compared to other carbon sources. Glucose has been found to be a suitable carbon source for the production of antibiotics by *Streptomyces* spp (Saurav and Kannabiran, 2010, Ravi and Dasari, 2011). The results showed the supplementation of glucose in the fermentation medium at different concentration, 1.0% w/v for NWU49, and 2.0% w/v for NWU14 and NWU91 were found to be optimal for antibiotic production by the isolates respectively. Within *Streptomyces* species with regards to carbon sources, species specific variations have been reported to occur for optimum secondary metabolites production (Jonsbu et al., 2002). Any variation in the concentration of the glucose on either side of the optimum concentration showed reduced antibacterial activity. Similar results have

been reported in different studies with 2.0% w/v glucose being the optimum concentration for maximum bioactive secondary metabolites production by *Streptomyces* spp (Ripa et al., 2009). High concentration of glucose is known to be a repressor of secondary metabolism and maximum cell growth rates can inhibit antimicrobial agents production (Demain, 1989). A catabolic repression of secondary metabolism produced by *Streptomyces* spp during the biosynthesis of bioactive compounds was reported after the increase of glucose in the fermentation medium (Gandhimathi et al., 2008).

Both the organic and inorganic nitrogen sources produced bioactive secondary metabolites, but organic nitrogen sources tend to exhibit more antibacterial activity than the inorganic sources. The supplementation of the oatmeal to the fermentation medium resulted in maximum antibacterial activity by NWU14 and NWU91 and yeast extract for NWU49. This is in tune with the earlier reports wherein oatmeal has been recognised as the best nitrogen source for the production of antibacterial metabolite by the actinomycetes (Selvin et al., 2009). The production of antibiotics in a nitrogen source supplemented medium depends on and varies with the requirement of nitrogen source by the organism for its primary and secondary metabolisms. Furthermore the varying concentration of the optimal nitrogen sources had a significant effect on the production of antibiotics produced by the isolates. The results showed that antibacterial activity was at its highest at 1% and 4% w/v concentration of the oat meal for NWU91 and NWU14 respectively while 1% w/v of yeast extract for NWU49. It was observed that further increase in concentrations decreased the antibacterial activity.

The temperature for the biosynthesis of secondary metabolites has been found to vary depending on the species of isolates (da Silva et al., 2012). In the current study also the temperature significantly affected the antimicrobial activity. However, maximum antibacterial activity for the 3 selected isolates was observed at 30°C which is within the

mesophilic range. At high temperatures less antibacterial activity was observed, this might be due to degradation of essential constituents are required for the production of antibiotics in fermentation medium at high temperature or the isolates might be rendered inactive at high temperature.

The pH of the fermentation medium is an important factor which affects the growth and bioactive secondary metabolites production. It has been reported that hydrogen or hydroxyl ion concentration may have a direct effect on cells or it may act indirectly by varying the degree of dissociation of substances that make-up the fermentation (Ravi and Dasari, 2011). The effect of pH of the fermentation medium on antibacterial activity was found to be an important parameter; differences in antibacterial activity were observed when the pH of the fermentation medium was varied. The optimum pH for the production of bioactive secondary metabolites by the isolates was pH 7, which may relate to the pH of the soil samples collected. The production of retamycin by *S. olindensis* was at its highest at pH 7.0 and other values were obtained at different pH (Guimarães et al., 2004). The importance of pH for antibacterial production by *Streptomyces* spp was reported by several investigators who observed that the optimum pH for antibiotic production ranges between 7.0 and 7.6 (Wang et al., 2010, Osman et al., 2011).

With regard to NaCl concentration, 2% was found to be optimum for production of the antimicrobial agent while concentration above the optimum was found to decrease the bioactive secondary metabolite production. Singh et al. (2009) reported that *S. tanashiensis* strain A2D isolated from soil of phoomdi in Loktak Lake of Manipur, India exhibited optimum growth at 2% (w/v) NaCl.

The inoculum size was important for bioactive secondary metabolite production. The results indicated that the variation of the inoculum size had a significant effect on antibacterial activity. For NWU14, 5.0% v/v inoculum volume was found to be the optimum

inoculum size for maximum antibacterial activity while for NWU49 and NWU91 it was 10.0% v/v. It was important to provide an optimum inoculum size in fermentation process, as a lower inoculum size may yield insufficient biomass resulting in low product formation, whereas a higher inoculum may produce too much biomass leading to poor product formation (Ravi and Dasari, 2011). And also as the concentration of inoculum increases, it is followed by an increase in biomass and after a certain period, metabolic waste interferes with the production of bioactive secondary metabolites due to which degradation of the product occurs.

The results showed a gradual increase in antibacterial activity with increase in the fermentation period from day 2 to day 10, while a further increase in fermentation period resulted in a steady decrease in antibacterial activity. The reduction in antibacterial activity after an optimum fermentation period might be due to reduced growth rate as a result of depletion of nutrients to the organism. As nutrients are depleted and waste products accumulate, the growth rate of the cells deviates from the maximum and ultimately growth ceases as the culture enters the stationary phase. After a period of time, the culture enters the death phase and the numbers of viable cells decrease resulting in the decrease in antibiotic production. A similar result was reported by (Ripa et al., 2009) wherein they reported 10 days as the optimum incubation time for the production of antibacterial metabolite.

8.5 Conclusion

According to the results, the actinomycete isolates showed great potential for the production of antibacterial agents. The optimization of the different parameters in the fermentation medium and conditions directly influenced the production of antibacterial agent by the isolates used in this study. The three selected isolates studied behaved differently, and exhibit great potential for the production of antibacterial agents that can inhibit clinical

pathogens. The optimization of the fermentation process is a vital step towards high antimicrobial activity that may lead to a high yield of the bioactive secondary metabolites and eventually in their identification. The results obtained in this study will be useful to develop better cultivation processes of the strains for efficient production of bioactive secondary metabolites on a large scale.

8.6 Acknowledgements

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CHAPTER 9

Isolation and Identification of Bioactive Compounds Produced by *Streptomyces* spp

Summary

This study investigated the bioactive secondary metabolites of actinomycete isolates in order to find novel compounds or effective components. Three *Streptomyces* isolates exhibiting broad spectrum antimicrobial activity against pathogenic organisms in previous studies were investigated by fermentation and by subsequent isolation, purification and compound identification of the bioactive secondary metabolites using Gas chromatography-mass spectrometry (GC-MS). Among the bioactive compounds identified were furosemide, phellopterin, 4,5-dihydroxyanthraquinone-2-carboxylic acid and milbemycin B. The compound 4,5-dihydroxyanthraquinone-2-carboxylic acid produced by NWU91 was not reported earlier from actinomycete. These results highlight the importance of *Streptomyces* isolates in bioactive compounds production.

