ISOLATION AND SCREENING OF SOIL ACTINOMYCETES FOR ANTIMICROBIAL ACTIVITY

BY

OLAKUNLE EBENEZER AJIBOLA

PG/LSC1003231

DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

BENIN CITY

DECEMBER, 2019

ISOLATION AND SCREENING OF SOIL ACTINOMYCETES FOR ANTIMICROBIAL ACTIVITY

BY

OLAKUNLE EBENEZER AJIBOLA

PG/LSC1003231

A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE, M.Sc. IN ENVIRONMENTAL AND PUBLIC HEALTH MICROBIOLOGY

DECEMBER, 2019

CERTIFICATION

I hereby certify that this work was carried out by **Olakunle Ebenezer AJIBOLA** at the department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, under my supervision.

Professor S.E. Omonigho **Date**
Supervisor

Professor (Mrs.) F. E. Oviasogie **Date**
Head of Department

APPROVAL

I hereby certify that this work was accepted in partial fulfillment for the award of Master of Science, M.Sc. in the department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City.

Professor F. E. Okieimen	**Date**
Dean, School of Post Graduate Studies	

ANTI PLAGIARISM

I attest and declared that the thesis titled Isolation and Screening of Soil Actinomycetes for Antimicrobial Activity has successfully passed the anti-plagiarism test and do not violate any copy right regulation.

Olakunle Ebenezer AJIBOLA **Date**

DEDICATION

This work is dedicated to God Almighty, in whom I live, move, and have my being.

ACKNOWLEDGEMENT

I wish to express my most profound gratitude to God Almighty.

I would like to render my warmest thanks to my supervisor, Professor S.E. Omonigho, who made this work possible. His friendly guidance and expert advice was invaluable throughout all the stages of this work.

I also wish to express my gratitude to Professor (Mrs) F.E. Oviasogie, and the entire staff of Microbiology department, University of Benin.

Special thanks to my mom, Mrs. Felicia Ajibola for her continuous support and understanding. God bless you all.

TABLE OF CONTENTS

TITLE PAGE……..……..ii

CERTIFICATION iii

APPROVAL iv

ANTI PLAGIARISM v

DEDICATION vi

ACKNOWLEDGEMENT vii

LIST OF TABLES, FIGURES AND PLATES xii

ABSTRACT xiii

CHAPTER ONE 1

1.0 INTRODUCTION 1

1.1 AIM AND OBJECTIVES 3

SPECIFIC OBJECTIVES 3

CHAPTER TWO 4

2.0 LITERATURE REVIEW 4

2.1 GENERAL OVERVIEW OF ACTINOMYCETES 4

2.2 TAXONOMY OF ACTINOMYCETES 4

2.3 SOIL ACTINOMYCETES 6

2.4 THE STREPTOMYCETES 7

2.5 SOIL ACTINOMYCETES AS SOURCES OF BIOACTIVE METABOLITES 8

2.5.1 ANTIBIOTICS 9

2.5.2 ANTIFUNGAL AGENTS 11

2.6 GENETIC ORGANIZATION OF ANTIMICROBIAL BIOSYNTHESIS IN ACTINOMYCETES 13

2.7 ANTIMICROBIAL RESISTANCE IN PATHOGENS 14

2.7.1 GENETIC BASIS FOR ANTIMICROBIAL RESISTANCE 16

2.7.2 BACTERIAL GENOME AND NEW ANTIBIOTICS 17

2.8 ISOLATION OF SOIL ACTINOMYCETES 18

2.8.1 PRE-TREATMENT TECHNIQUES 19

2.8.2 SELECTIVE INHIBITION OF NON-ACTINOMYCETES 20

2.8.3 NUTRITIONAL SELECTION FOR ACTINOMYCETES 21

2.8.4 ENRICHMENT METHODS 22

2.8.5 MEMBRANE FILTER METHOD 23

2.8.5 INTEGRATED METHOD 23

2.9 IDENTIFICATION OF ACTINOMYCETES 24

2.9.1 CLASSICAL APPROACH 24

2.9.2 CHEMOTAXONOMICAL APPROACH 25

2.9.3 MOLECULAR APPROACH 26

2.10 PRODUCTION OF ANTIBIOTICS FROM ACTINOMYCETES 26

2.10.1 LIQUID STATE FERMENTATION 26

2.10.2 SOLID STATE FERMENTATION 28

2.10.3 DOWNSTREAM PROCESSING 29

2.11 ANTIMICROBIAL ACTIVITY PROTOCOLS 30

CHAPTER THREE 33

3.0 MATERIALS AND METHODS 33

3.1 SAMPLE COLLECTION 33

3.2 PRE-TREATMENT OF SOIL SAMPLES 33

3.2 ISOLATION OF ACTINOMYCETES 33

3.3 SCREENING OF ISOLATES FOR ANTIMICROBIAL ACTIVITY 34

3.3.1 TEST MICROORGANISMS 34

3.3.2 PRIMARY SCREENING 34

3.3.3 SECONDARY SCREENING 35

3.4 IDENTIFICATION OF SELECTED ACTINOMYCETES ISOLATES 36

3.4.1 GRAM STAINING 36

3.4.2 MORPHOLOGICAL CHARACTERIZATION 36

3.4.3 PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION 36

3.4.4 MOLECULAR IDENTIFICATION 40

CHAPTER FOUR 41

RESULTS 41

CHAPTER FIVE 48

5.0 DISCUSSION AND CONCLUSION 48

5.1 DISCUSSION 48

5.2 CONCLUSION 52

REFERECES 54

APPENDIX A 62

MEDIA AND REAGENTS COMPOSITIONS 62

APPENDIX B 66

16S RIBOSOMAL RNA GENE PARTIAL SEQUENCE OF ISOLATE CYP1 66

LIST OF TABLES, FIGURES AND PLATES

TABLE	TITLE	PAGE
1	Physico-chemical properties of soil samples collected from different sites in Benin City and their average actinomycetes count	41
2	Primary screening of actinomycetes isolates for antimicrobial activity	42
3	Cultural characteristics of selected actinomycetes isolates on ISP-2 media	43
4	Antimicrobial activities of selected actinomycetes isolate crude extracts	44
5	Physiological and biochemical characterization of actinomycetes isolates	46

FIGURE	TITLE	PAGE
1	Antifungal activities of crude extracts compared to standard antifungals	45

PLATES	TITLE	
1	16S rRNA banding profile of isolate CYP1	47

ABSTRACT

Soil actinomycetes are recognized as promising sources of new antimicrobials. Antimicrobial resistance in pathogens has greatly increased in recent years, and has become a global public health problem. New antimicrobials are continuously required to combat these resistant strains. The aim of this study was to isolate and screen soil actinomycetes and evaluate their secondary metabolites for antimicrobial activities against selected pathogenic bacteria and fungi.

Four soil samples were collected, pre-treated with $CaCO_3$, serially diluted and spread plated on actinomycetes isolation agar (AIA) and international streptomyces project media 2 (ISP-2), supplemented with nystatin, neomycin and polymyxin B . Perpendicular streak method was used to check antagonistic activities of the isolated actinomycetes against test microorganisms. Small scale submerged fermentation system was used for the production of antimicrobial metabolites from the isolates. Agar well diffusion was then used to evaluate antimicrobial activities of the crude extracts against the test microorganisms.

The average aerobic actinomycetes plate count from the different soil samples ranged from $3.0\times10^4 \pm 2.4$ to $3.6\times10^4 \pm 1.9$ CFU/g. A total of 28 different microorganisms were isolated, characterized by cultural and morphological methods and identified as actinomycetes. Out of the 28 isolates, 10 (36%) showed antimicrobial activities on primary screening; from which isolates BYQ3, CYP1, CYP2, CYP4 and CYQ2 were selected for their wide spectrum of activities. Diameters of inhibition zones produced by these 5 isolates against the test microorganisms on secondary screening, ranged from 0 to 26 mm. Isolates CYP1 and CYP2 had the widest zones with CYP1 producing 26 mm against *Candida albicans*. The two promising isolates were further characterized by physiological and biochemical tests and identified as genus *Streptomyces*. Isolate CYP1 was then identified to the specie level by 16S

rRNA gene sequence analysis which confirmed that *Streptomyces* sp. CYP1 was homologous to *Streptomyces albus* (strain DSM 40313) of the order *Actinomycetales* and class *Actinobacteria*. Optimization of production conditions, further purification, structural elucidation and characterization are recommended to know the quality, novelty and commercial value of these antimicrobials.

CHAPTER ONE

1.0 INTRODUCTION

Actinomycetes are gram-positive bacteria with high guanine + cytosine content of over 55% in their DNA. They belong to the order *Actinomycetales* and form an important segment of the microflora of most natural environments. Soils, manures and composts, freshwater bodies such as lakes and river bottoms contain an abundance of these organisms. Actinomycetes are aerobic, spore forming organisms with a distinctive feature of possessing filamentous hyphae that do not normally undergo fragmentation. Due to their phenotypic similarities to fungi, actinomycetes are also known as ray fungi (Chaudhary *et al*., 2013).

Actinomycetes provide an excellent resource for the isolation and identification of therapeutically important secondary metabolites such as, antibiotic, antifungal, antiviral, anticancer, enzyme, immunosuppressant and other industrially useful compounds (Dhawane and Zodpe, 2017). These microbial compounds have been a source of life saving environment for many bacterial and fungal infections. Some effective antibiotics manufactured from actinomycetes includes: penicillin, streptomycin, tetracycline, erythromycin, amphotericin and vancomycin. These microbial natural products are notable not only for their potent therapeutic activities but also for the fact that they frequently pose desirable pharmacokinetic properties required for clinical development (Khasabuli and Kibera, 2014). Antibiotics of actinomycetes origin have a wide variety of chemical structure, including aminoglycosides, β-lactams, antracyclines, tetracycline, nucleosides, peptides, polyenes and actinomycins. Secondary metabolites isolated from soil actinomycetes have also been proven to be potent inhibitors of numerous plant pathogens (Agadagba, 2014).

A large number of actinomycetes have been isolated and screened from soil in the past several decades, accounting for 70 80% of relevant secondary metabolites available commercially. It has been estimated that approximately one-third of the thousands of naturally occurring antibiotics have been obtained from actinomycetes (Chaudhary *et al*., 2013). More than 70% of these antibiotics are attributed to two genera viz., *Streptomyces* and *Micromonospora* (Rai *et al*., 2018). The richness and diversity of actinomycetes present in any specific soil, is greatly influenced by the soil type, geographical location, cultivation and organic matter amongst other factors (Agadagba, 2014).

According to the World Health Organization, over-prescription and the improper use of antibiotics has led to the generation of antibiotic resistance in many bacterial pathogens (Kumar *et al*., 2010). Serious infections caused by microorganisms that have acquired resistance to commonly used antibiotics have become a major global healthcare problem in the 21st century (Jarallah and Rahaman, 2014). Some antibiotics like penicillin, erythromycin, and methicillin which used to be very effective treatment against infectious diseases are now less effective because pathogens are now more resistant to such antibiotics. Antibiotic resistant pathogens such as methicillin and vancomycin resistant strains of *Staphylococcus aureus* and others cause an enormous threat to the treatment of serious infections. These drug resistant strains emerge more quickly than the rate of discovery of new drugs and antibiotics (Kumar *et al*., 2010).

Also, increase in fungal infection happens because the available antifungal drugs are not very effective in treating fungal diseases. Fungal diseases are often difficult to diagnose and treat because antifungal drugs are often not very effective in the setting of impaired immunity (Casadevall *et al*., 2002). *Candida albicans* can develop resistance to antimycotic drugs such as fluconazole which is often used to treat candidiasis. The frequency of multiazole-resistant

strains belonging to *Candida* species other than *Candida albicans* is increasing (Hitchcock *et al*., 1993).

The global increase in antimicrobial resistant infections requires that efforts be intensified towards the discovery of new antibacterial and antifungal agents. Hence, many scientists and pharmaceutical companies are actively involved in isolation and screening of actinomycetes from different habitats, for the production of antibiotics. This is necessary for immediate replacement of the existing antibiotics and the development of novel drugs effective against resistant pathogenic bacteria and fungi.

1.1 AIM AND OBJECTIVES

The aim of this study was to isolate and screen antibiotic producing actinomycetes from the soil

SPECIFIC OBJECTIVES

The specific objectives of this study were to:

1. Isolate and enumerate actinomycetes populations from different soil samples
2. Screen the actinomycetes isolates for antibiotics production
3. Produce bioactive metabolites from the actinomycetes isolates through fermentation
4. Evaluate the antimicrobial activities of the bioactive metabolites against selected pathogens

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 GENERAL OVERVIEW OF ACTINOMYCETES

The actinomycetes (sing. actinomycete) are a large group of gram-positive, aerobic, high G-C bacteria that form branching filaments or hyphae and asexual spores. The name "Actinomycetes" was derived from Greek "atkis" (a ray) and "mykes" (fungus), having characteristics of both bacteria and fungi, but yet possess sufficient distinctive features to classify them as bacteria ((Das *et al*., 2008). Their resemblance to fungi in morphology may have resulted partly from adaptation to the same habitat (Willey *et al*., 2010). Majority of actinomycetes are free living organisms that are widely distributed in nature. They are primarily soil inhabitants (terrestrial habitat) but have been found in a diverse range of aquatic habitats, including sediments obtained from deep sea, even from great depths of the Mariana Trench. Their presence in extreme environments especially at cryophilic regions, such as Antarctica and desert soils has been reported (Bizuye *et al*., 2013; Adegboye and Babalola, 2012). Among microorganisms, actinomycetes gained special importance due to their capacity to produce bioactive secondary metabolites and enzymes. They are the most important producers of bioactive compounds such as antibiotic, antifungal, antiviral, anticancer, enzyme, immunosuppressant and other industrially useful compounds (Dhawane and Zodpe, 2017).

2.2 TAXONOMY OF ACTINOMYCETES

Actinomycetes belong to the domain Domain Bacteria and phylum *Actinobacteria*, which represents one of the largest taxonomic units among the 18 major lineages currently recognized within the Domain (Sharma *et al*., 2014). In volume 5 of Bergey's Manual of

Systematic Bacteriology 2nd edition, the phylum *Actinobacteria* is divided into 6 classes namely *Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria* and *Thermoleophilia*. The class *Actinobacteria* is further divided into 16 orders which are *Actinomycetales, Actinopolysporales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Frankiales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales, Incertae sedis* (Barka *et al*., 2016).

Actinomycetes belong to the order, *Actinomycetales* comprising of 14 suborders (Adegboye and Babalola, 2012). The order is also diverse in terms of morphology, phylogeny and chemotaxonomy (Kekuda *et al*., 2010). Actinomycetes (order *Actinomycetales*) were initially classified based on their filament branching (Willey *et al*., 2010). Thus, due to the presence of the filamentous forms, 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, mycelia pigment, cell wall composition, utilization of carbon and nitrogen sources, spores formation and percentage of G + C content in DNA (Willey *et al*., 2010). Modern phylogenetic and molecular techniques including 16S rRNA analysis and DNA-DNA hybridization are also commonly used for actionomycetes classification (Hirsch *et al*., 2010; Ventura *et al*., 2007). Based on the molecular and chemical composition data, the order *Actinomycetales* is divided into the following 14 suborders: *Actinomycineae, Actinopolysporineae, Catenulisporineae, Corynebacterineae, Frankineae, Glycomycineae, Jiangellineae, Kineosporineae, Micrococineae, Micromonosporineae, Propionibacterineae, Pseudonocardineae, Streptomycineae* and *Streptosporangineae* (Barka *et al*., 2016).