KEYWORDS: *Streptomyces* spp; bioactive compounds; antimicrobial activity; GC-MS

9.1 Introduction

The order Actinomycetales is made up Gram-positive filamentous bacteria that naturally inhabit soils. They are of great economical and industrial importance due to their ability to biosynthesize a vast number of bioactive secondary metabolites. The search for antibiotics of microbial origin is mainly based on the isolation of actinomycetes, from diverse sources and different strains (Chaudhary et al., 2013). Actinomycetes are the major producers of pharmaceuticals, pesticides and insecticides (Baltz, 2007, El-Khawagh et al., 2011). The biosynthesis of bioactive secondary metabolites by actinomycetes varies qualitatively and

quantitatively depending on the strains and species used as well as on their nutritional and cultural conditions (Knight et al., 2003, Zhang, 2005).

Among the actinomycetes, members of the genus *Streptomyces* are prolific producers of bioactive compounds, and have continued to provide a larger number and wider variety of new antibiotics than any other genus, suggesting that a substantial number of *Streptomyces* species or strains with novel antibiotic productivity exist in nature. *Streptomyces* spp produce about 80% of clinically important drugs in clinical use (Berdy, 2005). The search for novel bioactive compounds is a continuous process since the development of resistance to antibiotics is a major problem in the treatment of infectious diseases caused by pathogens (Adegboye et al., 2012).

In this study the partially purified fractions of the crude extracts of the *Streptomyces* isolates showed significant antimicrobial activity against test organisms. The isolation, purification, and structural identification of the bioactive compounds present in the extracts were reported.

9.2 Materials and Methods

9.2.1 Cultivation of the isolates

Each isolate of the selected actinomycetes (NWU14, NWU49 and NWU91) isolated from rhizospheric soil samples collected from Ngaka Modiri Molema district of North West Province, South Africa (Adegboye and Babalola, 2013). The isolates were maintained on Luria Bertani agar medium (Merck). Each actively growing pure culture of the isolates was used to inoculate 100 ml of Luria Bertani broth (Merck) in a 250-ml Erlenmeyer flask. After 48 h incubation at 30°C, the LB broth (10%) was used to seed culture in three 500-ml Erlenmeyer flasks each containing 100 ml of fermentation medium (glucose, oatmeal, yeast

extract, NaCl, $CaCO_3$ pH 7.0) The cultures were incubated at 30°C for 10 days under constant agitation of 220 rpm.

9.2.2 Extraction and partial purification of the crude extracts from the actinomycete isolates

The fermentation medium was centrifuged for 20 min at 8000× *g* to remove the mycelium. The supernatant was shared in 4 equal volumes of 60 ml, and then each was extracted with 60 ml of organic solvent. A range of extraction solvents was screened for effectiveness, including petroleum ether, *n*-hexane, chloroform, diethyl ether, ethyl acetate, benzene and *n*-butanol. The organic extracts were evaporated to dryness using a Rotary evaporator (Stuart RE300) and lyophilized using a BenchTop Pro 8L ZL-105 lyophilizer (Sp Scientific, USA). The resulting dry extracts were recuperated in 1 ml of methanol and subjected to biological assay against test organisms. The solvents which gave the highest inhibition diameter were then retained for the detection of antibiotics by bioautography.

9.2.3 Biological assay

Minimum inhibitory concentrations (MIC) of the partially purified fractions obtained from the crude extracts of the isolates were tested against the test organisms by agar-well diffusion method (Cappuccino and Sherman, 2011) using Mueller Hinton agar (Sigma Aldrich). Different concentrations of compounds (0 to 1000 µg/ml) were prepared in dimethyl sulphoxide (DMSO) and assayed against the test organisms. The organisms used in this assay are *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* ATCC 12344, *Campylobacter coli* ATCC 43478, *Bacillus subtilis* ATCC 11774, *B. cereus* ATCC 11778, *Proteus mirabilis* ATCC 49132, *Enterococcus faecalis* ATCC 14506, *Shigella boydii* ATCC 9207, *Klebsiella pneumoniae* ATCC 8308, *Pseudomonas aeruginosa* ATCC 10145 and *Salmonella typhimurium* ATCC 14208 which were obtained from Davies Diagnostics (Pty) Ltd, South Africa. The bacteria used for the tests were resistant to at least one known

antimicrobial agent (Adegboye and Babalola, 2013). Inhibition zones were measured after 24 h incubation at 37°C. This experiment was carried out in triplicate and negative controls were maintained as DMSO without compounds. Positive controls were tested with tetracycline. The lowest concentration of compounds that showed antibacterial activity in terms of growth inhibition against test organisms was recorded as MIC value.

9.2.4 Gas chromatography-mass spectrometry (GC-MS)

The partially purified active fractions were analysed by GC-MS. The analyses of the compounds in the active fractions were run on a GC-MS system (Agilent GC: 6890, with a 7683B Autosampler). The fused-silica Rxi-5Sil MS capillary column (30 m 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to a LECO Pegasus 4D MS. Oven temperature was programmed (35°C for 5 min, then 35-300°C at 10 °C/min) and subsequently, held isothermal for 20 min. The injector port; was 250°C, the transferline: 290°C, splitless. Volume injected: 0.2 ml and the column flow rate was 1 ml/min of 1 mg/ml solution (diluted in chloroform). The peaks of components in gas chromatography were subjected to mass-spectral analysis.

9.2.5 Mass spectrometer

The MS was a LECO Pegasus 4D recording with a EI-source at -70 eV; the solvent delay was 9 min. scan time 1.5 s; acquisition rate 10 spectra/second mass Range 50-1000 amu, detector voltage 1800 V, and Ion source temperature: 250 C. Data were recorded in TIC mode. The software adopted to handle the mass spectra and chromatograms was a LECO ChromaTOF optimized for Pegasus 4D V 3.34.

9.3 Results and discussion

9.3.1 Antibacterial activity

Chemical analysis of the bioactive secondary metabolites obtained from 10 days fermentation medium led to the isolation of 6 fractions that exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria after partitioning with different organic solvents (Table 9.1). The *in-vitro* antibacterial activity of the fractions against microorganisms employed was assessed by the presence or absence of inhibition zones diameters and MIC values. The results obtained showed that the compounds exhibited a broad spectrum of activity against the test organisms. They however generally exhibited better activity with the Gram-positive bacteria than the Gram-negative. From previous studies it is suggested that this is due to the presence of the cell wall which inhibits the permeability of hydrophobic compounds through the lipopolysaccharide cell wall of Gram-negative bacteria contrary to its Gram-positive counterpart (Salmi et al., 2008). In the case of Gram-positive bacteria however the absence of this cell wall allows for easy permeation of hydrophobic compounds within the cell of the bacteria. The results of both methods employed for the antibacterial activity are comparable (agar well-diffusion method and double dilution method for MIC). The MICs were between 7.8-1000 µg/ml (Table 9.2). MIC values of the active fractions ranged from 7.8-1000 µg/ml (acetyl acetate fraction of NWU14), 31.2-1000 µg/ml (acetyl acetate fraction of NWU49), 31.2-1000 µg/ml (n-hexane fraction of NWU91), 15.6-1000 µg/ml (benzene fraction of NWU91), 125-1000 µg/ml (petroleum ether fraction of NWU91), and 15.6-1000 µg/ml (acetyl acetate fraction of NWU91). Using the MIC values, there was a good comparism between the active fractions and the standard antibiotic (tetracycline). That of the active fractions ranged between 7.8-1000 µg/ml while that of the antibiotics was between 15.6-1000 µg/ml.

Table 9.1: Antibacterial activities of the active fractions against the test organisms

Test organisms	Zone of inhibition (mm)						
	1	**2**	**3**	**4**	**5**	**6**	**Tet**
Staph. aureus ATCC 29213	24	20	22	24	15	18	0
Strep. pyogenes ATCC 12344	30	22	12	18	12	10	18
Camp. coli ATCC 43478	18	12	18	18	14	12	16
B. subtilis ATCC 11774	14	16	22	20	18	22	18
B. cereus ATCC 11778	18	15	23	24	10	22	22
Pr. mirabilis ATCC 49132	12	16	16	18	10	14	20
Ent. faecalis ATCC 14506	18	19	18	14	0	16	16
Sh. boydii ATCC 9207	18	14	20	18	12	18	18
Kl. pneumoniae ATCC 8308	20	18	22	0	16	24	12
Ps. aeruginosa ATCC 10145	12	10	16	14	10	12	0
Salm. typhimurium ATCC 14208	14	10	16	18	10	10	0

1=NWU14 acetyl acetate fraction, **2**=NWU49 acetyl acetate fraction, **3**=NWU91 n-hexane fraction,

4=NWU91 benzene fraction, **5**=NWU91 petroleum ether fraction, **6**=NWU91 acetyl acetate fraction, **Tet**=Tetracycline

Table 9.2: Minimum inhibitory concentration (MIC) of the active fractions against test organisms

Test organisms	Zone of inhibition (mm)						
	1	**2**	**3**	**4**	**5**	**6**	**Tet**
Staph. aureus ATCC 29213	15.6	62.5	31.2	15.6	500	125	>1000
Strep. pyogenes ATCC 12344	7.8	31.2	1000	125	1000	1000	125
Camp. coli ATCC 43478	125	1000	125	125	500	1000	250
B. subtilis ATCC 11774	500	250	31.2	62.5	125	31.2	125
B. cereus ATCC 11778	125	125	31.2	15.6	1000	31.2	31.2
Pr. mirabilis ATCC 49132	500	250	250	125	1000	500	62.5
Ent. faecalis ATCC 14506	125	125	125	125	>1000	250	125
Sh. boydii ATCC 9207	125	500	62.5	500	1000	125	250
Kl. pneumoniae ATCC 8308	62.5	125	31.2	>1000	250	15.6	1000
Ps. aeruginosa ATCC 10145	1000	1000	250	500	1000	1000	>1000
Salm. typhimurium ATCC 14208	500	1000	250	1000	>1000	>1000	>1000

1=NWU14 acetyl acetate fraction, **2**=NWU49 acetyl acetate fraction, **3**=NWU91 n-hexane fraction, **4**=NWU91 benzene fraction, **5**=NWU91 petroleum ether fraction, **6**=NWU91 acetyl acetate fraction, **Tet**=Tetracycline

9.3.2 Chemical composition

The ethyl acetate, petroleum ether, benzene and n-hexane fractions of the crude extract from NWU91, ethyl acetate fractions of NWU49 and NWU14 were subjected to GC-MS analysis to establish the chemical components of these active fractions. The compounds obtained were classified based on the functional group of the parent compound and are shown in Table 9.3. Based on this criterion for classification fatty acids included fatty acids and their esters. The inorganic compounds consisted of compounds of boron (borates, boronic acid and borane), silicon (siloxane and silane) and phosphorus as phosphate and phosphonic acid derivatives. Aromatic components included the aromatic alcohols such as derivatives of phenol and the sulphur components sulfonic acid, sulphide, sulfoxide, solphone and octaatomic sulphur. Heterocyclic compounds also included the heterocyclic aromatic compounds. The results obtained indicated that although a great number of similar compounds were present in each fraction, some variations however existed for each. For example, all the fractions contained eicosane and hexadecanoic acid.

Specific compounds in the fractions for NWU91, for benzene are bergamotol, Z-α-trans, 2-(5-chloro-2-hydroxyphenyl)-6-iodo-4(3H)-quinazolinone, 4,5-dihydroxyanthraquinone-2-carboxylic acid, isoxazole and O,O',O"-tris(trimethylsilyl). The petroleum ether fraction consisted of specific compounds such as 3-carene, 4-acetyl- a mono terpene, 2-(7-hydroxymethyl-3,11-dimethyl-dodeca-2,6,10-trienyl)-[1,4] benzoquinone, 5,7,9(11)-androstatriene, 3-hydroxy-17-oxo- a steroid and noscapine. Compounds specific to the n-hexane fraction include milbemycin B, 5-demethoxy-5-one-6,28-anhydro-25-ethyl-4-methyl-13-chloro-oxime while that for the ethyl acetate fraction N-methyltaurine. In the case of 49 ethyl acetate they include topotecan, 3-phorbinepropanoic acid, 9-acetyl-14-ethyl-13,14-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-, 3,7,11,15-tetramethyl-2-hexadecenyl ester, mefloquine, D-xylofuranose, cyclic 1,2:3,5-bis(butylboronate,

furosemide, 2(1H)-isoquinolinecarboximidamide, 3,4-dihydro- and lycopene a polyene. For NWU14 ethyl acetate they include phellopterin, germacradienol, phenmetrazine, norandrostane, androstane, molybdenum, bis (N,N-diethyl methacrylamide)-dicarbonyl, 6-methoxy-1-(3-methoxybenzyl)-3,4-dihydroisoquinoline, 2-methyl-7-hydroxy-8-allyl-isoflavone, and tetraethyl-2,7,12,17-tetramethyl-21H,23H-porphine-3,8,13,18-tetrapropionate tin(IY) dichloride.

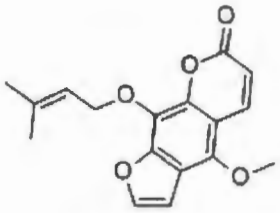
The major component of each fraction was the hydrocarbons with the n-hexane fraction of NWU91 the highest, 68.59% (Table 9.3). This was followed by the aromatic component with the petroleum ethyl fraction of NWU91 having the highest percentage. Generally the ethyl acetate fractions yielded more inorganic components unlike other fractions obtained from non-polar solvents. Some of the structures of the compounds are shown in Figure 9.1a-f. They also had comparatively similarly percentages for most of the compounds. The results obtained also showed that the ethyl acetate fraction of NWU49 exhibited an exceptional characteristic of having carbohydrate derivatives which was absent in all the other fractions.