Moreover, sequencing of 16S rRNA genes has led to the recognition of 39 families and 130 genera. Some of these genera include: *Tropheryma*, *Propionibacterium*, *Micromonospora*,

Salinispora, *Mycobacterium*, *Nocardia*, *Corynebacterium*, *Gordonia*, *Rhodococcus*, *Leifsonia*, *Bifidobacterium*, *Gardnerella*, *Streptomyces*, *Frankia*, *Thermobifida*, etc (Barka *et al*., 2016).

2.3 SOIL ACTINOMYCETES

Soil is a biologically active environment that is inhabited by varieties of microorganisms. It is a poorly characterized microbial environment. The genetic diversity of the soil has been estimated to be about 6400 to 38000 prokaryotic species per gram. On the contrary, less than 0.3% of the microscopically observed microorganisms present are culturable by standard techniques (Pettit, 2004).

Actinomycetes are primarily soil inhabitants, they are well known as soil saprophytes and are responsible for the characteristically "earthy" smell of freshly turned healthy soil due to the production of the organic compound, geosmin. *Streptomyces* species are the most dominant actinomycetes in soil although, other genera like *Norcardia, Microbispora*, *Micromonospora*, *Actinomyces*, *Actinoplanes*, *Streptosporangium*, etc. have also been isolated from soil samples (Adegboye and Babalola, 2012). The number and variety of actinomycetes present in any soil sample is determined by geographical location, soil type, soil temperature, soil pH, organic matter content, nutrient availability, aeration, moisture content, agricultural activities, and soil vegetation (Arifuzzaman *et al*., 2010). Average soil actinomycetes count has been estimated at about 10^7 to 10^8 organisms per gram of soil (Makut and Abdulazeez, 2012).

Some of actinomycetes are distributed in plant rhizosphere soils. The term rhizosphere is defined as the zone of soil that surrounds plant roots and influenced by root metabolism (Dhawane and Zodpe, 2017). The density of microorganisms is higher in this zone than in root-free soils (bulk soils). This difference has been linked to the secretion, by roots, of small organic compounds in the form of exudates that supply nutrition and energy sources for microbial growth. Microbial flora of the rhizosphere comprises mainly bacteria, fungi and

actinomycetes. Actinomycetes form thread-like filaments in the soil which give them an advantage in colonizing the rhizosphere effectively. As a rhizobacteria, they enhance plant growth, antagonize plant pathogens and make nutrients available for the plants (Maheshwari and Shimizu, 2011).

Actinomycetes have been isolated from diverse soil types and locations such as arid regions, tropical forest, mines, caves, swamps, deserts and savannahs. They are specifically abundant in slightly alkaline soils rich in organic matter. Soil actinomycetes produce several structurally diverse secondary metabolites of pharmaceutical and agricultural importance (Adegboye and Babalola, 2012). Actinomycetes play an important ecological role in the recycling and mineralization of nutrients in the soil by degrading vast numbers of soil organic matter, hence their common occurrence in composts. The presence of actinomycetes in the soil also promote plant growth by helping to fix atmospheric nitrogen, solubilization and immobilization of nutrients, siderophores production, biological control of pathogens and soil structure maintenance (Barka *et al*., 2016).

2.4 THE STREPTOMYCETES

Streptomyces is the largest genus of *Actinobacteria* and the type genus of the family *Streptomycetaceae*. Over 500 species of *Streptomyces* bacteria have been described (Raja and Prabakarana, 2011). The filaments and spores of *Streptomyces* species are very small usually 1 μm or less in diameter. Spore formation is by fragmentation and borne in straight, wavy, or helical chains (Sharma *et al*., 2014). The colonies are slow-growing and often have “earthy” odour due to the production geosmin. Early colonies are relatively smooth but later develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety. They produce a wide variety of pigments which determines the colour of the vegetative and aerial mycelia (Hasani *et al*, 2014). Streptomycetes are nonmotile, catalase positive, reduce nitrates

to nitrites and degrade adenine, esculin, casein, gelatin, hypoxanthine, starch, and Ltyrosine (Njenga *et al*., 2018). Their cell wall peptidoglycan contains major amounts of L-diaminopimelic acid (L-DAP). They have no mycolic acids but contain major amounts of saturated, iso- and anteiso-fatty acids. They usually possess either hexa- or octahydrogenated menaquinones with nine isoprene units as the major isoprenolog, and have complex polar lipid patterns that typically contain diphosphatidyl glycerol, phosphatidyl ethanolamine, phospha-tidyl inositol, and phosphatidyl inositol mannosides (Hasani *et al*, 2014).

Streptomyces has received particular attention for three main reasons. First, streptomycetes are abundant and important in the soil, where they play major roles in the cycling of carbon trapped in insoluble organic debris, particularly from plants and fungi. This action is enabled by the production of diverse hydrolytic exoenzymes. Second, the genus exhibits a fairly wide phylogenetic spread. Third, streptomycetes are among Nature's most competent chemists and produce a stunning multitude and diversity of bioactive secondary metabolites. Out of the approximately 10000 known antibiotics, 45-55% is produced by streptomycetes; consequently, they are of great interest in medicine and industry (Barka *et al*., 2016).

2.5 SOIL ACTINOMYCETES AS SOURCES OF BIOACTIVE METABOLITES

Soil actinomycetes account for a significant fraction of microbial metabolites. There are two general kinds of metabolites produced by microorganisms; primary and secondary metabolites. A primary metabolite is a kind of metabolite that is directly involved in normal growth, development, and reproduction. It usually performs physiological functions in the organism. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism. Apart from their non-involvement in growth and development, secondary metabolites also differ from primary

metabolites in the following ways; production of secondary is dependent on growth conditions liked type of culture media, also, they are often produced as groups of closely related molecules during the period of nutrient limitation or waste product accumulation, and lastly, it is often possible to overproduce these components (Willey *et al*., 2010).

The most important characteristic of soil actinomycetes is the ability to produce secondary metabolites with antibiotic, antifungal, antiviral and antitumoral properties. Other actinomycetes secondary metabolites include; bioherbicides, biopesticide, antiparasitic agents, hypercholesterolemia agents, immunostimulatory agents, immunosuppressive agents and therapeutic enzymes (Barka *et al*., 2016).

2.5.1 ANTIBIOTICS

Antibiotics are molecules that selectively inhibit bacterial growth without damaging the eukaryotic organisms. The selectivity of action of these substances is due to the fact that they interfere with processes essential for the bacterial cell and absent or different in the eukaryotic cell (Lo Grasso *et al*., 2016). Antibiotics are often referred to as 'wonder drugs' for their virtual success against pathogens. This remarkable group of compounds forms a diversified collection of biologically active molecules with different structures and modes of action. They attack basically every type of microbial processes such as membrane function, DNA, RNA, and protein synthesis, sporulation, germination, electron transport, etc. This makes them effective treatments for bacterial infections (Procópio *et al*., 2012).

Louis Pasteur was one of the pioneers of modern antibiotics knowledge, in 19th century. He discovered that, some of microorganisms are capable of inhibiting other microorganisms (Hasani *et al*., 2014). Penicillin was the first antibiotic to be discovered by Alexander Fleming from the fungus, *Penicillium notatum* in 1929 (Silva and Anne, 2004). The history of antibiotics obtained from actinomycetes began with the discovery of actinomycin from

Streptomyces antibioticus in 1940, streptothricin from *Streptomyces lavendulae* in 1942, and streptomycin from *Streptomyces griseus* in 1944, all of which were discovered by Waksman and colleagues (Barka *et al*., 2016). Between 1945 and 1960—the so-called "golden age of antibiotics", Microbiology and Chemistry worked hand in hand; bacteria were cultivated and their secondary metabolites extracted to yield a large number of compounds with antimicrobial activity (Hug *et al*., 2018). In particular, 80% of antibiotics of natural origin are sourced from the genus *Streptomyces* and other rare actinomycetes, such as *Actinomadura*, and only 20% is produced by fungal species (Lo Grasso *et al*., 2016).

The production of most antibiotics is species specific, and these secondary metabolites are essential for the actinomycetes to compete favourably with other microorganisms in its environment, even with organisms within the same genus. Symbiosis between actinomycetes and plants is another important process that involves the production of antibiotics, as the antibiotic protects the plant against pathogens, and plant exudates aid the development of the actinomycetes. Furthermore, some antibiotics originated as signal molecules, which are able to bring about changes in the expression of some genes that are not related to a stress response (Procópio *et al*., 2012).

The major classes of clinical antibiotics produced by actinomycetes are as follows:

(i) Aminoglycosides such as neomycin, kanamycin and streptomycin (Busscher *et al*., 2005; Park *et al*., 2013; Vakulenko and Mobashery, 2003) **(ii)** Angucyclines such as auricin; also, antitumor agents like landomycin and moromycin (Kharel *et al*., 2012) **(iii)** Ansamycins such as rifamycin and geldanamycin (Kang *et al*., 2012) **(iv)** Anthracyclines such as daunorubicin (Minotti *et al*., 2004; Nitiss, 2009) **(v)** β lactams such as cephamycins and also the important β lactamase inhibitor clavulanic acid (Liras, 1999; Jensen and Paradkar, 1999; Saudagar *et al*., 2008) **(vi)** Chloramphenicol (Vining and Stuttard, 1994) **(vii)** Glutarimides such as cycloheximide (Kominek, 1975) **(viii)** Glycopeptides such as vancomycin and teichoplanin

(Butler *et al*., 2014; Van Bambeke, 2006) **(ix)** Lipopeptides such as daptomycin (Baltz, 2010) **(x)** Lantibiotics such as mersacidin and actagardine (Willey and van der Donk, 2007) **(xi)** Macrolides such as clarythromycin, erythromycin, tylosin and clarithromycin (Gaynor and Mankin, 2003; Poehlsgaard and Douthwaite, 2003) **(xii)** Oxazolidinones such as cycloserine (Mulinos, 1995) **(xiii)** Streptogramins such as streptogramin (Johnston *et al*., 2002), and **(xiv)** tetracyclines (Okami and Hotta, 1998).

The producing capacity of individual actinomycetes can also vary enormously. Some *Streptomyces* species produce a single antibiotic, while others produce a range of different compounds and compound classes (Barka *et al*., 2016).

2.5.2 ANTIFUNGAL AGENTS

In 1949, Rachel Brown extracted the first antibiotic for treatment of fungal disease called Nystatin from *Streptomyces noursei* (Hasani *et al*., 2014). Afterwards, kasugamycin was extracted from a *Streptomyces kasugaensis* culture and was found to be a bactericidal and fungicidal agent, which acts by inhibiting protein biosynthesis in microorganisms but not in mammals (Umezawa *et al*., 1965). The systemically active kasugamycin was useful for the control of rice blast (*Pyricularia oryzae cavara*) and Pseudomonal diseases in several crops. In 1965, the first members of a new class of natural fungicides, polyoxins B and D were isolated from *Streptomyces cacaoi* var. asoensis (Isono *et al*., 1965). The polyoxins obstruct fungal cell wall synthesis by inhibiting chitin synthase (Endo and Misato, 1969). Polyoxin B is active against a number of fungal pathogens in fruits, vegetables, and ornamentals, while polyoxin D is used in the control of rice sheath blight, caused by *Rhizoctonia solani* (British Crop Protection Council. 1994). The validamycin family was discovered by researchers at Takeda Chemical Industries in a greenhouse assay for the treatment of sheath blight disease in rice plants caused by the fungus *Rhizoctonia solani*. Validamycin A is the most active

component of the validamycin complex and was isolated from *Streptomyces hygroscopicus* var. limoneus. When validamycin gets into the fungal cell, it is converted to validoxylamine A, a notably strong inhibitor of trehalase which catalyzes the conversion of trehalose to glucose (Kameda, 1987). Trehalose is a common storage carbohydrate hence trehalase plays an essential role in the transport of glucose in fungi and insects. Since vertebrates do not depend on the hydrolysis of trehalose for their metabolism, the ability to inhibit trehalose gives validamycin A a favorable biological selectivity (Elbein *et al*., 2003).

Antimycins are a family of well-identified antifungals that act by inhibiting electron flow in the mitochondrial electron transport chain (Sharma *et al*., 2014). These secondary metabolites have been identified in *Streptomyces* species isolated from the integument of attine ants. Antimycins A1–A4 have also been found in *Streptomyces* species isolated from workers of several *Acromyrmex* species. Compounds of this family may play an important role in the ant-microbe association. Another antifungal compound commonly found in *Streptomyces* species related to attine ants is candicidin (Schoenian *et al*., 2011; Seipke *et al*., 2011; Seipke *et al*., 2012). Urauchimycins are antifungals of the antimycin family. urauchimycins A and B have been shown to repress the morphological differentiation of *C. albicans*. Urauchimycin B has a wide spectrum of activity with MIC values similar to nystatin, which indicates the potential for medical use (Imamura *et al*., 1993). In 2006, two new urauchimycins C and D were isolated from *Streptomyces* sp. B1751 from marine sediment and *Streptomyces* sp. AdM21 from soil respectively (Yao *et al*., 2006). Antimycins were used for the treatment of human infections for many years, but due to its mechanism of action and side effects, its use in the treatment of human infections was discontinued. However, with the compelling need for new antifungal agents, it is essential to establish the toxicity obtainable by urauchimycin B, to evaluate whether it can be used as an antifungal agent for humans and animals (Sharma *et al*., 2014).

2.6 GENETIC ORGANIZATION OF ANTIMICROBIAL BIOSYNTHESIS IN ACTINOMYCETES

Genes responsible for the in the biosynthesis of antimicrobials and other secondary metabolites are usually clustered within the organism's genome and on rare occasions, in circular plasmids. A biosynthetic gene cluster involved in the production of a particular antibiotic will contain many genes located within a few thousand base pairs of each other. The size of a gene cluster can vary from a few to several hundred genes. Generally, 10−50 genes are required for the synthesis of an antibiotic (Lo Grasso *et al*., 2016). Biosynthetic gene clusters usually contain the following: genes involved in the precursor biosynthesis, genes involved in tailoring steps, as well as genes involved in export, resistance, and regulation respectively. Some peptide antibiotics are formed by assembling amino acid precursors by non-ribosomal machinery. This non-ribosomal peptide synthesis involves the use of non-proteinogenic amino acids, such as 3,5 dihydroxyphenylglycine (DPG) and 4-hydroxyphenylglycine (HPG). In most cases, the backbone of the antibiotic is modified by the tailoring steps; chlorination, methylation, glycosylation, N-acylation, etc. (Alduina *et al*., 2007).

Antibiotic gene clusters usually encode for regulatory genes, which are pathway specific, with positive or negative control on the cluster. There could also be some pleiotropic regulators that affect antibiotic production and primary metabolism of the bacteria. For example, the biosynthesis of actinorhodin, a benzoisochromanequinone polyketide antibiotic in *Streptomyces coelicolor* is regulated by the transcriptional activator ActII-ORF4 (Iqbal *et al*., 2012; Wietzorrek and Bibb, 1997), while the pathway for undecylprodigiosin, another antibiotic by the same organism is regulated by a mini cascade of two cluster specific regulators, with RedZ activating the expression of *redD*, an aberrant orphan response regulator, direct activator gene for the biosynthetic genes (White and Bibb, 1997).