A comparative analysis of the components of fractions for NWU91 indicated that the n-hexane, benzene and petroleum ether fractions exhibited more richness in the fatty acid than the ethyl acetate fraction. This result is in agreement with the chemistry of the solvents and the fact that "like dissolves like". Petroleum ether, benzene and n-hexane are non-polar solvents and as such have the tendency to dissolve less polar and non-polar compounds. This was corroborated by the result obtained for the hydrocarbon contents of the order n-hexane>benzene>petroleum ether>ethyl acetate (Table 9.3). Further supporting this theory is also the fact that the ethyl acetate fraction had the highest percentage for alcohol content.

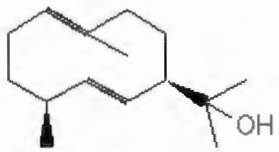
Table 9.3: Showing percentage composition of the chemical components obtained for the fractions.

Compound	1	2	3	4	5	6
Hydrocarbons	54.28	68.59	55.62	39.87	39.10	39.20
Alcohols	6.25	0.55	3.09	7.54	6.41	6.40
Aldehydes and ketone	7.42	2.21	4.21	9.91	8.76	12.20
Esters	3.02	2.21	9.83	-	8.76	8.00
Ethers	-	-	0.03	2.16	0.43	0.20
Inorganic compounds	0.46	4.97	-	5.61	5.35	1.20
Steroids	-	-	-	1.72	0.21	1.00
Sulphur	0.23	2.76	-	3.23	4.27	3.00
Heterocyclic	1.62	3.31	1.68	5.60	5.10	7.00
Aromatic	16.93	9.94	13.19	12.93	13.68	10.60
Fatty acids and esters	1.36	3.31	0.84	2.16	1.50	6.40
Macrocycles	0.23	1.11	-	0.65	0.21	0.20
Organic acid		-	-	0.22	0.85	0.2
Lactone		5.55	-	2.80	0.21	0.20
Amino acids		0.55		-	2.35	1.40
Selenium				0.43	-	-
Carbohydrate					0.85	-

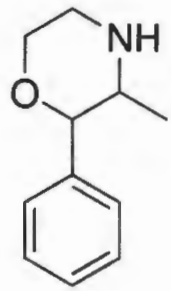
1=NWU91 Petroleum ether fraction, **2**=NWU91 n-hexane fraction, **3**=NWU91 benzene fraction, **4**=NWU91 ethyl acetate fraction, **5**=NWU49 ethyl acetate fraction, **6**=NWU14 ethyl acetate fraction



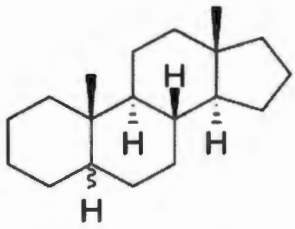
1 Phellopterin



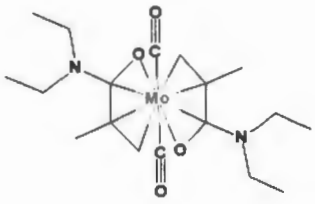
2 Gerrmacradienol



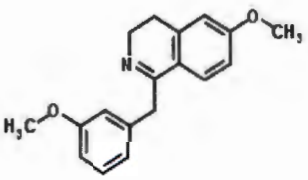
3 Phenmetrazine



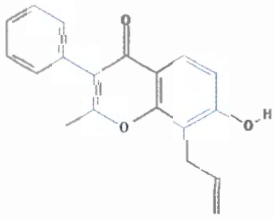
4 Androstane



5 Molybdenum, Bis (N,N-diethyl methacrylamide)-dicarbonyl

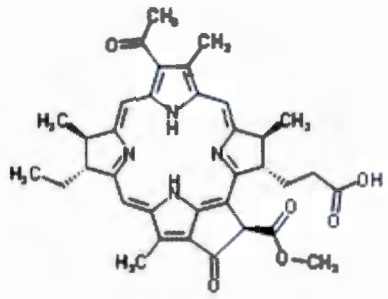


6 6-Methoxy-1-(3-methoxybenzyl)-3,4-dihydroisoquinoline

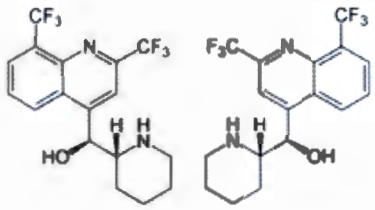


7 2-Methyl 7-hydroxy-8-allyl-isoflavone

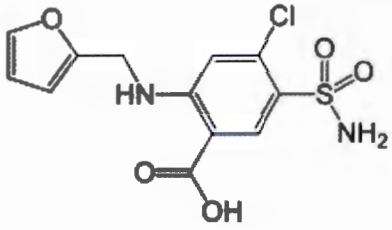
Figure 9.1: Structures of compounds from NWU14 ethyl acetate fraction



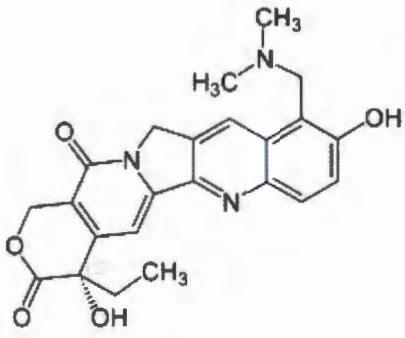
1 3-Phorbinepropanoic acid, 9-acetyl-14-ethyl-13,14-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl -20-oxo-, 3,7,11,15-tetramethyl-2-hexadecenyl ester



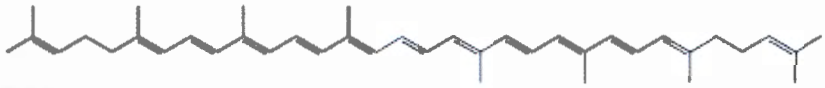
2 Mefloquine



3 Furosemide

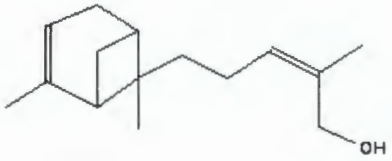


4 Topotecan

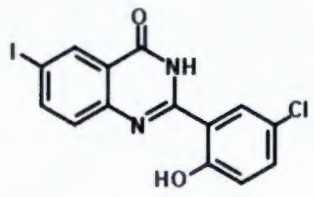


5 Lycopene

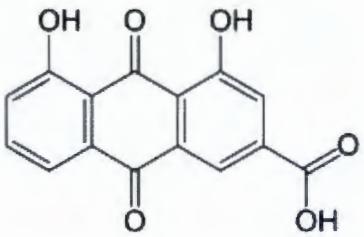
Figure 9.2: Structures of compounds from NWU49 ethyl acetate fraction



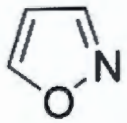
1 Bergamotol, Z-α-trans-



2 2-(5-Chloro-2-hydroxyphenyl-6-iodo-4(3H)-quinazolinone

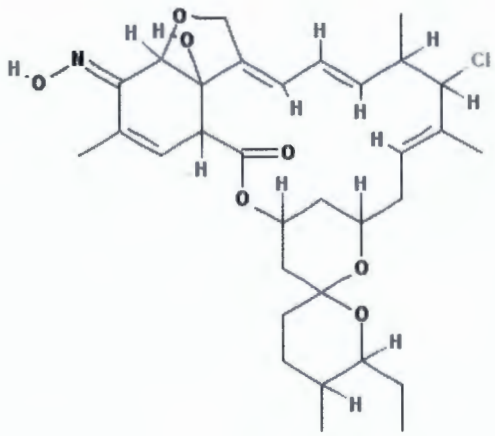


3 4,5-Dihydroxyanthraquinone-2-carboxylic acid



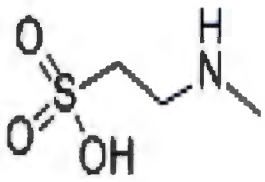
4 Isoxazole

Figure 9.3: Structures of compounds from NWU91 benzene fraction



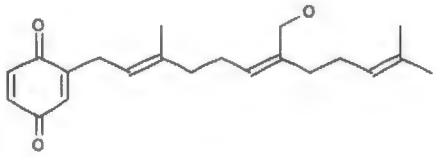
1 Milbemycin B, 5-demethoxy-5-one-6,28-anhydro-25-ethyl-4-methyl-13-chloro-oxime

Figure 9.4: Structure of compound from NWU91 n-hexane fraction

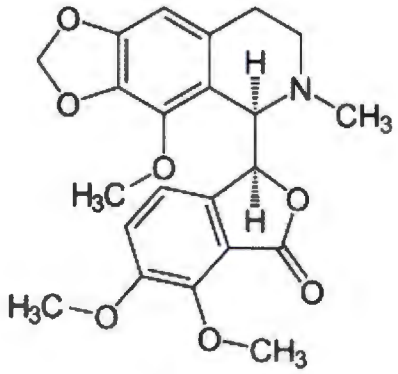


1 N-Methyltaurine

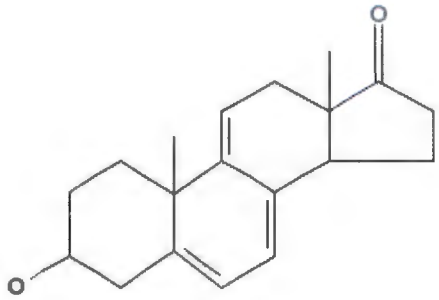
Figure 9.5: Structure of compound from NWU91 ethyl acetate fraction



1 2-(7-Hydroxymethyl-3,11-dimethyl-dodeca -2,6,10-trienyl)-[1,4]benzoquinone



2 Noscapine



3 Androstatriene

Figure 9.6: Structures of compounds from NWU91 petroleum ether fraction

9.3.3 Biological activity of the active compounds

In this study the activity of the individual fractions relative to the others may be ascribed to the effect of the compounds found specifically in each fraction. Therefore in a bid towards gaining insight into the individual antibacterial activity of the fractions, their potential activity was studied in relation to their chemical composition.

The 14 ethyl acetate fraction exhibited peculiar compounds that included 2-methyl-7-hydroxy-8-allyl-isoflavone, methoxy-1-(3-methoxybenzyl)-3,4-dihydroisoquinoline phellopterin and molybdenum, bis(N,N-diethylmethacrylamide)-dicarbony and are suggested to be a plausible reason for its peculiar antimicrobial activity in relation to the other fractions (Dastidar et al., 2004). Phellopterin also found in this fraction has been reported from previous studies to exhibit *in-vitro* inhibitory antimicrobial properties activities against *Shigella*, *Salmonella*, and *Bacillus* spp (Wagner et al., 2011). In addition, isoflavone derivatives have been reported to exhibit moderate antimicrobial agents (Morel et al., 2003), hence shedding light on the presence of 2-7-hydroxy-8-allyl-isoflavone in the fraction. Previous studies have shown that chelation enhances the hydrophobicity of biologically important molecules. As a consequence the permeability of the antimicrobial agent through the phospholipid bilayer of the cell wall of Gram-negative bacteria is enhanced. This is suggested to be the case for the coordination compound molybdenum, bis(N,N-diethyl methacrylamide)-dicarbonyl (El Husseiny et al., 2008, Chang et al., 2010, Aiyelabola et al., 2012). The probable activity of the fraction might be as a result of the chelation. The presence of the macrocyclic compound ethyl 2,7,12,17-tetramethyl-21H,23H-porphine-3,8,13,18-tetrapropionate tin(IY) dichloride further suggests complementary actives to the antimicrobial activity of this fraction as well. This is in accord with that proposed by Semenov et al. (2006) for similar compounds.

As a consequence the activity of the NWU49 ethyl acetate fraction is suggested to be as a result of lycopene a tetraterpene specific to the fraction, which has been reported to possess antibacterial properties (Chandra et al., 2007). Another peculiar compound within this fraction is furosemide, a sulphonamide. Sulphonamides are known for their broad spectrum of bactericidal activity. The size and charge distribution of this class of compound is very similar to that of para-aminobenzoic acid (PABA), therefore they may act as metabolism antagonists (Killmer et al., 1980, Gadad et al., 2000, Azab et al., 2013). According to(Kunin and Ellis, 2000), mefloquine, also specific to this fraction, has been reported to exhibit bactericidal activity against methicillin- and fluoroquinolone-susceptible and -resistant strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*; and gentamicin- and vancomycin-resistant strains of *Enterococcus faecalis* and *Enterococcus faecium*. Therefore it serves as one of the probable factors contributing to the activity of this fraction. Other compounds found exclusively in this fraction are 2-(7-hydroxymethyl-3,11-dimethyl-2,6,10-trienyl)-[1,4]benzoquinone, 2(1H)-isoquinolinecarboximidamide-3,4-dihydro, a quinone and benzopyridine respectively, and noscapine. The former have derivatives with reported reasonable antibacterial activities (Nishina et al., 1991, Drewes et al., 2005).