In antibiotics producing bacteria, resistance genes are imperative to avoid suicide, while transport genes are necessary for the export antibiotics outside the cell (Lo Grasso *et al*., 2016). Resistance to antibiotics is generally achieved in microorganisms by mechanisms such as; antibiotic inactivation or modification, increased efflux or decreased influx of the antibiotic, target site alteration and target amplification (Tenover, 2006). A typical example of antibiotic inactivation mechanism is the production of β-lactamase in many pathogens. This enzyme acts by hydrolysing the β-lactam ring of the antibiotic, thus inactivating it. The methylation of an adenine of the ribosomal RNA is an example of alteration of target site; this prevents the interaction between macrolides and ribosome. Resistance to glycopeptides is usually achieved by the presence of genes encoding for alternative forms of peptidoglycan, with low affinity for glycopeptides (Arthur *et al*., 1996). Glycopeptide resistance has been examined in *Streptomyces toyocaensis* and *Actinoplanes teichomyceticus*, which produce the glycopeptides A47934 and teicoplanin, respectively and also in *Streptomyces coelicolor*, which does not produce glycopeptide. For the producers of glycopeptides, activation of the resistance genes by the endogenously produced antibiotic prevents suicide, while in non-producing bacteria resistance may be due to the acquisition of resistance genes through horizontal gene transfer or mutation. As example of modification, acetylatation of the antibiotic by specific acetyl transferase inactivates its antibacterial property (Perry *et al*., 2014; Ramirez *et al*., 2003). The best known efflux system is that of tetracycline mediated by the gene *tetL,* carried by a transposon, which codes for a protein that transports the antibiotic outside the cell (Lo Grasso *et al*., 2016).

2.7 ANTIMICROBIAL RESISTANCE IN PATHOGENS

The discovery, commercialization and administration of antibiotics for the treatment of infections revolutionized modern medicine. It became one of the most important medical interventions needed for the development of complex medical approaches such as advanced

surgical procedures, solid organ transplantation and cancer management in patients. Unfortunately, the rapid emergence of resistant bacteria worldwide now threatens this therapeutic accomplishment and endangers the efficacy of antibiotics, which have saved millions of lives (Munita and Arias, 2016). The WHO recently carried out a study on global antibiotic resistance crisis. For that study, the new Global Antimicrobial Surveillance System (GLASS) implemented in May 2015 was used to support standardised antibiotic resistance surveillance globally. The main objective of the study was to track resistance-related issues of pharmaceutical drugs in use for treatment of hospital and community acquired infections across the 52 participating countries. The survey focused on *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Salmonella* spp., *Neisseria gonorrhoeae*, *Shigella* spp. and *Acinetobacter baumanii*. According to the report of that study, released in January 2018, *N. gonorrhoeae* is evolving into a superbug, as it is resistant to third generation cephalosporins and fluoroquinolones and is now classified by the WHO Priority Pathogens List of antibiotic-resistant bacteria. *Salmonella* spp., *S. aureus* and *N. gonorrhoeae* were prioritised for research and development of new and effective antibiotic treatments, while *A. baumanii*, *E. coli* and *K. pneumoniae* are classified as critical priority and *Shigella* spp. along with *S. pneumonaiae* are classified as medium priority (Hug *et al*., 2018).

The marked increase in antibiotic resistance among common bacterial pathogens has made the WHO to name antibiotic resistance as one of the three most important public health threats of the 21st century. Infections caused by multidrug-resistant (MDR) organisms are associated with increased mortality compared to those caused by susceptible bacteria and they carry an important economic burden. According to a recent report, antibiotic resistance is estimated to cause around 300 million premature deaths by 2050, with a loss of up to $100 trillion (£64 trillion) to the global economy. This situation is worsened by a lack of robust

antibiotic pipeline, resulting in the emergence of infections that are almost untreatable and leaving clinicians with no reliable alternatives to treat infected patients (Munita and Arias, 2016). From the late 1960s through the early 1980s, the pharmaceutical industry introduced many new antibiotics to solve the resistance problem, but after that the antibiotic pipeline began to dry up and fewer new drugs were introduced. As a result, in 2018, many decades after the first patients were treated with antibiotics; bacterial infections have again become a threat (Ventola, 2015).

2.7.1 GENETIC BASIS FOR ANTIMICROBIAL RESISTANCE

Bacteria have great genetic flexibility that allows them to adapt to a wide array of environmental threats, including antimicrobial molecules. Bacteria sharing the same ecological niche with antibiotic-producing organisms have evolved ancient mechanisms to survive the effect of the harmful antibiotic molecule and even thrive in its presence. From an evolutionary perspective, bacteria use two major genetic strategies to adapt to antimicrobial molecules: (***i***) mutations in gene(s) often associated with the mechanism of action of the compound, and (***ii)*** acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) (Munita and Arias, 2016).

Bacterial cells derived from a susceptible population often develop mutations in genes that affect the activity of specific antibiotics, resulting in preserved cell survival in the presence of the antibiotic molecule. Once a resistant mutant emerges, the antibiotic eliminates the susceptible population and the resistant bacteria predominate. Mechanisms of mutational antibiotic resistance includes the following; modifications of the antibiotic target to decrease the affinity for the drug, a decrease in the drug uptake, activation of efflux mechanisms to extrude the antibiotic molecule and global changes in important metabolic pathways via

modulation of regulatory networks (Lo Grasso *et al*., 2016). Thus, resistance arising due to acquired mutational changes is diverse and varies in complexity.

Acquisition of foreign DNA material through HGT is one of the most important drivers of bacterial evolution and it is frequently responsible for the development of antibiotic resistance. Classically, bacteria acquire external genetic material through three main mechanisms; transformation i.e. incorporation of naked DNA, transduction which is phage mediated and conjugation (Munita and Arias, 2016).

2.7.2 BACTERIAL GENOME AND NEW ANTIBIOTICS

With the increasing number of bacterial genome sequences, together with techniques that permit global transcriptional and proteomic profiling of bacterial cells under different conditions, hundreds of genes have been evaluated as targets for new antibiotics. A gene is recognized as essential when the bacterium cannot survive while the gene is inactive, and can become a target when a small molecule can alter its activity (Procópio *et al*., 2012).

Once bacterial targets with the desired characteristics are identified, these targets can be incorporated into *in vitro* biochemical assays that permit high throughput screening of chemical libraries for the identification of inhibitory compounds. The antibacterial activity of compounds identified during the screening can then be characterized using standard assays with bacterial cells. Compounds that demonstrate adequate antimicrobial activity can be further developed through preclinical and clinical studies into molecules similar to the ones identified using traditional discovery methods (Fields *et al*., 2017).

Although approaches based on genome sequencing data have the potential to contribute significantly to antibiotic development, initial efforts have not lived up to expectations, as few leads have been identified and new molecules discovered using these approaches have entered the market (Fields *et al*., 2017). The current antibiotic resistance crisis however

requires a continuous discovery and development of new antibiotics which traditional discovery methods like the screening of natural products synthesized by actinomycetes and different fungal species has provided over the decades.

2.8 ISOLATION OF SOIL ACTINOMYCETES

Discovery of novel actinomycetes metabolites from the soil can be enhanced by isolating diverse group of actinomycetes. It is pertinent to acquire new strains and avoid re-isolating strains that produce already known bioactive metabolites while preventing the growth of non-actinomycetes organisms, thus, novel isolation methods tend to be very critical (Hayakawa *et al.,* 2008). General bacterial isolation methods like; serial dilution, pour plate, streak plate and centrifugation techniques are also applicable for actinomycetes isolation. Centrifugation of soil sample followed by serial dilution of supernatant can increase the chances of actinomycetes growth on plate. But none of these methods can selectively isolate actinomycetes, making the purification of actinomycetes a challenging task (Kumar and Jadeja 2016).

Selective isolation of actinomycetes has been achieved using six approaches: (i) Pre-treatment of sample, in which soil samples are treated with physical or chemical method in order to decrease the number of non-actinomycetes organisms, (ii) selective inhibition, in which inhibitors such as antifungal agents and antibiotics are incorporated to inhibit non-actinomycetes organisms, (iii) nutritional selection, where media are formulated with nutritional components, which are preferentially utilized by actinomycetes, (iv) Enrichment method, in which nutrient media can be enriched with certain additional supplements, which favours the growth of actinomycetes or inhibit the growth of other microbes, (v) Membrane filter method, which does not dependent upon pre-treatment, specific media or antibiotics,

and (vi) Integrated method, in which any combination of different approaches can be applied (Kumar and Jadeja 2016).

2.8.1 PRE-TREATMENT TECHNIQUES

Pre-treatment of samples is very important for the selective isolation of actinomycetes, which grow slower than other bacteria and fungi. In general, pre-treatment processes select target actinomycetes by either promoting their growth or inhibiting unwanted microorganisms. Several chemical and physical pre-treatments have been used for the isolation of actinomycetes (Hayakawa, 2008).

Physical Treatments

Actinomycetes Spores are more resistant to desiccation than gram negative bacteria and hence, dry heating of soil samples at 120^{o}C for 1 hour favours the growth of *Streptomyces* and other rare genera. Other dry heat treatment temperatures and periods in literature includes: 110^{o}C for 10 minutes, 50-60^{o}C for 10 minutes, 45^{o}C for 3-12 hours, etc. (Dhawane and zodpe, 2017; Jiang *et al*., 2016; Rachdiati *et al*., 2016; Chaudhary *et al*., 2013). Suppression of bacteria and fungi has also been achieved by heating soil suspensions to temperatures ranging from 50^{o}C to 120^{o}C for periods ranging from 10 minutes to 1 hour accordingly (Singh *et al*., 2016; Jiang *et al*., 2016).

Nocardia species were selectively isolated using sucrose gradient centrifugation (Yamamura *et al.,* 2003). Preferential isolation of motile actinomycetes can also be achieved by centrifugation which eliminates *Streptomyces* species and other non-motile actinomycetes and facilitates motile actinomycetes, retained in the supernatant (Hayakawa *et al.,* 2008).

Sample suspensions can be treated with ultrasonic waves at 180 W for 40 seconds. This helps to release saprophytes fixed to soil granules into the suspension, increase account of actinomycetes, and reduced bacteria in the sample (Jiang *et al*., 2010).

Selective isolation of actinomycetes was also favoured by radiation (Bredholdt *et al.,* 2008). Super-high frequency irradiation favours the isolation of *Streptosporangium* and *Rhodococcus* species. Extremely high frequency irradiation was effective for *Streptosporangium* spp., *Nocardiopsis* and *Nocardia* while UV-irradiation was suitable for the isolation of *Nocardiopsis* and *Pseudonocardia* spp (Kumar and Jadeja, 2016).

Chemical Treatments

Chemicals treatments such as Calcium carbonate, chitin, calcium chloride, Phenol, sodium dodecyl sulphate (SDS), yeast extract, Germicides, Chemotactic agents, and Chloramine-T can be used for selective isolation of actinomycetes. Selective development of actinomycetes was increased when air-dried soil was re-moistened, mixed with calcium carbonate and incubated at 37ºC for 7 days (Rachdiati *et al*., 2016; Sharma and parihar, 2010).

Soil sample treated with 0.05% SDS and 5% yeast extract favoured the growth of *Streptomyces* and other genera (Hayakawa *et al.,* 1989).

Micromonospora and *Streptomyces* species have been selectively isolated by pre-treating soil samples with 1.5% phenol (Hayakawa *et al.,* 2004). Treatment of soil suspensions with phenol solution for 30 minutes was recommended by Singh *et al*. (2016).

Treatment of soil samples with chloramine-T was found to promote the growth of *Herbidospora, Microbispora, Microtetraspora, Nonomuraea and Strepto-sporangium* (Hayakawa *et al.,* 1997).

2.8.2 SELECTIVE INHIBITION OF NON-ACTINOMYCETES

Various antibiotics and antifungal agents can be used for the selective isolation of different actinomycetes. Achieving the suppression of bacteria while allowing growth of actinomycetes presents some difficulties since most antibacterial antibiotics also inhibit actinomycetes, hence, a need for careful antibacterial selection (Jiang *et al*., 2016). Dulaney

et al. (1955) recommended a combination of antibacterial and antifungal antibiotics to allow selective development of actinomycetes. Many workers have used different combinations antibiotics in media to achieve selective inhibition of various groups of organisms. The use of cyclohexamide and nystatin to inhibit bacteria and fungi respectively and increase the efficiency of media for isolating *Streptomyces* species was reported by Gong *et al*. (2018) and Chaudhary *et al*. (2013). The incorporation of nalidixic acid and nystatin into culture media was effective in the isolation of actinomycetes from soil samples (Priyadarshini *et al*., 2016; Njenga *et al*., 2017). A combination of amoxicilline and cyclohexamide was recommended by Bizuye *et al*. (2013) while Kumar *et al*. (2010) recommended the use of rifampicin and amphotericin B as antibacterial and antifungal agents respectively.

2.8.3 NUTRITIONAL SELECTION FOR ACTINOMYCETES

The design of selective isolation media for actinomycetes needs to colligate factors, such as isolation goals, target actinomycetes taxa and medium component (carbon and nitrogen sources). The component of selective isolation media can be formulated by using information from taxonomic databases and phenotypic databases (Jiang *et al*., 2016). Several carbon and nitrogen sources have been considered as selective substrates for actinomycetes. Kuster and Williams (1964) concluded that starch or glycerol, casein and nitrate were the most selective mixture for actinomycetes. The use of chitin as a sole carbon and nitrogen source was recommended by Lingappa and Lockwood (1962). Protein and amino acids as a nitrogen sources, play very crucial role in the differential isolation of actinomycetes. Porter *et al*, (1960) and El Nakeeb and Lechevalier (1963) separately reported that L- arginine as the sole source of nitrogen favours actinomycetes over bacteria. L-arginine can be replaced by glycine for non-Streptomyces actinomycetes (Kuster and Williams 1964).

Several selective media like starch casein agar (SCA) (Rai *et al*., 2018; Njenga *et al*., 2017; Atuanya *et al*., 2016; Cavalho and Van Der Sand, 2015; Janardhan *et al*., 2014; Khasabuli and Kibera, 2014; Bizuye *et al*., 2013; Kumar *et al*., 2010), actinomycetes isolation agar (AIA) (Dhawane and Zodpe, 2017; Chaudhary *et al*., 2013; Kumar *et al*., 2010; Pandey *et al*., 2011), starch nitrate agar (Njenga *et al*., 2017), basal salt agar (Jarallah and Rahaman, 2014), tryptic soy agar (TSA) (Rachdiati *et al*., 2016), chitin agar (Agadagba, 2014), international Streptomyces project (ISP) media 1-7 (Priyadarshini *et al*., 2016) and humic acid vitamin (HV) agar (Hayakawa *et al.,* 1991d) are popular for specific isolation of actinomycetes.

2.8.4 ENRICHMENT METHODS

Diverse genera of actinomycetes have been selectively isolated using various enrichment methods. Hayakawa *et al*. (1991c) developed an improved chemotactic method which utilizes the strong chemotactic response of actinomycete zoospores to γ-collidine. This method showed increased recovery of *Actinoplanes* and *Dactylosporangium* species from various soil samples. Hayakawa *et al*. (1995b) also found that vanillin is successful as a chemo attractant for isolating *Catenuloplanes* species from soil. The use of vanillin also enabled the isolation of members of the genus *Virgosporangium* (Tamura *et al*., 2001).

A technique which involved the use Pinus pollen grain baiting was used to isolate *Actinoplanes* spp. from soil (Hayakawa *et al*., 1991d). The bait bearing the sporangia in the dried soil particles is desiccated with silica gel and the spores are liberated upon immersion in water. A portion of the enriched zoospore sample is then plated on HV agar containing nalidixic acid. The desiccation stage almost completely eliminates contamination of bacteria from the colonized bait and resulted in 83% selective isolation of *Actinoplanes* species.

2.8.5 MEMBRANE FILTER METHOD

Membrane filter method described by Hirsch *et al*. (1983) is used for selective isolation of filamentous actinomycetes from natural mixed microbial populations without relying upon specific media and antibiotics. This is done by overlaying nutrient agar medium is with 0.22 to 0.45µm pore size cellulose ester membrane filter followed by inoculation of filter surface with mix cultures and incubation to allow the growth of bacteria. Actinomycetes produce highly branched mycelial networks and hence they have ability to penetrate the pores of membrane filter. During incubation, mycelium of actinomycetes penetrates the filter pores to the underlying agar medium, whereas non-actinomycetes bacteria are restricted to the filter surface. Removal of membrane filter and incubation of agar medium allow the development of the isolated actinomycetes colonies. Njenga *et al*. (2017) successfully used the membrane filter method to culture several actinomycetes isolates from the soil.

2.8.5 INTEGRATED METHOD

This is most preferred method for isolating actinomycetes. It involves a combination of physico-chemical methods with suitable antibiotics and other selective methods to promote the desired growth of actinomycetes. Primary treatment of soil with calcium carbonate followed by centrifugation and plating on tryptic soy agar (TSA) containing Amoxicillin and fluconazole showed tremendous increase in the number of the actinomycetes isolated as pure cultures (Rachdiati *et al*., 2016).

Calcium carbonate, rehydration and centrifugation methods were integrated and HV agar with fradiomycin, kanamycin, trimethoprim, nalidixic acid antibiotics were used by Otoguro *et al.* (2001) for the enrichment and selective isolation of *Actinokineospora* species from soil and plant litter. The combination of pollen-baiting and drying method using humic acid

vitamin agar with nalidixic acid promoted the growth of *Actinoplanes* (Hayakawa *et al.,* 1991d).

2.9 IDENTIFICATION OF ACTINOMYCETES

Classical, chemotaxonomical, numerical taxonomic and molecular approaches have been used to identify and classify actinomycetes to specie level.

2.9.1 CLASSICAL APPROACH

Classical approaches for identification and classification make use of morphological, physiological, and biochemical characteristics. The classical methodology defined within the identification key by Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974) is very much useful in the identification of actinomycetes.

The use of morphological characteristics is very important in identifying actinomycetes isolates. These characteristics have always been used in initial descriptions, especially of *Streptomyces* species. Characterization is best done by using a variety of standard culture media, including those recommended for the International *Streptomyces* Project (ISP) (Shirling and Gottlieb 1966; Singh *et al*., 2016). Morphological methods consist of both macroscopic and microscopic characterization. Macroscopically actinomycetes isolates are differentiated by their colony characters, e.g. size, shape, colour, consistency, melanoid pigments, reverse side pigments and soluble pigments. Microscopic characterization of isolates is done by cover slip culture, slide culture and Gram staining methods. Microscopic observations including colour of aerial mycelium, colour of substrate mycelium, and spore morphology have been used to identify actinomycetes. Formation of aerial mycelium, substrate mycelium and spores are studied by light microscopy and the spore surface and spore structure by scanning electron microscopy (Sharma *et al*., 2014; Dhawane and Zodpe, 2017; Rachdiati *et al*., 2016).

Physiological methods include; range of pH for growth, optimum temperature for growth and salinity test (Sharma *et al*., 2014). Biochemical tests generally used to identify actinomycetes include; starch hydrolysis, triple sugar iron (TSI) agar test, citrate utilization test, indole test, methyl red test, Voges-Proskauer test, catalase test, oxidase test, urea hydrolysis, hydrogen sulphide production, gelatin liquefaction, casein hydrolysis, nitrate reduction and motility test (Njenga *et al*., 2017; Sharma *et al*., 2014).

2.9.2 CHEMOTAXONOMICAL APPROACH

Chemotaxonomy is that the study of chemical variation in organisms and also the use of chemical constituents in the classification and identification of organisms (Sharma *et al*., 2014). It is an important tool in the classification of actinomycetes into different genera. Chemotaxonomy of actinomycetes focuses on the distribution of specific actinomycetes cell envelope chemicals such as amino acid, sugar, polar lipids, menaquinones, mycolic acid, and fatty acid by using chemical techniques, including the extraction, fractionation, purification, and resolution of the target compounds (Wang and Jiang, 2016).

Actinomycetes can be divided into broad groups at the generic level on the basis of cell wall composition (Wang and Jiang, 2016). For such grouping, the compositions of cell wall diaminopimelic acid isomers and whole cell sugars have become widely accepted as the taxonomic markers. A rapid, simple and inexpensive method which is quite suitable for the separation of diaminoacids of peptidoglycan from whole cells was described by Hasegawa *et al*. (1983). This procedure involves the use of the solvent system of thin-layer chromatography. However, for analysis of the amino acid in the peptide chains or inter-peptide bridge of the peptidoglycan, the cell wall extraction is required. Detailed cell wall extraction was described by Schleifer (1985).

2.9.3 MOLECULAR APPROACH

The most influential approach to identification and classification of microorganisms is through the study of nucleic acids. Identification based on 16S rDNA (16S rRNA gene) sequences has been very significant for actinomycetes identification (Yokota 1997). The phylogenetic tree constructed from 16S rDNA sequences allows the investigation of actinomycetes evolution. Analysis of the 16S rDNA begins by DNA extraction followed by the amplification of the gene coding for 16S rRNA using the polymerase chain reaction. The refined DNA fragments are directly sequenced with a DNA sequencer in order to determine the order in which the bases are arranged within the length of the sample. A computer is then used to compare the sequenced gene with the GenBank to obtain a match (Sharma *et al*., 2014).

2.10 PRODUCTION OF ANTIBIOTICS FROM ACTINOMYCETES

The first step in antibiotics production in the laboratory is usually preliminary (primary) screening of actinomycetes isolates for antimicrobial activities. Wadetwar and Patil (2013) used the perpendicular streak (cross streak) method to achieve this preliminary screening. Isolates were streaked as a single line at the centre of a solidified yeast malt extract agar (ISP 2 medium) in a Petri dish and were incubated at 28°C for seven days. Test organisms were then cross streaked perpendicular to the original streak of actinomycetes isolates. The plates were further incubated at 37°C for 24 to 48 hours for bacteria and at 25°C for 48 to 72 hours for fungi and only isolates with significant zones of clearance were taken for fermentation. A similar method was used by Bizuye *et al*. (2013) and Chaudhary *et al*. (2013).

2.10.1 LIQUID STATE FERMENTATION

Liquid state (submerged) fermentation has been an extremely important procedure in the discovery of new antibiotics. Different strains need different fermentation conditions,

including components, concentration, and pH of broth, and also the time, temperature, and aeration of fermentation. The first step in carrying out submerged fermentation is inoculum development which usually begins on solid media and subsequently liquid media are used; the media used are specific for inoculum development. The objective is usually to achieve a high level of viable biomass in a suitable growth stage and physiological form for use as an inoculum for the next stage (Jiang *et al*., 2016).

To prepare fermentation inoculum, Priyadarshini *et al*. (2016) incubated actinomycetes isolates on agar slant for 7 to 10 days until it was well sporulated. Spore suspension was made by adding sterile water to the slant, the suspension was then used to inoculate a 250 ml conical flask containing 50 ml of potato dextrose broth (PDB) which was used as the inoculum medium. The flask was incubated at 28°C on a rotary shaker (210- 220 rpm) for 48 hours. After incubation, 5 ml of the inoculum medium was transferred to a 250 ml shake flask containing 45 ml of the production medium. Sharma and Parihar (2010) used starch casein nitrate broth as inoculum medium which was inoculated with a loop full of purified actinomycetes growth and incubated at 28°C in shaking incubator at 150 rpm for 5 days.

Production medium for submerged fermentation with actinomycetes can either be defined (synthetic), semi synthetic or complex. It must have a suitable chemical composition; a source of carbon, nitrogen, growth factors and mineral salts. Some investigators reported that glucose decreases antibiotic production by suppressing the enzymes involved in antibiotic biosynthesis and recommended complex compounds such as starch and glycerol as the best carbon sources because they support a slow growth rate that is appropriate for antibiotic production. Many studies however support the use of glucose as carbon source for antibiotic production (Hasani *et al*., 2014). Complex nitrogen sources such as soybean meal, corn steep liquor and yeast extract are preferred to simple inorganic sources for antibiotic production; this can be attributed to slow breakdown of these compounds in the medium (Rafieenia,

2013). Minerals such as phosphorus, potassium, iron, zinc and manganese in trace increase level of antibiotic production (Martin, 2004; Gesheva *et al*., 2005).

Chaudhary *et al*. (2013) carried out submerged fermentation with actinomycetes isolates using ISP1 broth as production media. Fermentation was done in a 250-ml-capacity conical flask under sterile conditions and incubated at 30°C for 7 days at 150 rpm rotation. Jarallah and Rahaman (2014) reported the production of antimicrobial substances when *Streptomyces* sp. was inoculated into a one-litre-capacity flask containing 500 ml of fermentation medium consisting of (g/l): soluble starch, 20.0; $(NH_4)_2SO_4$, 2.0; $K_2 HPO_4$, 1.0; NaCl, 1.0; $MgSO_4$, 1.0; $CaCO_3$, 2.0; trace salt solution ($FeSO_4.7H_2O$, 0.1; $ZnSO_4.7H_2O$, 0.1 and 1000 ml distilled water), pH value of the medium was adjusted at 7.2 before sterilization. The flask was incubated at 25ºC for 5 days in an incubator. Other production media in literature for antibiotics production from actinomycetes include; yeast malt extract (ISP2) broth, nutrient broth, starch casein broth, and maltose yeast extract broth (Wadetwar and Patil, 2013).

2.10.2 SOLID STATE FERMENTATION

Solid state fermentation procedures have also been used for cultivation of actinomycetes at the research stage. The content of bioactive substances produced by actinomycetes in solid fermentation is more than in liquid fermentation. Methods of inoculum development for solid state fermentation are usually similar to the ones used in submerged fermentation (Jiang *et al*., 2016).

Bizuye *et al*. (2013) reported the use of solid state fermentation for antibiotics production from actinomycetes isolates. Cultures of promising isolates from primary screening were grown in starch casein broth (200 ml) at 37 C for 7 days. 10% of cultured broth was then inoculated into sterilize Erlenmeyer flask containing a natural media (40 g wheat grain and 20 ml milk) and incubated on thermostat water bath at 37 C for 7 days. Solid state fermentation

was also used by Rai *et al*. (2018) to the same end. The optimum component of solid medium can be different from one actinomycetes specie to the other. Also, it is has to be emphasized that not all of actinomycetes do well in solid fermentation (Jiang *et al*., 2016).

2.10.3 DOWNSTREAM PROCESSING

Downstream processing refers to the recovery and purification of biosynthetic products from fermentation media. Generally, the first step is removal of insoluble components such as cells, cell debris and other particulate matter from the fermented mixture. Typical operations to achieve this are filtration, centrifugation, sedimentation, etc. Additional operations such as grinding and homogenization required to recover products from solid sources are usually included in this group. The next step is product isolation which involves the removal of those components whose properties vary considerably from that of the desired product. Solvent extraction, adsorption, ultrafiltration and precipitation are some of the unit operations involved in this stage. Product purification is usually the final step in laboratory scale processing. This is done to separate those contaminants that resemble the product very closely in physical and chemical properties. Examples of operations at this stage include affinity, size exclusion, reversed phase chromatography, ion-exchange chromatography, adsorption chromatography, crystallization and fractional precipitation.

To recover and purify antibiotic metabolites, Singh *et al*. (2016) centrifuged fermented broth at 10,000 rpm for 20 minutes to separate the biomass, the active metabolite was then recovered from the fermented broth using two phase solvent extraction system with organic solvent. Solvents containing the active compounds were concentrated under vacuum to get dried crude extract. The obtained crude extract was treated with non-polar solvents like hexane or chloroform to separate the polar and non-polar components. The active components were finally purified by adsorption chromatography using silica gel (pore size

60Å, mesh size 230–400, particle size 40–63µm) as a stationary phase and gel filtration chromatography using sephadex LH- 20.

Sharma and Parihar (2010) harvested, filtered and centrifuged fermented broth to remove cells and debris. The filtrate was then mixed with ethyl acetate in the ratio of 1:1 (v/v) and shaken vigorously for 1 hour in a solvent extraction funnel. The solvent phase that contains antibacterial compound was separated from the aqueous phase. Solvent phase was evaporated to dryness in water bath at 80 - 90°C and the residue was used to check antibacterial activity. Janardhan *et al*. (2014) used a procedure similar to the one described above but differs only in the use of rota evaporator for concentration of extracts.

Jarallah and Rahaman (2014) also used ethyl acetate as solvent for extraction but concentrated the extracts by evaporation under vacuum to the least volume before using anhydrous Na_2SO_4 to achieve final dehydration. The extracted antimicrobial agent was then purified by thin layer chromatography on silica gel plate.

Rai *et al*. (2018) added an equal volume of ethyl acetate to a solid state fermented culture for 1 hour in thermostat water bath shaker at 37°C. The ethyl acetate containing active metabolite was then separated from the solid residue with Whatman No. 1 filter paper. The crude extracts obtained was re-dissolved in ethyl acetate and used as stock concentration for determination of antimicrobial activity against test pathogens using ethyl acetate as a control.

2.11 ANTIMICROBIAL ACTIVITY PROTOCOLS

A variety of laboratory methods can be used to evaluate or screen the *in vitro* antimicrobial activity of an extract or a pure compound. The most known and basic methods are; cross streak method, agar overlay method, disc diffusion method and agar well diffusion method (Hasani *et al*., 2014). The cross streak method is used to rapidly screen microorganisms for

antagonism and has been described in section 2.10 as a method mostly used for primary screening of isolates before fermentation.

Agar overlay method

In this method, actinomycetes isolates are spot inoculated on a solid medium and incubated for 7 days at 30°C. At the end of the incubation period, one ml of chloroform is added to the colony to arrest the growth of the cells. After 40 minutes, the plate is overlaid with 7 ml of semi-solid nutrient agar inoculated with 0.1 ml of overnight broth culture of test organism. Plates are then incubated again for 24 hours at 37°C and zone of inhibition is measured in millimetres (Hasani *et al*., 2014; Kumar *et al*., 2010).

Disc Diffusion assay

Disc diffusion is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, the antimicrobial agent diffuses from the disc into the agar and inhibits growth of the test microorganism, creating a zone of inhibition whose diameter is then measured (Muleta and Assefa, 2018; Gong *et al*., 2018; Khasabuli and Kibera, 2014; Rachdiati *et al*., 2016).