Isoxazole, a compound found in the 91 benzene fraction, is an azole with an oxygen atom next to the nitrogen. The bacterial activity of this compound and its derivatives are well established in literature (Shreenivas et al., 2011, Kumar and Jayaroopa, 2013). Derivatives of this compound include many beta-lactamase-resistant antibiotics currently in the market, for example cloxacillin, dicloxacillin and flucloxacillin (Yasuda et al., 1983). Therefore the possible antibacterial activity of the fraction may be ascribed to the presence of this compound. Another compound present in this fraction that may also be a contributory factor to the activity of the fraction is 2-(5-chloro-2-hydroxyphenyl)-6-iodo-4(3H)-

quinazolinonetrimethylsilyl)- and 4,5-dihydroxyanthraquinone-2-carboxylic acid (Anchel, 1949, Pandey et al., 2009). 5-dihydroxyanthraquinone-2-carboxylic acid is a bioactive compound that belongs to the class of anthraquinone. Anthraquinone is a prominent anticancer agent.

A compound that was specific to the n-hexane and petroleum ether fractions of NWU91 was milbemycin B, 5-demethoxy-5-one-6,28-anhydro-25-ethyl-4-methyl-13-chloro-oxime, a macrocylic compound. This is not surprising as it may be as a result of the relative polarity of the solvents. Milbemycin B has been previously reported in literature as antibacterial, antihelmintic and insecticidal.

In addition to the compounds peculiar to each fraction, the presence of certain classes of compounds is also contributory factors to the activity of the fractions. The presence of high aldehyde and ketone content in all the ethyl acetate fractions indicate enhanced antibacterial activity of the fractions. According to (Chang et al., 2001) aldehydes are known to have the best antibacterial activity. On the other hand the high percentage of fatty acids present in the n-hexane, petroleum ether, benzene fractions of NWU91 relative to its ethyl acetate fraction indicates their possibly better activity against Gram-negative bacteria than their counterpart. This is attributable to the bipolar nature of the fatty acids, and their having the long hydrophobic side chain, which may assist in their migration into the cell of the microbe with consequent disruption of the cell. Hence this explains the versatility of the fractions as broad spectrum antibacterial agents.

9.4 Acknowledgements

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CHAPTER 10

10.1 General discussion

The easy access and inappropriate use of antimicrobial agents has led to the occurrence of strains of resistant microorganisms. To prevent the emergence and re-emergence of antibiotic resistance by pathogens to the clinically available drugs already marketed, the continued search for new bioactive compounds to periodically replace the existing antibiotics is necessary. Among the well-studied and characterized pharmaceutically relevant microorganisms, actinomycetes continue to be the most important sources of novel, therapeutically relevant natural microbial products. Actinomycetes have been widely explored for bioactive compounds and enzymes of industrial importance. They continue to provide pharmaceutically important substances which are unique and novel chemical entities. Although heavily studied over the past decades, actinomycetes still continue to prove themselves as reliable sources of novel bioactive compounds (Parthasarathi et al., 2010). These bioactive compounds exhibit one or more biological activities and many of them have been developed into clinical drugs for the treatment of diseases in human, veterinary and agriculture sectors (Demain and Sanchez, 2009). The unearthing of microbial secondary metabolites has a great capability in the development of industrial microbiology.

Searching for actinomycetes with biological activities is crucial in natural product-based discovery. The actinomycetes diversity, which is an inexhaustible source, has not been fully exploited. Many reports describe that soil is a major source of actinomycetes but there are no reports available pertaining to actinomycetes diversity from rhizospheric soils in South Africa. The rhizosphere is the soil region influenced by plant roots and characterized with high microbial diversity, with actinomycetes as one of the major bacterial groups. Hence, this study isolated, identified and estimated the actinomycetes in an untapped resource of

rhizospheric soils collected from Ngaka Modiri Molema district of North West Province in South Africa, and screened them for their antimicrobial activities. Further, the identified antagonistic actinomycetes were characterised using conventional and molecular techniques.

In this study, 92 out of the 341 strains showed antagonistic activity against at least five of the 11 test organisms. All the actinomycete isolates were identified upto the species level. The rhizosphere hosts numerous actinomycetes strains expected to produce a wide variety of bioactive metabolites. The antagonistic activity seen in this study further suggests that actinomycetes hosted by the rhizosphere are a key source of bioactive compounds. In addition, the actinomycetes offer a novel source for such compounds. This study revealed that among the isolates, *Streptomyces* was the most dominant genera. The frequency and dominance of *Streptomyces* among actinomycetes in various soil types were reported by several researchers (Karthikeyan et al., 2008; Duraipandiyan et al., 2010). *Streptomyces* is considered as the most abundant plant-associated actinobacterial genus. The identification of the potent strains by molecular techniques revealed that out of 341 isolates, 21.4% of the isolates were assigned as potent *Streptomyces* spp, and 5.5% as potent rare actinomycetes. The ability to produce bioactive secondary metabolites is not a uniform trait within this order. It is also noted that the antimicrobial activity of the rhizospheric isolates were clearly different, indicating that antimicrobial activity is a strain, not a species specific property.

Antibiotics from actinomycetes have been shown to exhibit antimicrobial activity against clinical antibiotic resistant microbial strains and crop diseases. *Streptomyces* spp have been reported to be capable of producing enormous microbial antibiotics with a wide variety of pharmaceutical properties. Rare actinomycetes also produce diverse and unique chemical entities with biological activities. Therefore, attention has been given to actinomycetes to find the new potent producers of bioactive compounds. In addition to the *Streptomyces* isolates, many rare actinomycetes have broad and strong antimicrobial properties (Zhao et al., 2012).

In the analysis of rhizospheric actinomycetes, 22% of the rare actinomycetes were active against at least one of the test organisms. The rare actinomycetes strains were more limited in their antimicrobial scope. The broadest antimicrobial ranges and the strongest inhibitory effects were seen among *Streptomyces* isolates. The 10 isolates that exhibited the broadest and strongest spectrum antimicrobial analysis were considered candidates for screening for new antibiotics (Chapter 6). They inhibited the growth of at least seven of the test organisms making them a good candidate for further studies. The genus *Streptomyces* is a productive source of biologically important compounds.

Organisms currently classified into the group actinomycetes display a broad range of morphological, biochemical and physiological characteristics. Consistent with this considerable phenotype heterogeneity, comparative gene sequencing clearly showed that the order Actinomycetales is phylogenetically very diverse. The phylogenetic relationship between the isolates was determined by 16S rDNA sequence analysis. The 16S rDNA gene of the actinomycete isolates were PCR-amplified and sequenced. The partial nucleotide sequences of the 16S rDNA gene of the isolates were compared with nucleotide database of NCBI web server through BLAST tool. The BLAST search inferred that the isolates were members of the GC-rich actinomycetes. The 16S rDNA gene nucleotide sequence of different actinomycetes was obtained by BLASTN search; these reference sequences were selected based on high similarity (%) with good E value. The results of the 16S rDNA gene sequences analysis shows that query sequences were best pairwise aligned with 16S rDNA gene sequence of actinomycetes with sequence similarity and identity ranging between 79-100%, with E value of 0. Based on similarity criteria of the 16S rDNA gene sequences, antibiotic actinomycete isolates were sorted into 12 genera: *Streptomyces*, *Actinomadura*, *Nocardiopsis*, *Promicromonospora*, *Nocardia*, *Arthrobacter*, *Pseudonocardia*, *Micrococcus*, *Nonomuraea*,

Rhodococcus, *Streptosporangium* and *Saccharothrix*. These data indicated the considerable diversity of actinomycetes within the rhizosphere.