Agar well diffusion assay

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts. The procedure is similar to that of disc diffusion; the agar plate surface is inoculated by spreading a volume of the test organism inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8mm is punched aseptically with a sterile cork borer, and a volume (20–100 ml) of the antimicrobial agent or extract solution at desired concentration

is introduced into the well. The agar plates are incubated under suitable conditions depending on the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested (Bizuye *et al*., 2013; Cavalho and Van Der Sand, 2015; Chaudhary *et al*., 2013; Jarallah and Rahaman, 2014; Priyadarshini *et al*., 2016; Rai et al 2018).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

A total of four soil samples were collected from four different locations in Benin City (geographical coordinates: latitude 6.3350°N and longitude 5.6037°E), Edo State, Nigeria in June 2018. Every sample was a mixture of soil collected from 4 to 5 holes (from the same site) whose depth was around 1 to 10 cm. Surface layer of the soil was removed and the central portion was collected in sterile plastic bags with the help of a trowel, previously sterilised with ethanol. All samples were labelled accordingly, transported immediately to the laboratory and air dried in laminar airflow cabinet for 24 hours at room temperature and stored at 4°C for further analysis.

3.2 PRE-TREATMENT OF SOIL SAMPLES

The soil samples were pretreated with $CaCO_3$ to eliminate gram negative bacteria; 10 g of each sample was taken in a mortar and triturated with 1% $CaCO_3$ and incubated at 30°C for 2 days in a closed sterile Petri dish. A high relative humidity was maintained in the Petri dish by water saturated filter paper. Nystatin was later added to the $CaCO_3$-treated soil cultures to suppress the growth of fungi. Portions of each soil sample were also cultured without $CaCO_3$ treatment and a combination of nystatin and neomycin plus polymyxin B was used to inhibit fungi and bacteria respectively (Wadetwar and Patil, 2013; Chaudhary *et al*., 2013).

3.2 ISOLATION OF ACTINOMYCETES

One gram each, of both the air dried and $CaCO_3$-treated soil samples were suspended in 100 ml of sterile distilled water and the flasks were rotary shaken at 150 rpm for 30 min. From these suspensions, dilutions were prepared as 1:10, 1:100, and 1:1000 in distilled water.

Aliquots of 0.1ml of the highest dilutions were taken and spread evenly over the surface of two different types of media. The different media used for isolation were actinomycetes isolation agar (AIA) and International Streptomyces project media 2 (ISP-2). A solution nystatin (50µg/ml of media), prepared in ethanol and a solution of neomycin plus polymyxin B (50µg/ml of media) prepared in distilled water sterilized by autoclaving for 15 min at 121°C were added to the media accordingly. The plates were incubated at 28°C for 7 days. Typical actinomycetes colonies (tough, leathery or rough, chalky) were selected and further isolated by streak plate technique. Single isolated colonies were transferred to ISP 2 agar slants and stored at 4°C until further use (Muleta and Assefa, 2018; Njenga *et al*., 2017; Priyadarshini *et al*., 2016; Bizuye *et al*., 2013).

3.3 SCREENING OF ISOLATES FOR ANTIMICROBIAL ACTIVITY

3.3.1 TEST MICROORGANISMS

The test organisms used for antimicrobial screening were: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. typhi*, *Klebsiella pneumoniae*, *Candida albicans*, *Mucor* sp., *Aspergillus niger*, *Aspergillus fumigatus* and *Fusarium* sp. The test bacteria and fungi were obtained from University of Benin teaching hospital (UBTH) Microbiology laboratory and Mycofarms Research Laboratory, Benin City.

3.3.2 PRIMARY SCREENING

Primary screening for evaluating the antimicrobial potential of the axenic cultures was performed by perpendicular streak method against the test microorganisms. Isolates were screened for antagonism by inoculating a single streak of the pure actinomycetes isolates in the middle of the assay media plate; nutrient agar and sabouraud dextrose agar for bacteria and fungi respectively. The plates were incubated for 5 days at 28◦C and subsequently seeded

with test organism by a single streak at a 90° angle to the initial actinomycetes streak and finally the plates were incubated at 37°C for 24 to 48 hours for bacteria and at 25°C for 48 to 72 hours for fungi. The microbial interactions were analysed by determining the distance of inhibition measured in millimetres. Microbial strains showing moderate to good inhibition activity were selected for secondary screening (Wadetwar and Patil, 2013; Muleta and Assefa, 2018; Bizuye *et al*., 2013; Singh *et al*., 2016).

3.3.3 SECONDARY SCREENING

Metabolite Production

The isolates showing positive results in primary screening were subjected to secondary screening in a small scale submerged fermentation system. Well sporulated 7 to 10 days isolates were cultured in slants. 10ml of sterile water was added to each slant and the resulting spore suspension was added to a 250ml conical flask containing 100ml of the inoculum medium (potato dextrose broth) and incubated at 28°C on a rotary shaker (210-220 rpm). After 4 days of incubation, 10ml of the inoculum medium was transferred to another 250ml conical flask containing 100ml of the production medium (ISP-2 broth). The flasks were incubated at 28°C for 7 days on a rotary shaker. At the end of the fermentation period, 10ml of the production medium was collected into sterile centrifuge tubes and centrifuged at 5000 rpm for 15 minutes to separate the fermented broth and the mycelium. The clear supernatant was used for antimicrobial assay by agar well diffusion method (Cavalho and Van Der Sand, 2015; Priyadarshini *et al*., 2016; Singh *et al*., 2016; Nanjwade *et al*., 2009).

Agar Well Diffusion Assay

Molten sterile agar media (nutrient agar and Sabouraud dextrose agar) were poured into sterile Petri plates and inoculated with the test organisms by spread plate techniques. Wells were made using sterile cork borer and 50µl of the clear fermented broth supernatant was

added to each well. The plates were kept in a refrigerator for about 2 hours to allow the diffusion of the bioactive metabolite. After 2 hours, plates were incubated at 37°C for 24 to 48 hours for bacteria and at 28°C for 48 to 72 hours for fungi. The inhibition zones in millimetres (mm) were measured at the end of the incubation period. Actinomycetes isolates with good inhibition activity were then selected for morphological and molecular identification (Chaudhary *et al*., 2013; Priyadarshini *et al*., 2016; Jarallah and Rahaman, 2014).

3.4 IDENTIFICATION OF SELECTED ACTINOMYCETES ISOLATES

3.4.1 GRAM STAINING

Smear was prepared by spreading actinomycetes broth culture on a glass slide followed by heat fixing. The smear was covered with crystal violet for 60 seconds and washed off with water. It was then covered with Gram's iodine for another 60 seconds, decolorized with alcohol and washed with water. Finally the smear was stained with safranin counter stain for 60 seconds, washed, dried and viewed under a light microscope.

3.4.2 MORPHOLOGICAL CHARACTERIZATION

Actinomycete isolates were inoculated on ISP-2 media and incubated for 5 days at 28°C. The colonies formed were observed under a high-power magnifying lens and colony morphology was noted with respect to colour of mycelium, diffusible pigment, elevation and the nature of colony (Njenga *et al*., 2017; Chaudhary *et al*., 2013).

3.4.3 PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

After antimicrobial studies, the isolates which were found to be positive were selected for physiological and biochemical studies. Tests carried out are; gelatin hydrolysis, starch hydrolysis, urea hydrolysis, hydrogen sulphide production test, motility test, triple sugar iron

(TSI) agar test, citrate utilization test, indole test, methyl red test, Voges-Proskauer test, catalase test and oxidase test (Chaudhary *et al*., 2013; Mohan *et al*., 2014).

Catalase test

A colony of the selected actinomycetes isolate was placed on a microscopic slide. Using a Pasteur pipette, 1 drop of 3% Hydrogen peroxide was placed on the colony on the microscope slide and observed for immediate bubble formation. Positive reactions were evident by immediate bubble formation.

Oxidase test

Two drops of 1% tetra-methyl-p-phenylenediamine solution was placed on a piece of Whatman No. 1 filter paper. A colony of the actinomycetes isolate was transferred onto soaked filter paper using a sterile glass rod and observed for purple colouration. Positive tests were indicated by development of purple colour in 5-10 seconds.

Citrate utilization test

Actinomycetes isolate to be tested was inoculated into a test tube containing Simmon's citrate broth and incubated at 28°C for 4 days. If the organism has the ability to utilize citrate, the medium changes its colour from green to blue at the end of the incubation period.

Urea hydrolysis test

Slants of urea agar medium were prepared and inoculated with the actinomycetes isolates on the entire surface of the slant. The tubes were inoculated at 28°C and observed for a colour change after 24 hours and every day up to 6 days. Urease production was indicated by a bright pink (fuchsia) colour on the slant. Any degree of pink colour development was considered as a positive reaction. To eliminate protein hydrolysis as the cause of a positive test, a control medium lacking urea was used.

Indole test

Actinomycetes isolate to be tested was inoculated in peptone water, containing the amino acid tryptophan, and incubated at 28°C for 4 days. After incubation few drops of Kovac's reagent were added. Formation of a red or pink colour indicated the positive reaction.

Voges Proskaeur (VP) test

Actinomycetes isolate to be tested was inoculated into glucose phosphate broth and incubated for 4 days. 0.6 ml of alpha-naphthol was added to the test broth and shaked, followed by the addition of 0.2 ml of 40% KOH to the broth with shaking. The tube was allowed to stand for 15 minutes. Appearance of red colour was taken as a positive test. The tubes showing negative result were held for one hour, since maximum colour development occurs within one hour after addition of reagents.

Methyl red (MR) test

The actinomycetes isolate to be tested was inoculated into glucose phosphate broth containing glucose and phosphate buffer and incubated at 28°C for 4 days. Over the 4-day period the mixed-acid producing organisms produced sufficient acid to overcome the phosphate buffer and remain acidic. The pH of the medium was tested by addition of 5 drops of MR reagent. Development of red colour was taken as positive. MR negative organisms produced yellow colour.

Triple sugar iron (TSI) agar test

TSI agar slants were made, such that each tube contained both a slant (at the top) and a butt (at the bottom). Inoculation was done with a sterilized straight needle by first stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The test tube caps were left loosely on and incubated at 28°C for 4-5 days.

The tubes were then examined for the development of bubbles/cracks, yellow, red or black colouration.

Motility test

The actinomycetes isolates to be tested were inoculated on motility test agar. The tubes were then incubated at 28°C for 4 to 5 days. Motile organism spreads out from the line of inoculation and establish broad zone of growth in various patterns, while non-motile organisms do not exhibit any such motility patterns.

Starch hydrolysis

Starch agar plates were prepared inoculated with the actinomycetes isolates, by making a single streak line at the centre of the plate before incubation at 28°C for 5 days. After incubation, iodine was added to see whether the starch in the agar around the growing organism had been hydrolyzed by α- amylase or not. Iodine reacts with starch to produce a blue/black colour. Where starch had been hydrolyzed there was clear zone around the bacterial growth because the starch was no longer in the agar to react with the iodine. Where starch had not been hydrolyzed, the agar turns blue/black colour.

Gelatine liquefaction / gelatine hydrolysis test

Gelatin hydrolysis was tested using a nutrient gelatin medium, which contained peptone, beef extract, and gelatin. Gelatin served as both solidifying agent and substrate for gelatinase activity. Actinomycetes isolate to be tested was stab-inoculated into tubes containing the medium and incubated at 28°C for 5 days. Gelatinase positive organisms produced gelatinases which resulted in the liquefaction of the medium. Since gelatin was digested and unable to form gel, the medium remained liquid when placed in an ice bath. The same inoculated with a gelatinase negative bacterium remained solid after the cold treatment.

3.4.4 MOLECULAR IDENTIFICATION

The actinomycetes isolate with the best antimicrobial activity was selected for molecular identification by 16S ribosomal RNA gene sequencing (Priyadarshini *et al*., 2016).

CHAPTER FOUR

RESULTS

A total of 28 different actinomycete isolates were recovered from soil samples collected at a depth of 1 to 10 cm from four different locations in Benin City. Of these 28 isolates, 7 were isolated from clayey, water lodged soil with sparse vegetation, 6 from loamy soil with forest canopy cover, 11 from sandy soil with grasses cover and 4 from a refuse dump site with sandy soil, as presented in table 1.

Table 1: Physico-chemical properties of soil samples collected from different sites in Benin City and their average culturable actinomycetes counts

Sample sites	Description of sample area	Soil pH	Soil temperature (°C)	Average actinomycetes count (CFU/g)	Number of actinomycetes isolates
A	Clayey, water lodged soil with sparse vegetation	5.74 ± 0.02	25 ± 0.5	$3.0\times10^4 \pm 2.4$	4
B	Loamy soil with forest canopy cover	6.21 ± 0.02	25 ± 0.5	$3.3\times10^4 \pm 1.2$	6
C	Sandy soil with dense grasses cover	5.65 ± 0.006	26 ± 0.5	$3.6\times10^4 \pm 1.9$	11
D	Refuse dump site with sandy soil	5.47 ± 0.02	26 ± 0.5	$3.1\times10^4 \pm 0.7$	7

Values are means±SD

Out of the 28 actinomycetes isolates subjected to primary screening, 10 isolates showed varying levels of antimicrobial activities against the test organisms (Table 2). The morphological and cultural characteristics of the 10 isolates which were selected for further analysis are presented in table 3

Table 2: Primary screening of actinomycetes isolates for antimicrobial activity.

Isolates	**Test microorganisms**									
	E. coli	***P. aeruginosa***	***S. aureus***	***S. typhi***	***C. albicans***	***K. pneumoniae***	***Mucor* sp.**	***A. niger***	***A. fumigatus***	***Fusarium* sp.**
AXP1	+	-	-	+	-	+	-	-	-	-
BYQ3	+	-	+	-	-	+	+	-	-	-
BYQ4	+	+	-	-	-	+	-	-	-	-
CYP1	+	-	+	+	+	+	+	+	+	+
CYP2	+	+	+	-	+	+	+	+	+	+
CYP4	+	-	+	+	+	+	-	-	-	-
CYQ1	-	-	-	-	+	-	-	+	+	-
CYQ2	+	-	-	+	-	-	-	+	+	-
CYQ7	-	+	-	-	+	-	-	-	-	+
DYQ4	-	-	+	-	+	-	-	-	-	-

+ Active against test organism; - inactive against test organism.

Table 3: Cultural characteristics of selected actinomycetes isolates on ISP-2 media

Isolates	Aerial mycelium	Diffusible pigment	Elevation	Surface
AXP1	grey	-	flat	smooth
BYQ3	grey	-	raised	rough
BYQ4	grey	-	raised	rough
CYP1	white	+	raised	smooth
CYP2	brown	+	raised	rough
CYP4	white	+	raised	rough
CYQ1	grey	-	raised	rough
CYQ2	brown	+	flat	rough
CYQ7	black	-	raised	smooth
DYQ4	grey	-	flat	rough

+ Diffusible pigment present; - Diffusible pigment absent.

Upon primary screening, 5 isolates were identified based on spectrum activity against test organisms, and the antimicrobial activity of secondary metabolite extract of these isolates confirmed through secondary screening (Table 4).

Table 4: Antimicrobial activities of selected actinomycetes isolate crude extracts

Test microorganisms	**Antimicrobial activity (Zones of inhibition in mm)**				
	BYQ3	**CYP1**	**CYP2**	**CYP4**	**CYQ2**
E. coli	3 ± 0.6	2 ± 0.0	4 ± 1.0	2 ± 0.0	3 ± 0.1
P. aeruginosa	-	-	2 ± 0.6	-	-
S. aureus	2 ± 0.6	2 ± 0.0	3 ± 1.8	3 ± 1.0	-
S. typhi	-	3 ± 1.0	-	4 ± 1.7	2 ± 0.0
C. albicans	-	26 ± 0.5	20 ± 0.6	-	-
K. pneumoniae	3 ± 0.0	2 ± 0.6	-	3 ± 0.6	-
Mucor sp.	2 ± 0.0	23 ± 1.5	18 ± 2.0	-	-
A. niger	-	17 ± 1.0	12 ± 1.5	-	4 ± 1.0
A. fumigatus	-	18 ± 1.5	14 ± 1.5	-	3 ± 1.8
Fusarium sp.	-	9 ± 2.0	-	-	-

- Inactive against test organism; Values are means±SD

Based on the result of secondary screening, only extracts from 2 out of 5 isolates gave significant zones of inhibition against 5 fungal test organisms. Antifungal activities of the 2 effective isolates were then compared with standard antifungal drugs (figure 1).

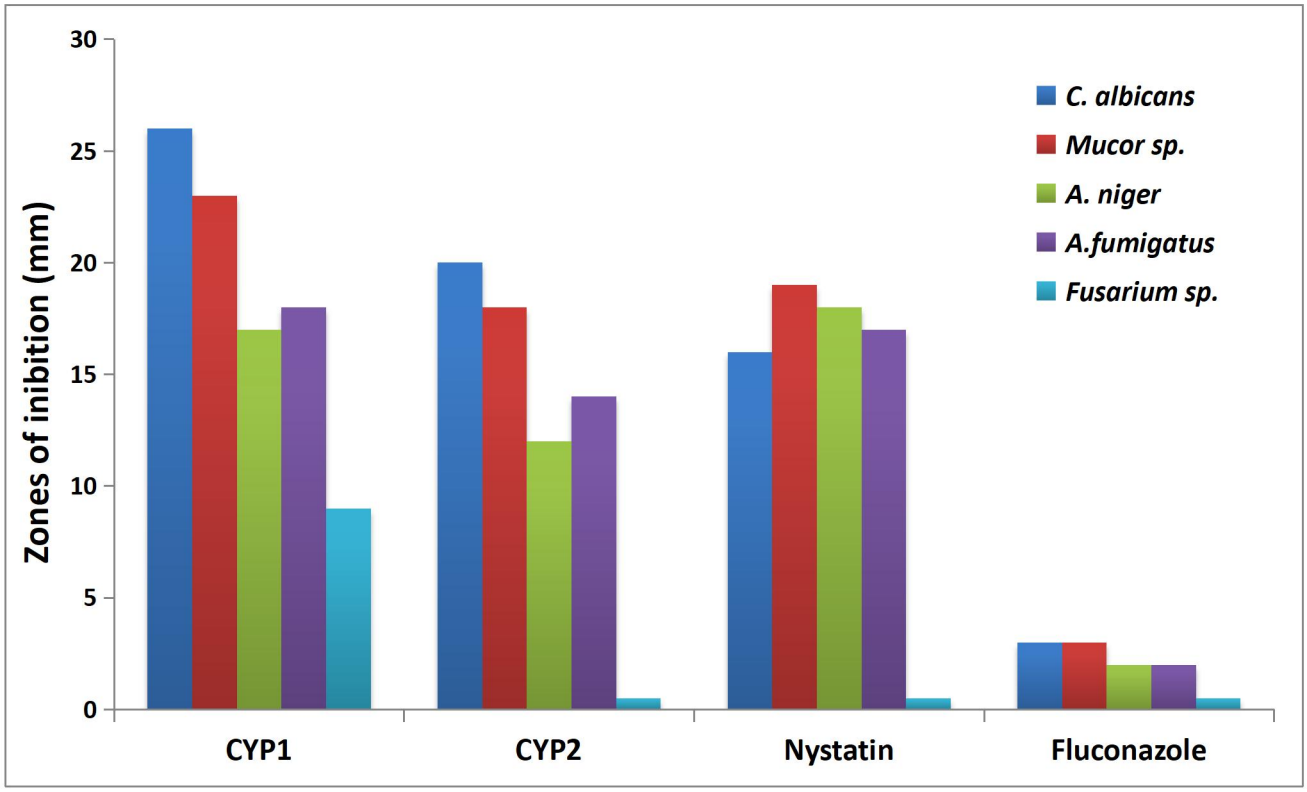

Figure 1: Antifungal activities of crude extracts compared to standard antifungals

The two actinomycetes isolate whose crude extracts produced significant zones of inhibition against fungal test organisms were subjected physiological and biochemical tests as shown in table 5. Furthermore, the actinomycetes isolate with the best antimicrobial activity was selected for molecular identification by 16S ribosomal RNA gene sequencing; rRNA banding profile is shown in plate 1 and partial gene sequence in appendix B.

Table 5: Physiological and biochemical characterization of actinomycetes isolates

Tests		Isolates	
		CYP1	CYP2
Gram reaction		+	+
Catalase		+	+
Oxidase		+	+
Citrate		-	-
Indole		-	-
Methyl red		+	+
Urease		+	-
Voges-proskauer		-	+
Motility		-	-
Gelatin hydrolysis		+	+
Starch hydrolysis		+	+
TSI	**Gas production**	-	-
	H_2S production	-	+
	Glucose fermentation	+	+
	Lactose fermentation	+	+

+ Positive, - Negative

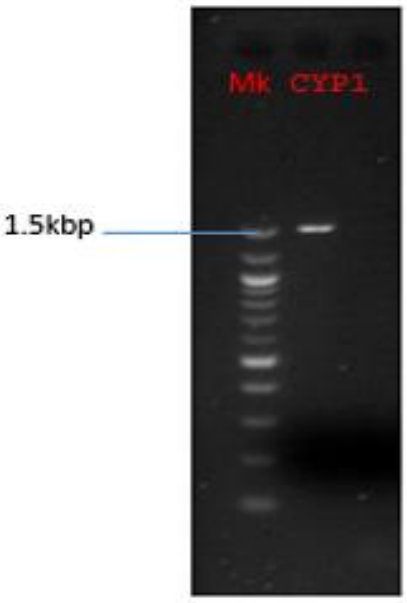

Plate 1: 16S rRNA banding profile of isolate CYP1

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Antibiotics and antifungals are the most important bioactive compounds for the treatment of infectious diseases, but the emergence of multi-drug resistant pathogens pose a major threat to effective treatment of these diseases. Thus, there has been increasing interest for searching effective antimicrobials from soil actinomycetes in diversified ecological niches. Antimicrobial-producing actinomycetes have been intensively studied in several underexplored environments, niche, and extreme habitats in various parts of the world in the last few years. Yet, there is no report regarding isolation of antimicrobial-producing actinomycetes from Benin City, Nigeria. Therefore, an attempt has been made to isolate actinomycetes from this region in order to find novel species.

In this present study, out of 28 different actinomycete isolates recovered from the soil samples, the greatest percentage (39%) and also the highest average count ($3.6\times10^4 \pm 1.9$ CFU/g) came from sample site C which is characterized by sandy soil with dense grasses cover (table 1). This relatively high population can be attributed to high plant roots density of the sample site. Muleta and Assefa (2018) and Geetanjali and Jain (2016) also reported that greater percentages of actinomycetes were found in rhizospheric soils. Sample sites B (Loamy soil with forest canopy cover) and D (Refuse dump site with sandy soil) had similar percentages of the recovered isolates, 21% and 25% and average counts $3.3\times10^4 \pm 1.2$ CFU/g and $3.1\times10^4 \pm 0.7$ CFU/g respectively (table 1). These figures are higher those obtained from sample site A (Clayey, water lodged soil with sparse vegetation) with 14% of the recovered isolates and an average count of $3.0\times10^4 \pm 2.4$ CFU/g. Sparse vegetation, lower organic matter content as well as higher moisture content of the water lodged, clayey soil of sample

site A, might have contributed to the lower figures obtained from the site (Hasani *et al*., 2014; Nanjwade *et al*., 2010; Williams *et al*., 1972).

It has been reported that 1g of soil when plated, harbours up to 10 billion microorganisms, of which about 4.2 × 10^6 CFU/g (dry weight) are accounted for by bacteria species (Torsvik, and Ovreas, 2002). The highest, average actinomycetes count ($3.6\times10^4 \pm 1.9$ CFU/g) obtained in this study was however lower than expectation. This may be partly attributed to the slightly acidic pH of the soil samples.

Out of the 28 actinomycetes isolates subjected to primary screening, 10 (36%) isolates showed varying levels of antimicrobial activities against the 10 test microorganisms with 80% of the potent isolates recovered from the rhizospheric soils (Table 2). The present result is in agreement with that of Dhawane and Zodpe (2017) who reported that microorganisms isolated from rhizospheric soil could be an interesting source of antimicrobial bioactive substances. Abo-Shadi *et al*. (2010) also reported that rhizospheric soil can serve as an effective source of antimicrobial compounds. However, the percentage of potent isolates (36%) obtained in this present study is higher than the 12.82 % and 26.7% reported by Kumar *et al*. (2010) and Bizuye *et al*. (2013) respectively, but lower than 60% reported by Muleta and Assefa (2018). These differences may be attributed to differences in antibiotic resistant capacity of test organisms, the genetic differences of the antibiotic producing isolates, and their capacity to produce more than one secondary metabolite (Muleta and Assefa, 2018).

Out of the 10 different actinomycetes isolates that showed antimicrobial activities, 5 isolates (50%); BYQ3, CYP1, CYP2, CYP4 and CYQ2, showed a wide spectrum of antibiosis against the test organisms. Likewise, 4 (40%) and 1 (10%) of isolates were found to inhibit any 3 and 2 of the test organisms respectively. Isolates that inhibited 3 test organisms were AXP1, BYQ4, CYQ1 and CYQ7 while isolate DYQ4 was found to inhibit only 2 of the test organisms (Table 2). The actinomycetes isolates also showed variations, in inhibiting the test

organisms. Consequently, most of the actinomycetes of isolates 7 (70%) inhibited *E. coli*, followed by 6 (60%) of the isolates inhibiting *C. albicans* and *K. pneumonia*, 50% inhibited *S. aureus* while 40% inhibited *S. typhi, A. niger* and *A. fumigatus*. The most resistant test organisms were *P. aeruginosa, Mucor* sp. and *Fusarium* sp. which were inhibited by only 30% of the potent actinomycetes isolates (Table 2).

All the 10 isolates selected for primary screening grew on ISP-2 agar showing morphology typical of actinomycetes, since the colonies were slow growing, aerobic, tough, leathery or rough/smooth, chalky and with aerial mycelia of different colours (table 3). Diffusible pigment was observed in 4 of the isolates (CYP1, CYP2, CYP4 and CYQ2) on ISP-2 agar. The cultural (Macroscopic) characteristics of aerial mycelium of each isolate range from white, grey, brown to black. These results reveal that the actinomycetes isolates were related to the genus *Streptomyces* (Muleta and Assefa, 2018).

The five (5) actinomycetes isolates that showed wide spectrum of antimicrobial activity in the primary screening were further screened for bioactive compound production through fermentation and agar well diffusion of crude extracts. Observation of clear inhibition zones around the wells on the inoculated plates is an indication of antimicrobial activities of the extracted bioactive compounds against the test organisms.

The range of recorded inhibition zones of crude extracts from isolates against test organisms in the present study was 0 26 mm (table 4). This is higher than figures from Chaudhary *et al.* (2013), Muleta and Assefa (2018) and Priyadarshini *et al*. (2016), who reported highest inhibition zones of 15mm, 17mm and 22mm respectively. It is however lower than 40mm and 30mm reported by Bizuye *et al*. (2013) and Pandey *et al*. (2011) respectively. These differences may be due to genetic differences of the antibiotic producing isolates and differences in fermentation conditions.

Some of the putative isolates of primary screening when subjected to secondary screening, showed different activity from that of primary screening; some of the active isolates showed little activity in the secondary screening while some showed improved activity. According to Pandey *et al*. (2011), during screening of novel secondary metabolites, actinomycetes isolates are often encountered which show antibiotic activity on agar but not in liquid culture. Out of the 5 selected isolates in this present study, only extracts from 2 isolates (CYP1 and CYP2) showed significant activities against 5 fungal test organisms.

Crude extract from isolate CYP1 gave the highest zones of inhibition; 26mm, 23mm, 18mm, 17mm and 9mm against *Candida albicans*, *Mucor* sp., *Aspergillus fumigatus*, *Aspergillus niger* and *Fusarium* sp., respectively (table 4). These figures are higher than those obtained by Mohan *et al*. (2014) who reported inhibition zones of 20mm, 16mm and 12mm against *Candida albicans*, *Aspergillus niger* and *Aspergillus fumigatus* respectively, by antifungal gents extracted from actinomycetes isolated from marine sediments. Wadetwar and Patil (2013) also reported narrower zones of 10mm against *Candida albicans* and 8mm against *Aspergillus niger*. Sharma and Parihar (2010) however recorded wider zones of 23mm and 19mm against *Candida albicans* and *Fusarium* sp respectively. Wider zones of inhibition recorded by Sharma and Parihar (2010) may largely be due to solvent extraction of the crude fermentation broth before its antimicrobial assay.

Comparing the antifungal activities of extracts from isolates CYP1 and CYP2 with those of standard antifungals, nystatin and fluconazole, extract from isolate CYP1 gave wider zones of inhibition than nystatin (20µ/ml) and fluconazole (10µ/ml) as shown in table 5 and fig 1. Lower susceptibility of the test fungi to standard antifungal agents may be attributed to the increasing number of antifungal resistant strains.

Physiological and biochemical characterization of isolates CYP1 and CYP2 showed the same pattern of results (table 5) obtained by previous works done by researchers on actinomycetes

(Chaudhary *et al*., 2013; Mohan *et al*., 2014). These results show that the isolates have been grouped under *Streptomyces* genus (Priyadarshini *et al*., 2016). Further molecular study was carried out for complete identification of isolate CYP1. This isolate was chosen for molecular identification based on its good antimicrobial results. To confirm the identity of the isolate as *Streptomyces* sp., molecular study was done based on 16S rDNA analysis, because nuclear 16S rRNA gene have been revealed to have some variable regions with sequence divergence (Priyadarshini *et al*., 2016). The results obtained from partial sequencing of the 16S rRNA gene (see appendix) supported the fact that isolate CYP1 is closely related (99% identical) to *Streptomyces albus* (strain DSM 40313) which belongs to genus *Streptomyces* in family *Streptomycetaceae*, order *Actinomycetales* and class *Actinobacteria*. Hence, the *Streptomyces* sp. CYP1 is identified as *Streptomyces albus*.

5.2 CONCLUSION

Antimicrobial resistant pathogens have emerged as serious concern in recent years, particularly the acquired multi-drug resistant strains that cause serious public health problems throughout the world. The study of actinomycetes from different environments throughout the world has yielded a lot of antimicrobial agents that are of great value to the treatment of many infectious diseases. Hence, the need to isolate and screen soil actinomycetes from unexplored habitats for new bioactive secondary metabolites.

The findings of this study showed that the antimicrobial compound obtained from isolate CYP1 (identified as *Streptomyces albus*) has antifungal activities. The data, in general, showed that the antimicrobial compounds obtained from the isolate demonstrated broad spectrum and a remarkable antimicrobial activity against *Candida albicans*, *Mucor* sp., *Aspergillus fumigatus*, *Aspergillus niger* and *Fusarium* sp.