The analysis of the 16S rDNA gene sequences of the isolates from the rhizosphere revealed that some of the isolates formed a highly clustered group of populations, with a phylogenetically closer relationship to known antibiotic producing actinomycetes while others formed distinct phyletic lines. It can be assumed that phylogenetically distant strains are more likely to have different genetic makeup than phylogenetically related strains; since secondary metabolites are produced by the concerted action of several genes, it is reasonable to expect an increased number of novel genes and gene combinations from isolates phylogenetically unrelated to strains highly screened for antibiotics. Contrary to this finding, very similar antibiotic biosynthesis genes have been found in distantly related actinomycetes and diverse antibiotic genes have been found in closely related species (Metsä-Ketelä et al., 2002). The genetic identity of an isolate using 16S rDNA gene sequence was not predictive of its antibiotic potential since the ability to produce antimicrobial agents is strain dependent and not species specific. Previous findings showed that actinomycete isolates might belong to the same genus and have similar 16S rDNA gene but possess different antibiotic genotype (Davelos Baines et al., 2007). Genetic characterization focusing only on 16S rDNA gene sequence analyses will provide incomplete information on the specific interaction potential of individual actinomycete isolates. Although the phylogenetic analysis using 16S rDNA gene sequences may predict that an isolate possesses antimicrobial activity through its closest related relative on the tree, screening for biosynthetic gene clusters better ascertains the antibiotic production potential of the isolates.

In recent years, scanning the genes encoding polyketide synthases and nonribosomal peptide synthetases that synthesize most of the biologically active polyketide and peptide compounds have been broadly applied in assessing the biosynthetic potential of culturable

microorganisms and culture-independent samples (Zhao et al., 2008; Ichikawa et al., 2013; O'Brien et al., 2014). Polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) pathways biosynthesize a large number of biologically active compounds and exist widely in the genomes of actinomycetes (Wenzel and Müller, 2005; Chen et al., 2011). A PCR-based screening for the presence of genes associated with biosynthetic pathways can rapidly predict the isolates that have the potential to produce secondary metabolites. Therefore, in parallel with antimicrobial activity testing, the PCR-based approach to detect type I polyketide synthase (PKS-I), PKS-II and nonribosomal peptide synthase (NRPS) genes were also carried out.

Degenerate primers of PKS-I, PKS-II and NRPS genes are used to analyse the antibiotic potential capacity of the actinomycetes isolates. In this study, a total of 341 actinomycete isolates were screened for the presence of biosynthetic gene clusters by amplifying the genes encoding polyketide synthases (PKS-I, PKS-II) and nonribosomal peptide synthetase (NRPS) using three sets of degenerate primers. The PKS and NRPS primers used in this study successfully detected the targeted fragments of these genes in a number of the isolates. Twenty-six percent (26%) of the isolates carried at least one of the biosynthetic enzyme genes involved in the biosynthesis of secondary metabolites. The percentage of isolates with PKS-I, PKS-II and NRPS gene fragments matching the expected gene size were 26.0%, 45.8% and 62.6% respectively. These results suggested that the NRPS pathway was widely spread in the rhizosphere derived actinomycete isolates. Several amplified gene fragments were sequenced, and the sequence analysis of the PCR amplicons of PKS-I, PKS-II and NRPS genes of the actinomyete isolates confirmed that all the partial sequences encoded parts of biosynthetic enzymes screened for.

Results from previous studies and this study suggest that for the culturable actinomycetes, the antimicrobial potential may only be assessed by screening of antimicrobial

activity against the test organisms. The percentages of strains with PKS-I, PKS-II and NPRS and the percentage of strains showing antimicrobial activity do not correlate, and there is an ample number of examples of strains possessing the functional genes showing no antimicrobial activity and vice versa (Wood et al., 2007). Some of the isolates showed correlation between the antimicrobial activity and successfully amplified secondary metabolite biosynthetic gene fragments from their genomes. Some of the isolates showed antimicrobial activity but none of the functional genes was detected in them. For example isolate NWU91 did exhibit broad spectrum antibacterial activity but could not amplify the PKS-NRPS genes from it. Possibly the code genes of antimicrobial products were not PKS or NRPS synthetical genes, or the primers amplifying PKS/NRPS genes were not suitable or had low homology with these isolates. One of the isolates NWU73 belonged to genus *Promicromonospora*, from previous reports it can be noted that members of this genus do not amplify the PKS-I, PKS-II and NRPS genes, yet they possess antimicrobial activity (Gontang et al., 2010). Some isolates did not show any antimicrobial activity, yet there was amplification of the PKS-NRPS genes fragments. For this phenomenon there are several plausible explanations, either the antimicrobials of these isolates were effective against pathogens not tested or they produced them in quantities too low to inhibit the test organisms. It may also be due to the fact that the PKS-NRPS genes of the isolates were silent or the PKS-NRPS gene clusters of the isolate were incomplete.

Sequencing the recovered PKS and NRPS PCR amplicons provided additional information on the phylogenetic position of the isolates and in some cases the potential function of the gene products as well. Phylogenies were constructed to study the structural and functional relatedness between these identified genes to other reference genes in the GenBank. The amplified portion of the PKS and NRPS genes from the isolates formed clades with other known antibiotic producing actinomycetes, this suggests that these isolates may

demonstrate the potential to produce bioactive compounds related to that class of antibiotic produced by the known producer. Most of the isolates formed phylogenetically distinct clades, indicating that the isolates containing these genes have not been analysed for their biosynthetic gene pathways yet. Thus, this group of isolates has the potential to produce a structurally novel class of bioactive compounds. These results revealed that the actinomycetes associated with the rhizosphere possess the potential ability to produce diversely bioactive secondary metabolites.

Furthermore, in contrast to the genus *Streptomyces*, only a limited number of secondary metabolite biosynthesis gene clusters in rare actinomycetes have been studied. This work suggests that genetic characterization may provide insight into the genetic make-up of the isolates, specifically identifying those isolates with potential. The biosynthetic gene from the actinomycetes isolates may provide novel material for genetic manipulation and combinatorial biosynthesis, which may lead to the generation of pharmaceutical compounds. It should be noted that the fact that a strain produces a known bioactive compound does not imply that it is the only bioactive compound that will be produced by such a strain, or that all bioactive compounds produced by the strain will be known bioactive compounds.

The use of a variety of media and fermentation conditions to induce the expression of secondary metabolite biosynthetic gene clusters in isolates for the production of secondary metabolites is fundamental. Optimization of the culture media and conditions are essential for high yields of the bioactive secondary metabolites. In this study, the isolates (NWU14, NWU49 and NWU91) were found to produce high levels of antimicrobial metabolites in the fermentation medium supplemented with glucose as the carbon source. Addition of glucose up to 2% resulted in the highest antimicrobial activity of the isolates, but significantly in many fermentation processes a higher concentration of glucose has a suppressive effect on production of bioactive metabolites (Gandhimathi et al., 2008). It was reported that organic

nitrogen sources were the best nitrogen sources for the production of bioactive secondary metabolites compared to inorganic nitrogen sources. Similar to the results from this study, oatmeal was found to be suitable for optimal production of antibiotic production, followed by yeast extract (Narayana and Vijayalakshmi, 2008). Maximum bioactive secondary metabolites production by the isolates was found at the mesophilic range of temperature. Similarly, Vijayakumar et al. (2012) also reported the isolation of antibacterial and antifungal agents from *Streptomyces afghaniensis* at 30°C. Studies have shown that low temperature may cease the metabolic activity and high temperature kills the cells of the microorganism (Gogoi et al., 2008).

The pH of culture media is one of the determining factors for the metabolism and hence for the biosynthesis of secondary metabolites. The pH is related to permeability characteristics of the cell wall and membrane and thus has an effect on ion uptake or loss to the nutrient medium (Gogoi et al., 2008). The optimal pH of the isolates for maximal production of bioactive secondary metabolites is at pH range of neutral to slightly alkaline. These results were in accordance to Sousa et al. (2008) who reported that the majority of actinomycetes isolated from rhizosphere and non-rhizosphere soil grows at a pH range varying from 6.5 to 8.0. Regarding the effect of NaCl concentration, 2% was found to be the optimum for production of the antimicrobial agents while concentrations above the optimum were found to decrease the bioactive secondary metabolite production. Singh et al. (2009) reported that *S. tanashiensis* strain A2D isolated from soil of phoomdi in Loktak Lake of Manipur, India exhibited optimum growth at 2% (w/v) NaCl. The size of the inoculum also influenced the ability of the isolates to produce antimicrobial metabolites. An inoculum size of 10% v/v was found to be the optimal inoculum for maximum antibiotic production by the isolates. The present result is in accordance with Thakur et al. (2009), who also reported the importance of inoculum size in increasing mycelial growth and metabolite production by

Streptomyces sp. A fermentation period of up to 10 days was observed to be optimum for maximum bioactive secondary metabolite production. Bioactive secondary metabolite production began to decrease after 10 days of incubation. Ripa et al. (2009) also reported the same result for antimicrobial metabolites production from a new *Streptomyces sp.* RUPA-08PR isolated from Bangladeshi soil. After optimization of the constituents and cultural conditions of production medium the zone of inhibition increased, this indicates that a significant increase in antibiotic production was achieved after optimization.

Analytical techniques continue to improve to allow the rapid elucidation of structures and make microbial products valuable components of modern drug discovery. The extraction of antimicrobial compounds from the CFS with equal volumes of different organic solvents resulted in antimicrobial activity against bacteria. These findings envisaged that the extraction method had a definite effect on the isolation of bioactive principles. It has also been reported that organic solvents always provide a higher efficiency in extracting compounds for antimicrobial activities compared to water based methods. In this study, the active solvent fractions from the crude extract of the actinomycete isolates showed a mixture of compounds in GC-MS analysis. A bioactive compound dibutyl phthalate has been reported in culture broth of *S. albidoflavus* by GC-MS (Roy et al., 2006). Narayana et al. (2008) analyzed benzyl alcohol, phenyl ethyl alcohol, and 2H-1, 4-benzoxazin-3(4H)-one from *Streptomyces sp.* ANU6277 by GC-MS analysis.

One of the active compounds identified from the ethyl acetate fraction of NWU14 was phellopterin. Phellopterin was first isolated from the root of a plant (*Angelica dahurica*), and was reported in previous research to exhibit pharmaceutical properties such as antimicrobial and immunosuppressant (Dekermendjian et al., 1996; Wagner et al., 2011). For NWU49 ethyl acetate fraction, the peculiar compound with activity is furosemide, a

sulphonamide. Sulphonamides are known for their broad spectrum of bactericidal activity (Willey et al., 2010).

In this study, the presence of 4,5-dihydroxyanthraquinone-2-carboxylic acid and milbemycin B from NWU91 was observed by GC-MS analysis. 4,5-dihydroxyanthraquinone-2-carboxylic acid is a bioactive compound that belongs to the class of anthraquinone. type II polyketide synthase is responsible for the biosynthesis of anthraquinone. The gene encoding for PKS-II was not amplified in NWU91, this might be a false negative result, i.e the isolate may possess the biosynthetic genes for the antibiotic in question, but no PCR amplicon was seen. Another explanation is that this might be as result of variations in primer target sequences preventing the primers from binding efficiently (Wood et al., 2007). This compound was first isolated from the leaves of *Cassia reticulate* and reported for having antibiotic activity (Anchel, 1949; Yu et al., 2008). This is the first time it will be reported in the literature for being produced by actinomycetes. A new anthraquinone, 1,8-dihydroxy-2-ethyl-3-methylanthraquinone isolated from Australian *Streptomyces sp* exhibited anticancer activity but not antibacterial activity (Clark et al., 2004). Anthraquinones are an interesting class of molecules that display a wide spectrum of biological applications, including antibacterial and anticancer properties. The possible mechanism of action of this compound includes being bacteriostatic or bactericidal. Milbemycin B belongs to a family of novel macrolide antibiotics with antibacterial, antihelmintic and insecticidal properties. This compound was first isolated from *S. hygroscopicus* from soil samples (Wang et al., 2009).

10.2 Conclusions

In conclusion, 341 isolates were screened for antibacterial activity, with one-third of the strains showing antibacterial activity. This study suggested that rhizospheric soil samples are a potent source of actinomycetes with biological activity against pathogenic bacteria.

Diverse actinomycete genera were isolated, 23 representative isolates were sorted into 18 phylotypes belonging to 12 different genera. They were screened for the presence of biosynthetic gene clusters and antimicrobial activities. Of the 341 isolates, 27% exhibited antibacterial activity against at least five of the 11 test organisms. All the active isolates have activities against *Staph. aureus*. Some of the isolates have at least one biosynthetic gene, and some isolates (NWU14) possess all the 3 biosynthetic genes screened for while some isolates (NWU91) do not amplify any of these genes but exhibit strong and broad spectrum activity. The presence of high aldehyde and ketone content in all the ethyl acetate fractions indicates enhanced antibacterial activity by the actinomycete isolates. Among the bioactive compounds identified are furosemide, phellopterin, 4,5-dihydroxyanthraquinone-2-carboxylic acid and milbemycin B, some of which are not previously reported in literature from actinomycete. These results highlight the importance of *Streptomyces* isolates in the search for novel bioactive compounds.

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