The findings also showed that actinomycetes isolates recovered from rhizospheric soil samples have the potential to produce antimicrobial compounds. It is suggested that the other isolates be further processed to fully realize their antibiotic property on different test microorganisms. There is need for further studies to optimize the production conditions of the bioactive compounds from the potent actinomycetes isolates.

REFERECES

Abo-Shadi, M. A., Sidkey, N. M. and Al-Mutrafy, A. M. (2010). Antimicrobial Agent Producing Microbes from some Soils' Rhizosphere in Al-Madinah Al-Munawwarah, KSA. *Journal of American Science* **6**: 915-925.

Adegboye, M. F. and Babalola, O. O. (2012). Taxonomy and ecology of antibiotic producing actinomycetes. *African Journal of Agricultural Research* **7**(15): 2255-2261.

Agadagba, S. K. (2014). Isolation of Actinomycetes from Soil. *Journal of Microbiology Research* **4**(3): 136-140.

Alduina, R., Lo Piccolo, L., D'Alia, D., Ferraro, C., Gunnarsson, N., Donadio, S. and Puglia, A. M. (2007). Phosphate controlled regulator for the biosynthesis of the dalbavancin precursor A40926. *Journal of Bacteriology* **189**(22): 8120-8129.

Arifuzzaman, M., Khatun, M. R. and Rahman, H. (2010). Isolation and screening of actinomycetes from Sundarbans soil for antibacterial activity. *African Journal of Biotechnology* **9**: 4615-4619.

Arthur, M., Reynolds, P. E., Depardieu, F., Evers, S., Dutka-Malen, S., Quintiliani, R. Jr. and Courvalin, P. (1996). Mechanisms of glycopeptide resistance in *Enterococci*. *Journal of Infection* **32**(1): 11-16.

Atuanya, E. I., Dunkwu-Okafor, A. and Udochukwu, U. (2016). Production of Biosurfactants by Actinomycetes Isolated from Hydrocarbon Contaminated Soils and Ikpoba River Sediments in Benin-City, Nigeria. *Nigerian Journal of Basic and Applied Science* **24**(2): 45-52

Baltz, R. H. (2010). Genomics and the ancient origins of the daptomycin biosynthetic gene cluster. *Journal of Antibiotics* **63**:506–511.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H. P., Clément, C., Ouhdouch, Y. and van Wezel, G. P. (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiology and Molecular Biology Reviews* **80**: 1– 43.

Bizuye, A., Moges, F. and Andualem, B. (2013). Isolation and screening of antibiotic producing Actinomycetes from soils in Gondar town, North West Ethiopia. *Asian Pacific Journal of Tropical Disease* **3**(5): 375-381

Bredholt, H., Fjaervik, E., Jhonsen, G. and Zotechev, S. B. (2008). Actinomycetes from sediments in the Trondhein Fjrod, Norway: Diversity and biological activity. *Marine Drugs* **6**: 12-24.

British Crop Protection Council (1994). *The pesticide manual*. 10th edition. British Crop Protection Council, Alton, United Kingdom.

Buchanan, R. E. and Gibbons, N. E. (1974). *Bergey"s manual of determinative bacteriology*. 8th edition. The Williams and Wilkins Co., Baltimore. pp747 - 842.

Busscher, G. F., Rutjes, F. P. and van Delft, F. L. (2005). 2-Deoxystreptamine: central scaffold of aminoglycoside antibiotics. *Chemical Reviews* **105**: 775–791.

Butler, M. S., Hansford, K. A., Blaskovich, M. A., Halai, R. and Cooper, M. A. (2014). Glycopeptide antibiotics: back to the future. *Journal of Antibiotics* **67**: 631–644.

Casadevall, A., Feldmesser, M. and Pirofski, L. (2002). Induced Humoral Immunity and Vaccination against Major Human Fungal Pathogens. *Current Opinion in Microbiology* **5**: 386-391.

Cavalho, T. and Van Der Sand, S. (2016). Evaluation of Antimicrobial Activity of the Endophytic Actinomycete R18(6) against Multiresistant Gram-negative Bacteria. *Annals of the Brazilian Academy of Sciences* **88**(1): 155-163.

Chaudhary, H. S., Yadav, J., Shrivastava, A. R., Singh, S., Singh, A. K. and Gopalan, N. (2013). Antibacterial activity of Actinomycetes isolated from different soil samples of Sheopur (A city of central India). *Journal of Advanced Pharmaceutical Technology and Research* **4**(2): 118-123.

Das, S., Lyla, P. S. and Khan, S. A. (2008). Distribution and generic composition of culturable marine actinomycetes from the sediments of Indian continental slope of Bay of Bengal. *Chinese Journal of Oceanology and Limnology* **26**(2): 166-177.

Dhawane, V. P. and Zodpe, S. N. (2017). Screening and Isolation of Pigment Producers and Non-pigment Producers Actinomycetes from Rhizosperic Soil Sample. *International Journal of Current Microbiology and Applied Sciences* **6**(5): 1570-1578.

Dulaney, E., Larsen, A. and Stapley, E. (1955). A Note on the Isolation of Microorganisms from Natural Sources. *Mycologia* **47**: 420-422.

Elbein, A, D., Pan, Y. T., Pastuszak, I. and Carroll, D. (2003). New Insights on Trehalose: a Multifunctional Molecule. *Glycobiology* **13**:17R–27R.

El-Nakeeb, M. A., and Lechevalier, H. A. (1963). Selective Isolation of Aerobic Actinomycetes. *Applied Microbiology* **11**(2): 75-77.

Endo, A. and Misato, T. (1969). Polyoxin D, a Competitive Inhibitor of UDPN-acetylglucosamine: Chitin N-acetylglucosaminyltransferase in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **37**: 718–722.

Fields, F. R., Lee, S. W. and McConnell, M. J. (2017). Using Bacterial Genomes and Essential Genes for the Development of New Antibiotics. *Biochemical Pharmacology* **134**: 74–86.

Gaynor, M. and Mankin, A. S. (2003). Macrolide Antibiotics: Binding Site, Mechanism of Action, Resistance. *Current Topics in Medicinal Chemistry* **3**: 949–961.

Geetanjali, J. P. (2016). Antibiotic production by rhizospheric soil microflora - a review. *International Journal of Pharmaceutical Sciences and Research* **7**(11): 4304-4314.

Gesheva, V., Ivanova, V. and Genava, R. (2005). Effects of nutrients on the Production of Microbial Antibiotic by *Streptomyces hygroscopicus*. *Microbial Research* **160**(3): 243-248.

Gong, B., Chen, S., Lan, W., Huang, Y. and Zhu, X. (2018). Antibacterial and Antitumor Potential of Actinomycetes Isolated from Mangrove Soil in the Maowei Sea of the

Southern Coast of China. *Iranian Journal of Pharmaceutical Research* **17** (4): 1339-1346.

Hasani, A., Kariminik, A. and Issazadeh K. (2014). Streptomycetes: Characteristics and Their Antimicrobial Activities. *International journal of Advanced Biological and Biomedical Research* **2**(1): 63-75

Hasegawa, T., Takizawa, M. and Tanida, S. (1983). A rapid analysis for Chemical Grouping of Aerobic Actinomycetes. *Journal of General and Applied Microbiology* **29**: 319–22.

Hayakawa, M. (2008). Studies on the Isolation and Distribution of Rare Actinomycetes in Soil. *Actinomycetologica* **22**: 12–19.

Hayakawa, M., Iino, H., Takeuchi, S. and Yamazaki, T. (1997). Application of a Method Incorporating Treatment with Chloramine-T for the Selective Isolation of *Streptosporangiaceae* from Soil. *Journal of Fermentation and Bioengineering* **84**: 599-602.

Hayakawa, M., Ariizumi, M., Yamazaki, T. and Nonomura, H. (1995b). Chemotaxis in the Zoosporic Actinomycete *Catenuloplanes japonicus*. *Actinomycetologica* **9**: 152–163.

Hayakawa, M. and Nonomura. H. (1989). A New Method for the Intensive Isolation of Actinomycetes from Soil. *Actinomycetol*ogica **3**: 95-104.

Hayakawa, M., Tamura, T., Iino, H. and Nonomura, H. (1991d). Pollen-baiting and Drying Method for Highly Selective Isolation of *Actinoplanes* spp. from soil. *Journal of Fermentation and Bioengineering* **72**: 433-438.

Hayakawa, M., Tamura, T. and Nonomura, H. (1991c). Selective Isolation of Actinoplanes and Dactylosporangium from soil by using Collidine as the Chemoattractant. *Journal of Fermentation and Bioengineering* **72**: 426-432.

Hayakawa, M., Yoshida, Y. and Iimura, Y. (2004). Selective Isolation of Bioactive Soil Actinomycetes belonging to the *Streptomyces violaceusniger* phenotypic cluster. *Journal of Applied Microbiology* **96**: 973-981.

Hirsch, P. R., Mauchline, T. H. and Clark, I. M. (2010). Culture-independent Molecular Techniques for Soil Microbial Ecology. *Soil Biology and Biochemistry* **42**: 878-887.

Hitchcock, C., A., Pye, G. W., Tork, D. F., Johnson, E. M. and Warnok, D. W. (1993). Fluconazole Resistance in *Candida glabrata*. *Antimicrobial Agents Chemotherapy* **37**: 1962-1965.

Hug, J. J., Bader, C. D., Remškar, M., Cirnski, K. and Müller, R. (2018). Concepts and Methods to Access Novel Antibiotics from Actinomycetes. *Antibiotics* **7**(44): 1-47.

Imamura, N., Nishijima, M., Adachi, K., and Sano, H. (1993). Novel Antimycin Antibiotics, Urauchimycins A and B, Produced by Marine Actinomycete. *Journal of Antibiotics* **46**(2): 241– 246.

Iqbal, M., Mast, Y., Amin, R., Hodgson, D. A., Wohlleben, W. and Burroughs, N. J. (2012). Extracting Regulator Activity Profiles by Integration of De novo Motifs and Expression Data: Characterizing Key Regulators of Nutrient Depletion Responses in *Streptomyces coelicolor*. *Nucleic Acids Research* **40**: 5227-5239.

Isono, K., Nagatsu, J., Kawashima, Y. and Suzuki, S. (1965). Studies on Polyoxins, Antifungal Antibiotics. Part I. Isolation and Characterization of Polyoxins A and B. *Agriculture and Biological Chemistry (Tokyo)* **29**: 848–854.

Janardhan, A., Kumar, A. P., Viswanath, B., Saigopal, D. V. R. and Narasimha G. (2014). Production of Bioactive Compounds by Actinomycetes and Their Antioxidant Properties. *Biotechnology Research International* **2014**: 1-9.

Jiang, Y., Li, Q., Chen, X. and Jiang, C. (2016). Isolation and Cultivation Methods of Actinobacteria. In: *Actinobacteria - Basics and Biotechnological Applications*. D. Dhanasekaran and Y. Jiang, IntechOpen, DOI: 10.5772/61457.

Jarallah, E. and Rahman, H. N. (2014). Extraction and Purification of Antimicrobial agent Produced from Actinomycetes Isolated from Agriculture Soils. *Pure and Applied Sciences* **22**: 746-758.

Jensen, S. E. and Paradkar, A. S. **(**1999). Biosynthesis and Molecular Genetics of Clavulanic acid. *Antonie Van Leeuwenhoek* **75**: 125–133.

Johnston, N. J., Mukhtar, T. A and Wright, G. D. (2002). Streptogramin Antibiotics: Mode of Action and Resistance. *Current Drug Targets* **3**: 335–344.

Kameda, Y. (1987). Validoxylamines as Trehalase Inhibitors. *Journal of Antibiotics* **40**: 563-565.

Kang, Q., Shen, Y. and Bai, L. (2012). Biosynthesis of 3,5-AHBA-derived Natural Products. Natural Product Reports **29**: 243–263.

Kekuda, T. R. P., Shobha, K. S. and Onkarappa, R. (2010). Fascinating Diversity and Potent Biological Activities of Actinomycete Metabolites. *Journal of Pharmacy Research* **3**(2): 250-256.

Kharel, M. K., Pahari P., Shepherd, M. D., Tibrewal, N., Nybo, S. E., Shaaban, K. A. and Rohr, J. (2012). Angucyclines: Biosynthesis, Mode-of-Action, New Natural products, and Synthesis. Natural Product Reports **29**: 264–325

Khasabuli, O. Y. and Kibera, A. N. (2014). Isolation, Characterization and Primary Screening of Soil Actinomycetes from Kenyatta University Arboretum Grounds for Antibacterial Activities. *Journal of Applied Biosciences* **74**: 6072– 6079.

Kominek, L. (1975). Cycloheximide Production by *Streptomyces griseus*: Control Mechanisms of Cycloheximide Biosynthesis. *Antimicrobial Agents Chemotherapy* **7**: 856–860.

Kuster, E., and Williams, S. (1964). Selection of Media for Isolation of Streptomycetes. *Nature* **202**(4935): 928-929.

Kumar, R. R. and Jadeja, V. J. (2016). Isolation of Actinomycetes: A Complete Approach. *International Journal of Current Microbiology and Applied Sciences* **5**(5): 606-618.

Kumar, N., Singh, R. K., Mishra, S. K., Singh, A. K. and Pachouri, U. C. (2010). Isolation and Screening of Soil Actinomycetes as Source of Antibiotics Active against Bacteria. *International Journal of Microbiology Research* **2**(2): 12-16.

Linagarppa, Y. and Lockwood, J. (1962). Chitin Media for Selective Isolation and Culture of Actinomycetes. *Phytopathology* **52**: 317-323.

Liras, P. (1999). Biosynthesis and Molecular Genetics of Cephamycins. Cephamycins Produced by Actinomycetes. *Antonie Van Leeuwenhoek* **75**:109–124.

Lo Grasso, L., Martino, D. C. and Alduina, R. (2016). Production of Antibacterial Compounds from Actinomycetes. In: *Actinobacteria - Basics and Biotechnological Applications*. D. Dhanasekaran and Y. Jiang, IntechOpen, DOI: 10.5772/61525.

Madigan, M. T., Martinko, J. M., Dunlap, P. V. and Clark, D. P. (2009). *Brock biology of microorganisms*.12th Edition, Pearson, SanFrancisco.

Makut, M. D. and Abdulazeez, U. S. (2012). A survey of Antibiotic-producing Actinomycetes in the Soil Environment of Keffi metropolis, Nasarawa State, Nigeria. *Journal of Microbiology and Antimicrobials* **4**(5): 74-78

Maheshwari, D. K. and Shimizu, M. (2011). Endophytic Actinomycetes: Biocontrol Agents and Growth Promoters. In *Bacteria in Agrobiology: Plant Growth Responses*. Springer Berlin Heidelberg. pp 201-220.

Martin, J. F. (2004). Phosphate Control of the Biosynthesis of Antibiotics and other Secondary Metabolites is mediated by the PhoR-PhoP System: An Unfinished story. *Journal of Bacteriology* **186**(16): 5197-5201.

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G. and Gianni, L. (2004). Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity. *Pharmacological Reviews* **56**:185–229.

Mohan, V. J., Sirisha, B., Prathyusha, K. and Rao P. (2014). Isolation, Screening and Characterization of Actinomycetes from Marine Sediments for their Potential to Produce Antifungal Agents. *Global journal of Biology, Agriculture and Health Sciences* **3**(4):131-137.

Muleta, A. and Assefa, F. (2018). Isolation and screening of antibiotic producing actinomycetes from rhizosphere and agricultural soils. *African Journal of Biotechnology* **17**(22): 700-714.

Mulinos, M. G. (1955). Cycloserine: An Antibiotic Paradox. *Antibiotics Annual* **3**:131–135.

Munita, J. M. and Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology Spectrum* **4**(2): 1-37.

Nanjwade, B. K., Chandrashekhara, S., Shamarez, A. M., Goudanavar, S. P. and Manvi, V. F. (2010). Isolation and Morphological Characterization of Antibiotic Producing Actinomycetes. *Tropical Journal of Pharmaceutical Research* **9**(3): 231-236.

Nitiss, J. L. (2009). Targeting DNA Topoisomerase II in Cancer Chemotherapy. *Nature Reviews Cancer* **9**: 338–350.

Njenga, P. W., Mwaura F. B., Wagacha, J. M. and Gathuru, E. M. (2017). Methods of Isolating Actinomycetes from the Soils of Menengai Crater in Kenya. *Archives of Clinical Microbiology* **8**(345): 1-7.

Nonomura, H. (1974). Key for Classification and Identification of 458 Species of the Streptomycetes included in ISP. *Journal of Fermentation Technology* **52**(2): 78 - 92.

Okami, Y., Hotta, K. (1988). Search and Discovery of New Antibiotics. In: *Actinomycetes in biotechnology*. Goodfellow, M., Williams, S. T., Mordarski, M (eds). Academic Press, San Diego, CA. pp 33–67.

Otoguro, M., Hayakawa, M., Yamazaki, T. and Iimura, Y. (2001a). An integrated Method for the Selective Isolation of *Actinokineospora* spp. in Soil and Plant Litter. *Journal of Applied Bacteriology* **91**: 118-130.

Pandey, A., Ali, I., Butola, K. S., Chatterji, T. and Singh, V. (2011). Isolation and Characterization of Actinomycetes from Soil and Evaluation of Antibacterial Activities of Actinomycetes against Pathogens. *International Journal of Applied Biology and Pharmaceutical Technology* **2**(4): 384-392

Park, S R., Park, J. W., Ban, Y. H., Sohng, J. K. and Yoon, Y. J. (2013). 2-Deoxystreptamine- containing Aminoglycoside Antibiotics: Recent Advances in the Characterization and Manipulation of their Biosynthetic Pathways. *Natural Product Reports* **30**: 11–20.

Perry, J. A., Westman, E. L. and Wright, G. D. (2014). The Antibiotic Resistome: What's New? *Current Opinion in Microbiology* **21**: 45-50.

Pettit, R. K. (2004). Soil DNA Libraries for Anticancer Drug Discovery. *Cancer Chemotherapy and Pharmacology* 54: 1-6

Poehlsgaard, J. and Douthwaite, S. (2003). Macrolide Antibiotic Interaction and Resistance on the Bacterial Ribosome. *Current Opinion in Investigational Drugs* **4**:140–148.

Porter, J., Wilhelm, J., Tresner, H. (1960). Method for the Preferential Isolation of Actinomycetes from Soil. *Applied Microbiology* **8**:174-178.

Priyadarshini, A., Singdevsachan, S. K., Tripathy, S. K., Mohanta, Y. K., Patra, J. K. and Sethi, B. K. (2016). Isolation and Identification of Actinomycetes from Mangrove Soil and Extraction of Secondary Metabolites for Antibacterial Activity. *British Biotechnology Journal* **12**(2): 1-13.

Procópioa, R. E. L., da Silvaa, I. R., Martinsa, M. K., de Azevedoa, J. L. and de Araújo J. M. (2012). Antibiotics Produced by Streptomyces. *The Brazilian Journal of Infectious Diseases* **16**(5): 466–471.

Rachdiati, H., Zakariya, N. A. B, and Dash, G. K. (2016). Isolation and Screening of Antibiotic Producing Actinomycetes from Soils in Manong, Perak, Malaysia. *Der Pharmacia Lettre* **8**(15): 226-229.

Rafieenia, R. (2013). Effect of Nutrients and Culture Conditions on Antibiotic Synthesis in Streptomycetes. *Asian Journal of Pharmaceutical and Health Sciences* **3**(3): 810-821.

Rai, K., Khadka, S. and Shrestha, B. (2018). Actinomycetes: Isolation, Characterization and Screening for Antimicrobial Activity from Different Sites of Chitwan, Nepal. *International Journal of Microbiology and Biotechnology* **3**(1): 25-30.

Raja, A. and Prabakarana, P. (2011). Actinomycetes and Drug-An Overview. *American Journal of Drug Discovery and Development* **1**(2): 75-84.

Ramirez, M. S., Nikolaidis, N. and Tolmasky, M. E. (2003). Rise and Dissemination of Aminoglycoside Resistance: The aac(6')-Ib Paradigm. *Frontiers in Microbiology* **4**: 121

Saudagar, P. S., Survase, S. A. and Singhal, R. S. (2008). Clavulanic Acid: A Review. *Biotechnology Advances* **26**: 335–351.

Schleifer, K. H. (1985). Analysis of the Chemical Composition and Primary Structure of Murein. *Methods in Microbiology* **18**:123 –56

Schoenian, I., Spiteller, M., Manoj, J., Rainer, W., Hubert, H. and Dieter, S. (2011). Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proceedings of the National Academy of Sciences of the United States of America* **108**(5): 1955–1960.

Seipke, R. F., Barke, J., Brearley C., Hill, L., Douglas, W. Y., Goss, R. J. M. and Hutchings, M. I. (2011). A single *Streptomyces* symbiont makes multiple antifungals to support the fungus farming ant *Acromyrmex octospinosus*. *PLoS ONE* **6**(8): 1-8

Seipke, F. R., Gruschow, S., Goss, R. J. and Hutchings, M. I. (2012). Isolating Antifungals from Fungus-growing Ant Symbionts using a Genome-guided Chemistry Approach. *Methods in Enzymology* **517**: 47–70.

Silva, M., and Anne, D. (2004). The Best Penicillin for Resistant Bacteria. *Journal of antibiotics* **48**: 562-569.

Sharma, M., Dangi, P. and Choudhary, M. (2014). Actinomycetes: Source, Identification, and Their Applications. *International Journal of Current Microbiology and Applied Sciences* **3**(2): 801-832.

Sharma, H. and Parihar, L. (2010). Antifungal Activity of Extracts obtained from Actinomycetes. *Journal of Yeast and Fungal Research* **1**(10): 197 – 200.

Shirling, E. B. and Gottlieb, D. (1966). Methods for Characterization of *Streptomycetes* species. *International Journal of Systematic Bacteriol*ogy **16**: 313 – 340.

Singh, V., Haque, S., Singh, H., Verma, J., Vibha, K., Singh, R., Jawed, A. and Tripathi, C. K. M. (2016). Isolation, Screening and Identification of Novel Isolates of Actinomycetes from India for Antimicrobial Application. *Frontiers in Microbiology* **7**: 1921-1928.

Tamura, T., Hayakawa, M. and Hatano, K. (2001). A new genus of the order *Actinomycetales*, Virgosporangium gen. nov., with descriptions of Virgosporangium ochraceum sp. nov. and Virgosporangium auranticolor sp. nov. *International Journal of Systemic and Evolutionary* Bacteriology **51**: 1809–1816.

Tenover, F. C. (2006). Mechanisms of Antimicrobial Resistance in Bacteria. *The American Journal of Medicine* **119**(6A): S3-S10.

Torsvik, V. and Ovreas, L. (2002). Microbial Diversity and Function in Soil: from Genes to Ecosystems. *Current Opinion in Microbiology* **5**: 240 – 245.

Umezawa, H., Okami, Y., Hashimoto, T., Suhara, Y., Hamada, M. and Takeuchi, T. (1965). A New Antibiotic, Kasugsmycin. *Journal of Antibiotics* **18**: 101–103.

Vakulenko, S. B. and Mobashery, S. (2003). Versatility of aminoglycosides and prospects for their future. *Clinical Microbiology Reviews* **16**: 430–450.

Van Bambeke, F. (2006). Glycopeptides and Glycodepsipeptides in Clinical Development: A Comparative Review of their Antibacterial Spectrum, Pharmacokinetics and Clinical Efficacy. *Current Opinion in Investigational Drugs* **7**: 740 –749.

Ventola, C. L. (2015). The Antibiotic Resistance Crisis, Part 1: Causes and Threats. *Pharmacy and Therapeutics* **40**(4): 277-283.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G. F., Chater, K. F. and Van Sinderen, D. (2007). Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum. *Microbiology and Molecular Biology Reviews* **71**: 495-548.

Vining LC, Stuttard C. 1994. Chloramphenicol, genetics and biochemistry of antibiotic production. Butterworth-Heinemann, Boston, MA.

Wadetwar, R. N. and Patil, A. T. (2013). Isolation and characterization of Bioactive Actinomycetes from soil in and around Nagpur. *International Journal of Pharmaceutical Sciences and* Research **4**(4): 1428-1433.

Wang, Y. and Jiang, Y. (2016). Chemotaxonomy of Actinobacteria. In: *Actinobacteria - Basics and Biotechnological Applications*. D. Dhanasekaran and Y. Jiang, IntechOpen, DOI: 10.5772/61482

White, J. and Bibb, M. (1997). bldA dependence of undecylprodigiosin production in *Streptomyces coelicolor* A3(2) involves a pathway-specific regulatory cascade. *Journal of Bacteriology* **179**(3): 627-633.

Wietzorrek, A. and Bibb, M. (1997). A Novel Family of Proteins that Regulates Antibiotic Production in Streptomycetes appears to contain an OmpR like DNA-binding fold. *Molecular Microbiology* **25**: 1181-1184.

Willey, J. M., Sherwood, L. M. and Woolverton, C. J. (2010). *Prescott, Harley and Klein's Microbiology*. 7th edition edition. McGraw-Hill, New York.

Willey, J. M. and Van Der Donk, W. A. (2007). Lantibiotics: Peptides of Diverse Structure and Function. *Annual Review of Microbiology* **61**:477–501.

Williams, S. T., Shameemullah, M., Watson, E. T. and Mayfield C. I. (1972). Studies on the Ecology of Actinomycetes in Soil: The Influence of Moisture Tension on Growth and Survival. *Soil Biology and Biochemistry* **4**: 215-225.

Yamamura, H., Hayakawa, M. and Iimura, Y. (2003a). Application of Sucrose-gradient Centrifugation for Selective Isolation of *Nocardia* spp. from Soil. *Journal of Applied Microbiology* **95**: 677-685.

Yokota, A. (1997). Phylogenetic Relationship of Actinomycetes. *Atlas of actinomycetes*. Asakura Publishing Co. Ltd., Japan. pp.194 – 197.

APPENDIX A

MEDIA AND REAGENTS COMPOSITIONS

Actinomycete Isolation Agar

Agar	15.0g
Glycerol	5.0g
Sodium propionate	4.0g
Sodium caseinate	2.0g
K_2HPO_4	0.5g
Asparagine	0.1g
$MgSO_4 \cdot 7H_2O$	0.1g
$FeSO_4 \cdot 7H_2O$	1.0mg

International Streptomyces Project Medium- 2 (ISP-2)

Yeast extracts	4 g
Malt extracts	10 g
Dextrose	4 g
Agar	20 g
Distilled water	1000.0 ml

Sabouraud Dextrose Agar

Neopeptone	30.0g
Agar	20.0g
Distilled water	1000.0 ml

Potato dextrose agar (PDB)

Potato	200.0 g
Dextrose	20.0 g
Distilled water	1000.0 ml

Nutrient Agar

Agar	15.0g
Peptone	5.0g
NaCl	5.0g
Yeast extracts	2.0g

Beef extract	1.0g
Distilled water	1000.0 ml

Gram's crystal violet

Solution A

Crystal violet	2.0 g
Ethanol	20.0 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Gram's iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	50.0 ml

Counter stain

Safranin	0.25 g
Ethanol	10.0 ml
Distilled water	90.0 ml

Gelatin medium

Peptone	5.0 g
Beef extract	3.0 g
Gelatin	10.0 g
Distilled water	1000.0 ml

Starch agar medium

Peptone	10.0 g
Beef extract	3.0 g
Soluble starch	1.5 g
Sodium chloride	5.0 g
Agar	18.0 g
Distilled water	1000.0 ml

Christensen's urea agar

Peptone	1.0 g

Glucose	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Phenol red	0.12 g
Urea	40.0%
Agar	18.0 g
Distilled water	1000.0 ml

Triple Sugar Iron Agar (TSI Agar)

Peptone	20.0g
Agar	12.0g
Lactose	10.0g
Sucrose	10.0g
NaCl	5.0g
Beef extracts	3.0g
Yeast extracts	3.0g
Glucose	1.0g
Ferric citrate	0.3g
$Na_2S_2O_3$	0.3g
Phenol Red	0.025g
Distilled water	1000.0 ml

Motility Medium

Pancreatic digest of casein	10.0g
Glucose	5.0g
Agar	3.0g
Na_2HPO_4	2.5g
Yeast extracts	2.5g
Distilled water	1000.0 ml

Simmon's citrate agar medium

NH_4HPO_4	1.0 g
K_2HSO_4	1.0 g
NaCl	5.0 g
$MgSO_4$	0.2 g

Bromothymol blue	0.08 g
Sodium citrate	2.0 g
Agar	18.0 g
Distilled water	1000.0 ml

Indole broth

Peptone	20.0 g
Sodiun chloride	5.0 g
Distilled water	1000.0 ml

Kovac's reagent

p-Dimethylaminobenzaldehyde	5.0 g
Isoamyl alcohol	75.0 ml
Conc. HCl	25.0 ml

MR-VP medium

Peptone	7.0 g
Dextrose	5.0 g
K_2HPO_4	5.0 g
Distilled water	1000.0 ml

Methyl red Reagent

Methyl red	1.0 g
Ethanol	300.0 ml
Distilled water	200.0 ml

Voges-Proskauer reagents

Barrits A

a-naphthol	5.0 g
Ethanol	95.0 ml

Barrits B

KOH	40.0 g
Creatine	0.3 g
Distilled water	100 ml

APPENDIX B

16S RIBOSOMAL RNA GENE PARTIAL SEQUENCE OF ISOLATE CYP1

AGCCTATGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAACCCGCTTCGGT
GGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCT
GGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACACGGGATCGCATGG
TCTCCGTGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGT
TGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGA
CCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGA
CGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCGAGTGACGGTAC
CTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
TGCGAGCGTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGT
CGGATGTGAAAGCCCGGGGCTTACCCCGGGTCTGCATTCGATACGGGCAGGCTA
GAGTTCGGCAGGGGAGATTGGAATTCCTGGTGTAGCGGGAAATGCGCAGATATC
AGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACCCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGTTGGGCACTAGGTGTGGGGGGCATTCCACGTCGTCCGTGCCGCAGCTAACGC
ATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTG
ACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAG
AACCTTACCAAGGCTTGACATACACCGGAAAGCCGTAGAGATACGGCCCCCCTT
GTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCAACTCCTTTC
GGGGAGGTTGGGACTCACGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGG
GACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATG
GCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGT
CTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAA
TCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCTTGTACACACCGCCCG
TCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGG
GGAGTCGTCGAAGGTGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCG
ATTTAC